Genetic structure and phenotypic differences among and within extant populations of *Chrysanthemum arcticum* L. and *C. a.* subsp. *arcticum*

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Chapter 1 Literature review

History of chrysanthemum.

Chrysanthemums (mums) are native to the northern hemisphere, the majority of species are from Asia (primarily China, Korea, and Japan) with a few annual species from Europe (the Mediterranean region, centered in Algeria and the Canary Islands) (Ackerson, 1957, 1967a, 1967b; Anderson, 2006; Hemsley, 1889). Chrysanthemum history was recorded as early as the 15th century BC in China, with the plant noted for being cultivated for ornamental and medicinal applications (Anderson, 2006; Hemsley, 1889). A gift from the King of Korea to the Japanese Emperor as a tribute contained red, white, yellow, blue, and black chrysanthemums in the year 386 AD, was the first record that chrysanthemums were brought into Japan (Anderson, 2006; Bailey et al., 1896). “Chu” is the Chinese character for chrysanthemum and “kiku” has the same meaning in Japanese, although the characteristics are similar (Ackerson, 1967a). Chrysanthemum were introduced into the western world via the Netherlands in 1688, and cultivated as *Matricaria japonica* maxima. The plant was taken to Great Britain in 1754 (Anderson, 2006; Hemsley, 1889). The name of chrysanthemum was given by the Swedish botanist Carl Linnaeus, combining the Greek word “chrysos” for “golden” color and “anthemon” for flower (Morton, 1891). In 1789, “Old Purple”, the only surviving cultivar from one of three brought from China by a French merchant Pierre Louis Blancard, became the first named cultivar grown in the western world (Smith, 2013).

Although cultivated chrysanthemum was described as early as 500 BC in “Li-Ki” by Confucius, the first successful hybridization of chrysanthemum and

germination of seedlings was produced in France by Bernet in 1827 (Anderson, 2006; Bretschneider, 1895; Smith, 2013). Considerable numbers of new cultivars have been developed by amateur breeders in Europe, such as Robert Fortune (Follwell, 1907; Royal Horticultural Society, 1890). According to the 1937 USDA Yearbook (1937), chrysanthemums were introduced into the United States in 1798; a new cultivar, named 'William Penn', bred by the earliest known chrysanthemum breeder in the United States, was shown at the Pennsylvania Horticultural Society in 1841 (Anderson, 2006; Emsweller et al., 1937; Smith, 2013). After decades of efforts by breeders, by the 1930s over 3,000 cultivars existed (Anderson, 2006).

Commercial cultivated chrysanthemum.

Cultivated chrysanthemums (*Chrysanthemum* x*grandiflorum* Tzvelv. and *C.* x*hybridum* Anderson) (Asteraceae) are one of the world's most important ornamental crops (Anderson, 2006), ranking second worldwide in the commercial floriculture market after roses, *Rosa* x*hybrida* (Xia et al., 2006). In regards to comprehensive product use for the crop in farmgate values in 2018, US chrysanthemum production included $147.49M in sales of over 53 million containers of herbaceous perennials, $10.459M (w) of cut flowers from 6.9M bunches of pompon types, as well as ~7.7M flowering potted plants valued at nearly $30M (United States Department of Agriculture, 2019). Thus, the total wholesale value of chrysanthemums produced in the US during 2018, by large growers in the top 15 states, totaled nearly $187M (United States Department of

Agriculture, 2019). The species is the No. 1 selling herbaceous perennial in the USA.

The market's demand for chrysanthemum has increased year by year, encouraging researchers and breeders to develop new cultivars with novel appearance and tolerance to abiotic and biotic stress (Anderson, 2006; Su et al., 2019). Accordingly, in term of commercial chrysanthemum breeding, a wide range of studies have been exploring and improving ornamental traits and stress tolerance, including plant architecture (Klie et al., 2016), floret types, size (Song, Gao, et al., 2018; Song, Zhao, et al., 2018), early flowering time (Shulga et al., 2011), medicinal compounds (Fang et al., 2012), waterlogging tolerance (Su et al., 2017), winter hardiness (Anderson et al., 2008; Anderson & Gesick, 2004), salt tolerance (Liu et al., 2013), and drought tolerance (Li et al., 2018).

Cultivated chrysanthemums (*C.* x*grandiflorum*) are allopolyploid (2*n*=6*x*=54) interspecific hybrids, resulting from thousands of years of hybridization. These hybridizations involving ten or more species, particularly: *C. indicum*, *C. koreanum*, *C. lavandulaefolium*, *C. vestitum*, *C. weyrichii*, and *C. zawadskii* (Dowrick, 1952a, 1952b, 1953, 1958; Dowrick & El-Bayoumi, 1966), dating in cultivation as far back as King Solomon (Schweinfurth, 1919) and the Chinese Shang dynasty (Way, 2020).

In classical breeding, breeders mainly use phenotypes for selection which may be affected by individual x environment (G x E) (Romagosa & Fox, 1993; Su et al., 2019). Furthermore, the hexaploid nature of the species (Dowrick, 1952a, 1952b, 1953, 1958; Dowrick & El-Bayoumi, 1966), 2*n*=6*x*=54; (Dowrick, 1952a,

1952b, 1953, 1958; Dowrick & El-Bayoumi, 1966), and the forced outcrossing of the species, due to a complex sporophytic self incompatibility system (Anderson et al., 1988; Zagorski et al., 1983), have complicated the effectiveness of traditional breeding.

Chrysanthemum arcticum, C. arcticum L. subsp. *arcticum* and *C. arcticum* L. subsp. *polaré* Hultén

Until 1741, it was assumed that all *Chrysanthemum* species were native to Eurasia. In that year, Dr. Georg Wilhelm Steller, a physician and natural historian was engaged on Captain-Commander Vitus Bering's second Kamchatka expedition to map the easternmost coastline of Imperial Russia (Steller, 1993). While, Bering's first expedition of 1782 had failed expectations, it still brought back the first map of the shores of Kamchatka, Russia, and the northeastern end of Asia (Collins, Jr., 1947). Steller was the first European botanist to step onto American soil in the Arctic and subarctic regions. Steller went ashore on Kodiak Island -- the easternmost of the Aleutian Island chain. As the islands of the Aleutian chain, Bering Island didn't have any trees and, instead, had predominantly flowering plants, grasses, and mosses. Steller collected plant and animal species on Bering Island. Steller is the only naturalist who ever saw *Rhytina stelleri*, a large sea cow, alive; currently it has been recorded as extinct for centuries (Collins, Jr., 1947). On their way back to Kamchatka, the boat stopped at Bird Island, part of the Shumagin Islands of the Aleutian Island chain, where *C. arcticum* subsp. *arcticum* is native to select Aleutian Islands including

Kodiak Island and the Bird Islands (Hultén, 1937). It was most likely collected by Steller there (Lauridsen, 1885).

Chrysanthemum arcticum L., Arctic daisy, (=*Arctanthemum arcticum*; =*Dendranthema arcticum*) and its two subspecies (*C. arcticum* L. subsp. *arcticum*, *C. arcticum* L. subsp. *polaré* Hultén) are the only chrysanthemum species native to North America (Hultén, 1968; Steller, 1993). Both *C. arcticum* and *C. arcticum* subsp. *polaré* are only found in N. America whereas *C. arcticum* subsp. *arcticum* has two remnant populations occurring in Eurasia adjacent to the Aleutian Islands (in the Kamchatka peninsula, Russian Federation and in Hokkaido, Japan).

The genus *Chrysanthemum* (Asteraceae) consists of a range of species, from 40 (Liu et al., 2012), 75 (Ohashi & Yonekura, 2004), or more (Anderson, 1987), depending on whether taxonomists lump or split members of the *Chrysanthemum* species are classified into three sections of the genus: *Chrysanthemum*, *Ajania* and *Arctanthemum*. The genus was once changed to *Dendranthema* instead of *Chrysanthemum,* based on genetic and sectional perspectives (Anderson, 1987; Anderson et al., 1988; Iwatsuki et al., 1995; Koyama, 1995; Tzvelv, 1985). *Chrysanthemum arcticum* L. subsp. *arcticum* was initially considered as an infraspecific taxon in the section *Chrysanthemum* (Kitamura, 1940). Subsequently, Bremer and Humphries (1993) recognized the genus *Arctanthemum* and the name changed to *Arctanthemum arcticum* L. Currently, the name of the species and subspecies has been changed back to *Chrysanthemum* and assigned into the generic section *Arctanthemum* by Ohashi

and Yonekura (2004). Thus, the species in this study are correctly referred to as *C. arcticum, C. arcticum* subsp. *arcticum,* and *C. arcticum* subsp. *polaré*.

According to Hultén's taxonomic treatise (Hultén, 1937), the center of origin and diversity of *C. arcticum* is the subarctic region of North America, specifically the state of Alaska, USA (Fig. 1). Most of the populations of both *C. arcticum* and the two subspecies are found growing only in maritime regions along coastal areas (Hultén, 1937, 1968). From the mid 18th century to the present day, the extensive historic (herbaria) accessions of *C. arcticum* and its two subspecies reflect the great effort made by taxonomists to document the taxonomic group throughout N. America, the Russian Federation and Japan. *Chrysanthemum arcticum* subsp. *arcticum* overlaps in distribution with *C. arcticum* along the seashore of the Pacific coast of North America (Hultén, 1968; Uehara et al., 2017). *Chrysanthemum arcticum* and, to a lesser extent, sympatric *C. arcticum* subsp. *arcticum,* also extends southward into western Canada, specifically the province of British Columbia.

Chrysanthemum arcticum subsp. *arcticum* spreads from the subarctic coast of Alaska westwards in some, but not all, of the Aleutian Islands to the Pribilof Islands, Afognak Island, and Shumagin Islands as well as far as the westernmost point of N. America on Attu Island (Hultén, 1937, 1968) (Fig. 1). Two remnant populations of *C. arcticum* subsp. *arcticum* are found in Eurasia, namely the Russian Federation on the Kamchatka Peninsula (discovered by Steller after returning from his trip with Bering, post 1741) as well as one population in

eastern Asia, specifically on the Island of Hokkaido, Japan (Hultén, 1968; Uehara et al., 2017).

Chrysanthemum arcticum subsp. *polaré* is restricted to the arctic regions of Alaska in the area around Nome and Utqiaġvik (formerly Barrow), as well as in the subarctic and arctic regions along the shores of Hudson and James Bays and eastern Yukon beyond the Mackenzie River in Canada (Hultén, 1937, 1968; Porsild, 1957; Porsild & Cody, 1980) (Fig. 1). The distribution of *C. a.* subsp. *polaré* is not known to be sympatric with the *C. a.* subsp. *arcticum* in North America (Budd, 1987).

All members of *C. arcticum* species are cold hardy perennial herbaceous with winter hardiness of USDA Zone 1 or air temperatures below -45.5° C (-50° F) to USDA Z5 (B & T World Seeds, 2019). The species grow in coastal marine environments prone to extreme depths of snow with a groundcover or dwarf habit, often exposed to a length of cold, damp and rainy periods during late spring and fall, which delays breaking dormancy, regrowth and flowering (Anderson, N. O., 2018-2019, unpublished data) (Hultén, 1968; Porsild, 1957; Porsild & Cody, 1980). According to herbarium records for the species, a range of flowering times from as early as May 21 (week 21) to as late as September 25 (week 39), across the distribution range from the high latitudes of 46.47°N to 68.15 °N and longitudes of 171.71 °W to 173.97 °E (Liu and Anderson, 2019, unpublished data), indicate this to be a long day plant for flower bud initiation and development.

Advantageous traits of the *Chrysanthemum arcticum* species complex.

Taxa within *Chrysanthemum arcticum* species complex share many phenotypic traits, differentiated in the dichotomous keys by species-specific diagnostic traits (qualitative) (Hultén, 1937, 1968). *Chrysanthemum arcticum* species all possess glabrous or nearly wedge-shaped leaves and daisy-like flowers with white ray head floret (petals) with short, yellow disc florets assembled in the classic Asteraceae inflorescence. The florets are 2-5 centimeters in diameter, generally solitary, although instances of 2-3 florets on branched inflorescence stems have been noted (Hultén, 1968). The involucral bracts below the inflorescence have black tips (Bremer & Humphries, 1993; Hultén, 1968; Porsild & Cody, 1980). Leaves from *Chrysanthemum arcticum* species are tripartite with primarily regularly toothed leaf margins with leaves tending to have five-segmented leaves and a deep sinus. *Chrysanthemum a.* subsp. *arcticum* has leaves with a fine, shallow sinus (Nishikawa & Kobayashi, 1989). The number of mid veins in the ray floret petals also differs among the species and subspecies (Hultén, 1937, 1968). Meanwhile, some quantitative differentiation of the taxa within the C. *arcticum* complex also can be used to distinguish the taxa, such as, *C. a.* subsp. *arcticum* flowering stems are 30-40 cm tall whereas *C. a.* subsp. *polaré* has the shortest stems of 6-20 cm (Iwatsuki et al., 1995; Johnson, 1987). However, these quantitative measurements may be highly affected by environmental factors, which were not collected from individuals growing in identical environments (Anderson, 2006; Dole & Wilkins, 1999).

Common and outstanding features of *Chrysanthemum arcticum* and its subspecies, such as salt tolerance, ground cover plant habit, and winter hardiness make it of interest as a resource in breeding. According to the US Salinity laboratory, saline soils are referred to as ones with electrical conductivity (EC) >4 mS/cm NaCl and an exchangeable sodium percentage (ESP) <15 (Regional Salinity Laboratory, 1954). The majority of cultivated chrysanthemums are recognized as being less than moderately salt tolerant displaying salt injury under saline condition. Under saline conditions, significant symptoms are observed: such as, retarded growth; whitish, rolled, dry leaves, or death (Rai et al., 2017; Regional Salinity Laboratory, 1954). In contrast, *Chrysanthemum arcticum* individuals from Anchor Point, Kenai-1, Kenai-2 and Old Valdez-4 collection sites from mainland Alaska, grow robustly even in high saline conditions (Chapter 3).

Genetic diversity within and among populations and conservation strategies.

Genetic diversity is defined as the amount of genetic variability among individuals of a variety or population of a species (Brown, 1983). The variation sources are mutation and recombination among individuals, as well as selection, genetic drift and gene flow acting among different populations (Suneson, 1960; Frankel, 1977; Nevo et al., 1984; Hamrick et al., 1992). According to previous studies, the genetic variation within and among populations is generally considered to be structured in space and time (Loveless & Hamrick, 1984). It is

widely accepted that the population-level of genetic diversity within species is important for species resistance (Hughes & Stachowicz, 2004; Neel, 2008). Genetic losses can be occurred by gradually reducing alleles enrichment and heterozygosity in small and isolated populations, or more suddenly by loss of the entire population (Neel, 2008). Considering conservation efforts, especially in the case of *in situ* conservation, the structure of genetic diversity within and between populations is an important factor (Wilcox, 1984; Gole, 2003; Volis & Biecher, 2010). The different aspects of genetic diversity among populations in a species, e.g. the extent and distribution of populations and how they are genetically structured, would be an essential prerequisite to determine the conservation strategies (Rao & Hodgkin, 2002).

DArTseqLD (Diversity Arrays Technology low density).

DArTseqLD (Diversity Arrays Technology; https://www.diversityarrays.com/) is a useful individual by sequencing method to generate single nucleotide polymorphisms (SNPs) since it combines DArT complexity reduction methodology with next generation sequencing (NGS) platforms (Kilian et al., 2012). This method has been widely used in polyploid species and those without any sequenced genome or classical genetic maps (Zaitoun et al., 2018; Baloch et al., 2017; Barilli et al., 2018). Genome complexity is reduced by the restriction enzyme and the short fragments will randomly select as a subset from a normal genotyping, which is usually producing an order of magnitude, smaller representations and correspondingly smaller number of

markers. The low density (LD) assay produces thousands of markers and, therefore, is highly appropriate for representing among and within species differences, regardless of whether the DNA was derived from herbarium specimens or fresh samples.

The aim of this study.

Based on Anderson's 2017-2018 collection expeditions to over 25 sites recorded for herbarium specimen collections (Anderson, 2017-2019, unpublished data) (Parks, 2018), it was found that the range has decreased significantly for both *C. arcticum* and *C. a.* subsp. *arcticum*, since its first discovery by Steller in 1741 (Steller, 1993) and subsequent mapping by Hultén (1968). Meanwhile, *C. arcticum* subsp. *polaré* has been listed on the rare vascular plants of Manitoba, Canada since 1980 by White and Johnson (White & Johnson, 1980), which may require implementation of conservation strategies. This trend is in agreement with the previous warming simulation studies (Kaplan & New, 2006), which indicates that major northward shifts and significant reductions of the tundra biomes in the Arctic in response to warming. Due to climate change, the Arctic plant communities are faced with challenges and many plant species are threatened with extinction, especially for high latitude plant communities (Swann et al., 2010). Populations of threatened species are considered to be vulnerable to a loss of genetic diversity, causing by the degradation of population size and reduced gene flow among populations (Lowe et al., 2005; Yokogawa et al., 2013).

The aim of study is to examine the genetic structure of extant *C. arcticum* and *C. a.* subsp. *arcticum* populations collected in different geographic locations on the Alaska mainland and Attu Island (the western-most Aleutian Island). Single nucleotide polymorphism (SNP) markers were employed to illustrate the genetic diversity and genetic structure of relatedness and variation among and within species and populations. A better understanding of how the genetic diversity is distributed may provide insights for facilitating conservation, enhancing genetic diversity of species and populations, as well as selection of individuals with unique SNP markers for use in breeding.

From the standpoint of utilizing this advantageous plant resource with outstanding traits, the aim of this study is also to establish phenotypic differences among wild *C. arcticum* and *C. a.* subsp. *arcticum* species and populations. The SNP markers, derived from DArTseq genotyping technique, would reveal the genetic variation within and among extant populations of *C. arcticum* and *C. a.* subsp. *arcticum*. Possible linkage of these SNP markers with phenotypic traits is of great interest, particularly species-specific traits and those of commercial interest, such as salt tolerance. Phenotypic differentiation among *C. arcticum* and *C. a.* subsp. *arcticum* species and populations will provide a comprehensive foundation for marker assisted selection, aided by genomic sequencing of all *C. arcticum* species.

List of figures.

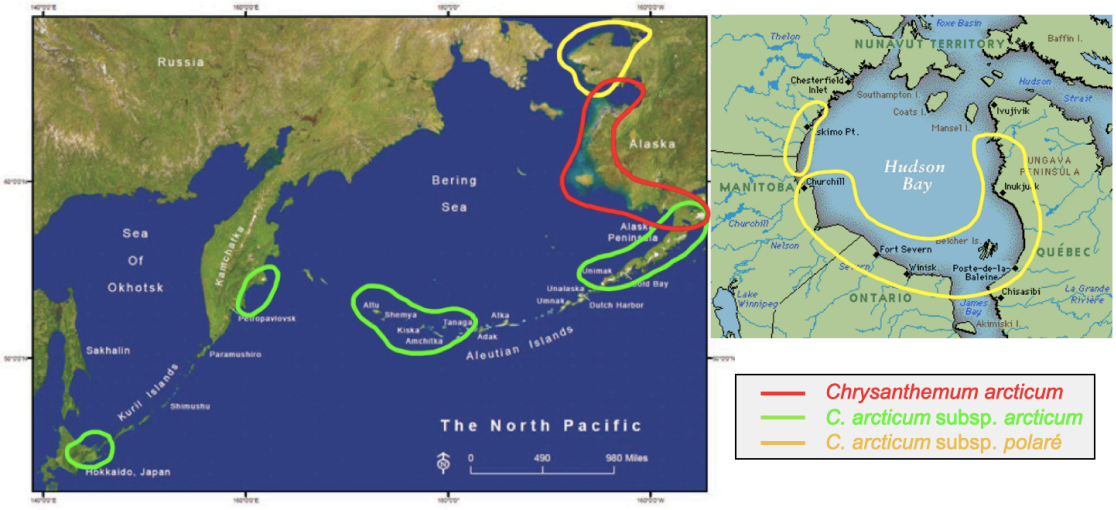

Figure 1. Chapter 1 Distribution of *Chrysanthemum arcticum, C. arcticum* subsp. *arcticum* and *C. arcticum* subsp. *polaré* (Hultén, 1937, 1968; Uehara et al., 2017; Porslid & Cody, 1980).

Chapter 2 SNP-based estimates of genetic structure among and within extant populations of *Chrysanthemum arcticum* L. and *C. a.* subsp. *arcticum*

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Abstract

Understanding the genetic diversity in natural plant populations is important in order to develop conservation strategies and utilize valuable germplasm resources. *Chrysanthemum arcticum* L., Arctic daisy, (=*Arctanthemum arcticum*; =*Dendranthema arcticum*) and its two subspecies (*C. arcticum* L. subsp. *arcticum*, *C. arcticum* L. subsp. *polaré* Hultén) are the only chrysanthemum species native to North America. We collected 529 individuals in nine *C. arcticum* and 21 *C. a.* subsp. arcticum populations from the state of Alaska mainland and Attu Island (the westernmost Aleutian Island). Evidence of declination in population size and decreasing range of distribution were detected for both species and subspecies. Population genetic diversity was analyzed using 7,449 SNP markers developed using low density DArTseq technology. Three distinct genetic clusters within *C. arcticum* populations were detected by STRUCTURE 2.3.4 that were consistent for results obtained by principal coordinate analysis (PCoA), discriminant analysis of principal components (DAPC), and unweighted pair group method with arithmetic mean (UPGMA). SNP data showed a clear taxonomic distinction among *C. arcticum* and *C. a.* subsp. *arcticum.* However, within *C. a.* subsp. *arcticum* populations, two subgroups occurred in genetic cluster analyses that were a mixture of individuals

from different populations, which may be the result of gene flow in nearby regions.

Key words: chrysanthemum, *Chrysanthemum arcticum*, conservation, DArTseqLD, gene flow, genetic diversity, population structure, Single nucleotide polymorphisms (SNPs)

Introduction

Cultivated chrysanthemums (*Chrysanthemum* x*grandiflorum* Tzvelv. and *C.* x*hybridum* Anderson) (Asteraceae) are one of the most important ornamental crops (Anderson, 2006; Anderson et al., 2008), ranking second worldwide in the commercial floriculture market after roses, *Rosa* x*hybrida* (Xia et al., 2006). In terms of comprehensive product use for the crop in 2018, US chrysanthemum production included $147.49M (w, farmgate value) in sales of herbaceous perennials sold in 53.05M pots, $10.459M (w) of cut flowers from 6.88M bunches of pompon types, as well as $28.999M (w) (United States Department of Agriculture, 2019) of 7.68M flowering potted plants sold. Thus, the total wholesale value of chrysanthemums produced in the US during 2018, by large growers in the top 15 states, totaled $186.948M (United States Department of Agriculture, 2019). The species is the No. 1 selling herbaceous perennial in the USA. The species is a hexaploid, 2*n*=6*x*=54 (Dowrick, 1952a, 1952b, 1953, 1958; Dowrick & El-Bayoumi, 1966) and is an outcrossing species due to a complex sporophytic self incompatibility system (Anderson et al., 1988; Zagorski

et al., 1983). Chrysanthemums have been involved in a wide range of worldwide research, including breeding, reproductive biology, plant physiology, and postharvest studies as a floral, medicinal, and food crop (Anderson, 2006).

Chrysanthemums are native to the northern hemisphere, with a few annual species from Europe (the Mediterranean region, centered in Algeria and the Canary Islands) and the majority of species from Asia minor and major (primarily China, Korea, and Japan) (Ackerson, 1957, 1967a, 1967b; Anderson, 2006; Hemsley, 1889). They were introduced into the western world via the Netherlands in 1688, later spread to Great Britain in 1754 and the United States in 1798 (Anderson, 2006; Hemsley, 1889). Cultivated chrysanthemums (*C.* x*grandiflorum*) are allopolyploid interspecific hybrids, resulting from thousands of years of hybridization involving ten or more species, particularly *C. indicum, C. koreanum, C. lavandulaefolium, C. vestitum, C. weyrichii,* and *C. zawadskii* (Dowrick, 1952a, 1952b, 1953, 1958; Dowrick & El-Bayoumi, 1966), dating in cultivation as far back as King Solomon (Schweinfurth, 1919) and the Chinese Shang dynasty (Way, 2020).

Until 1741, it was assumed that all Chrysanthemum species were native to Eurasia. In that year, Dr. Georg Wilhelm Steller, a physician and natural historian on Captain-Commander Vitus Bering's second Kamchatka expedition to map the easternmost coastline of Imperial Russia and discover the Bering Strait (Steller, 1993), was the first European botanist to step onto American soil in the Arctic and subArctic regions. Steller went ashore on Kodiak Island -- the easternmost of the Aleutian Island chain -- and collected plant and animal species. On their way

back to Kamchatka, Russia, the boat stopped at Bird Island, part of the Shumagin Islands, where *C. arcticum* subsp. *arcticum* is native to select Aleutian islands including Kodiak and the Bird Islands (Hultén, 1937) and was most likely collected by Steller (Lauridsen, 1885).

Chrysanthemum arcticum L., Arctic daisy, (=*Arctanthemum arcticum*; =*Dendranthema arcticum*) and its two subspecies (*C. arcticum* L. subsp. *arcticum*, *C. arcticum* L. subsp. *polaré* Hultén) are the only chrysanthemum species native to North America (Hultén, 1968a; Steller, 1993). Both *C. arcticum* and *C. arcticum* subsp. *polaré* are only found in N. America whereas *C. arcticum* subsp. *arcticum* has two remnant populations occurring in Eurasia adjacent to the Aleutian Islands (in the Kamchatka peninsula, Russian Federation and in Hokkaido, Japan). The genus *Chrysanthemum* (Asteraceae) consists of a range of species, 40 (Liu et al., 2012), 75 (Ohashi & Yonekura, 2004) or more (Anderson, 1987), depending on whether taxonomists lump or split members of the *Chrysanthemum.* Species are classified into three sections of the genus: *Chrysanthemum*, *Ajania* and *Arctanthemum*. The genus was once changed to *Dendranthema* instead of *Chrysanthemum,* based on genetic and sectional perspectives (Anderson, 1987; Anderson et al., 1988; Iwatsuki et al., 1995; Koyama, 1995; Tzvelv, 1985). *Chrysanthemum arcticum* L. subsp. *arcticum* was initially considered as an infraspecific taxon in the section *Chrysanthemum* (Kitamura, 1940). Subsequently, Bremer and Humphries (1993) recognized the genus *Arctanthemum* and the name changed to *Arctanthemum arcticum* L. Currently, the name of the species and subspecies has been changed back to

Chrysanthemum and assigned into the generic section *Arctanthemum* (Ohashi & Yonekura, 2004). Thus, the species are correctly referred to as *C. arcticum, C. arcticum* subsp. *arcticum,* and *C. arcticum* subsp. *polaré*.

Based on Anderson's 2017-2018 collection expeditions to obtain extant specimens (Anderson N. O., 2017-2019, unpublished data) (Parks, 2018), it was found that for >25 previously collected historic (herbaria) populations of both *C. arcticum* and *C. a.* subsp. *arcticum,* the range of distribution has decreased from its first discovery by Steller in 1741 (Steller, 1993) and mapped by Hultén (Hultén, 1968). Meanwhile, *C. arcticum* subsp. *polaré* has been listed on the rare vascular plants of Manitoba, Canada since 1980 by White and Johnson (White & Johnson, 1980). Now it is of interest for conservation as well as for evolutionary and genetic studies, in addition to being a potential source of phenotypic traits for new product development.

DArTseq (Diversity Arrays Technology; https://www.diversityarrays.com/) is a useful method to generate single nucleotide polymorphisms (SNPs) since it combines DArT complexity reduction methodology with next generation sequencing platforms (Kilian et al., 2012) and is widely used in polyploid species and those without any sequenced genome or classical genetic maps. Genome complexity is reduced with restriction enzyme digesting and sequencing the short fragments. Either DArTseq high (HD) or low density (LD) are used to identify SNP markers and create a pool of mutation silicoDArTs with dominant traits. Prior to analyses, SNP generation is optimized for each submitted organism of a pilot plate representing the diversity of the species' genome, known as the

complexity reduction method. In the present study, DArTseqLD will be used to generate SNP markers to study genetic structure.

The objective of this study is to examine the genetic structure of extant *C. arcticum* and *C. a.* subsp. *arcticum* populations collected in different geographic locations on the Alaska mainland and Attu Island (the western-most Aleutian Island). Single nucleotide polymorphism (SNP) markers, derived from DArTseqLD (low density), are employed to illustrate the genetic diversity and genetic structure of the species and subspecies, relatedness among and within populations and how the genetic variability is distributed since these have implications for facilitating conservation strategies or enhancing genetic diversity of *C. arcticum* and *C. a.* subsp. *arcticum* populations. The null hypothesis tested was: Ho: There is no difference in genetic variation (SNPs) within and among extant populations of *C. arcticum* and *C. arcticum* subsp. *arcticum.*

Materials and methods

Locations of Extant Populations. This study focused on extant *C. arcticum* collected by Dr. Neil Anderson (University of Minnesota) during 2017-2018 from the coastline of southwest Alaska mainland (59° 46'N to 61° 6'N, -146° 16'W to -151° 51'W) and *C. arcticum* subsp. *arcticum* collected from the coastline of the westernmost Aleutian Island, Attu Island (52° 48'N to 52° 50'N, 173° 9'E to 173° 18'E) (Table 1). Extant populations of *C. arcticum* subsp. *polaré* were not available at this time so they could not be included. Where relevant, collection permits were issued for the collection and research of *C. arcticum* germplasm (USFWS No. 74500-17-018). Herein we define a collection site as the main location that contained one or more populations within which the species were collected; populations were defined as discrete groupings of sympatric plants capable of gene exchange; each population was at least 100 m apart. There are four collection sites on the Alaska mainland for nine extant populations of *C. arcticum* (n=225 individuals in total; Table 1): Anchor Point (n=1 population), Kenai (n=3 populations), Ninilchik (n=1 population), and Old Valdez (n=4 populations) (Fig. 2). Among these sites, Old Valdez was geographically isolated a significant distance away from the other sites by the Kenai Peninsula and mountain ranges. Old Valdez sites were approximately 275.82 km away from Kenai sites; 320.64 km away from Ninilchik site; and 341.69 km away from Anchor Point site. Old Valdez, Alaska is the site of the former city of Valdez, which was devastated in the 1964 earthquake (https://www.valdezmuseum.org/category/education/old-town-walking-tour/); the

current city of Valdez is 8.05 km south of this location. Nonetheless, the other remaining *C. arcticum* populations, while significantly closer than the Old Valdez site, are still geographically isolated from each other. Collections at each site consisted of running a transect directly through smaller populations ($n \leq 10$ individuals) with sampling of individuals occurring every 1 m on center (OC). In larger populations ($n > 10$ individuals), two perpendicular transects were run through the approximate center of each population and plants were collected along transects at intervals of 1 m OC. One large rhizome (underground stem) was collected from each selected individual, bagged in resealable plastic bags (1.75 mil, 1 Quart Get Reddi® Reclosable Food Service Bags, https://www.usplastic.com/catalog/item.aspx?itemid=128308&catid=) and put on ice in a portable cooler. Once collections were completed each day, the specimen bags were placed in a refrigerator (~3-5°C) until eventual transport to the lab (within 2-3 weeks after collection). Once the specimens arrived in the lab, two leaf samples were taken and frozen in -80°C freezer for subsequent DNA extraction. Each rhizome was then transplanted into square 754 cm^3 plastic pots (Landmark Plastic, Akron, Ohio) in Sunshine LC8 soilless potting medium (Sun Gro Horticulture, Agawam, MA) and rooted in a mist house with an intermittent mist system, at a mist frequency of 10 minutes intervals (mist nozzles, reverse osmosis water) during 0600-2200 HR with a 7 sec. duration, in a glass greenhouse (21/21°C, day/night, 16 hrs; 0600–2200 HR) with lighting at a minimum set point of 150 μmol m^{-2} s^{-1}. Once rooted (1-2 weeks), the plants were moved to a greenhouse under the following environmental conditions:

24.4±3.0/18.3±1.5°C day/night daily integral and a 16 hr photoperiod (0600–2200 HR; long days). Supplemental lighting was supplied during the winter months and cloudy days by 400 w high pressure sodium high intensity discharge (HPS-HID) lamps, at a minimum of 150 µmol m^{-2} s^{-1} at plant level. The computerized greenhouse was in the St. Paul campus Plant Growth Facilities (University of Minnesota, St. Paul, MN). Fertigation water was applied twice daily, between 0700-0800 HR and 1600-1700 HR, using a constant liquid feed (CLF) of 125 ppm N supplied from a water-soluble 20N–4.4P–16.6K fertilizer (Scotts, Marysville, OH). Monthly rotational fungicide drenches were administered.

Old Valdez-1 was the first *C. arcticum* population collected, located off McKinley Street (n=34 plants; Table 1). Old Valdez-2 population was across the road (SW) from the Old Valdez-1 site, in a triangular piece of land, next to the shoreline near the old pier (n=18; Table 1). The Old Valdez-3 site is due west of the Old Valdez-1 site (n=23; Table 1). Old Valdez-4 (n=15; Table 1) is ~4.023 km from the Old Valdez-1 site and geographically isolated from Old Valdez-1 to -3 populations. All Old Valdez sites consist of coastal salt marsh tidal flats (20m-30m from the Bering Sea) with constant salt winds, specifically the Wet Graminoid Herbaceous plant community or Halophytic grass wet meadow (Code IIIA3h) (Nolan & Ross, 2006). Species dominating the plant community are *Triglochin maritimum* L.*, Plantago maritima* L.*, Elymus arenarius* L.*,* and *Puccinellia nutkaensis* J. Presl (Nolan & Ross, 2006), although in addition *Potentilla palustris* L. (Old Valdez-1 & 2) and *Achillea millefolium* L. (Old Valdez-3) also predominated. Soil samples were collected from each site, when

possible, for full profile testing (Spurway) at the University of Minnesota Soil Testing Laboratory for greenhouse, florist and nursery samples (http://soiltest.cfans.umn.edu/testing-services/greenhouse-florist-and-nursery-tests). The Old Valdez sites (Fig. 3c) were punctuated with solitary Arctic daisy flowers from most plants rising above *Elymus arenarius* L. The Old Valdez-4 *C. arcticum* population collection site was a depressed marshy area, dominated by *Geranium erianthum* DC. and *Achillea millefolium* species. This population had sympatric *Leucanthemum vulgare* L. located 0.4 km away, although it is unknown whether the two species are cross-compatible.

The Anchor Point *Chrysanthemum arcticum* collection site was at the State Recreational Area, off Old Sterling Highway (#1) in Anchor Point, AK (n=40; Table 1), a distance of 30.09 km from Ninilchik and 30.57 km from Kenai. The plant community is a lll.A.3.i. Halophytic Sedge Wet Meadow (Viereck et al., 1992). Dense, nearly monotypic swards of coarse sedges growing ~1 m in height (particularly *Carex lyngbyaei* Hornem.) near the seaward coastal side, (~25-45 m from the ocean edge. This is a successional community since it was not entirely monotypic sedges. *Elymus arenarius* was also growing along with *Chrysanthemum arcticum.* The Anchor Point-1 population is located in standing saltwater on the inland side of the parking area of the State Recreational Area. Plants were growing on hummocks and hip waders were necessary during plant collection.

The Ninilchik site (Ninilchik-1, n=13; Table 1) of *Chrysanthemum arcticum* is located at the Deep Creek State Recreational Area, Ninilchik, AK. in a sedge

meadow and salty marsh area. It is also a lll.A.3.i. Halophytic Sedge Wet Meadow plant community (Viereck et al., 1992), dominated by *Carex lyngbyaei* as reported for the Anchor Point-1 site. Ninilchik is located 61.76 km from the Kenai sites.

The three Kenai populations of *Chrysanthemum arcticum* collected (Kenai-1, -2, -3) are located along the Kenai Spur Highway north of Soldotna, AK in close proximity (sympatric) near the mouth of the Kenai River at the Cook Inlet. At these locations, the Kenai River is known as the "Lower River", due to a gentle current. Both the Kenai-1 (n=32; Table 1) and Kenai-2 (n=22; Table 1) collection sites were along the east side of the Kenai River and south of the bridge on the Kenai Spur Highway (Fig. 3b). The Kenai-3 site (n=25; Table 1) was approximately 2.012 km east of the Kenai-1 and Kenai-2 sites with a similar habitat. At all three locations, the river is influenced by the changing tides, resulting in an admixture of fresh and salt waters permeating the sedge meadows and marshes. All three sites were also lll.A.3.i. Halophytic Sedge Wet Meadow plant communities (Viereck et al., 1992), dominated by *Carex lyngbyaei*. Numerous caribou (*Rangifer tarandus granti* from the Kenai lowlands herd; https://www.adfg.alaska.gov/index.cfm?adfg=caribou.main) bedding sites were observed adjacent to or on top of *C. arcticum* plants growing on hummocks or semi-protected areas.

Attu Island is the western-most Aleutian Island of North America (Heusser, 1990; Talbot & Talbot, 1994) frequently classified in the Arctic (Hultén, 1960) or Hypoarctic zones (Yurtsev, 1994). Attu Island's climate is cool (3.8°C mean

annual temperature) with 90% of the days having measurable precipitation (average rainfall=1,372mm/yr) (Leslie, 1989). Classically, two types of vegetation predominate throughout the Aleutian Islands (all are treeless): meadows (in valleys, hollows among hills, plateaus or ridges) and heaths (found on more exposed locations) (Hultén, 1960). Individuals were collected in twenty-one populations on the eastern end of Attu island (Fig. 4). Attu-1 to Attu-7 populations were located along the eastern shoreline of Massacre Bay; the fourteen sites of Attu-8 to Attu -21 were located along the western shoreline of Massacre Bay, known as Casco Cove or further inland (Fig. 4). Attu-1, -2 and -3 sites were sympatric (<50 m apart) on adjacent cliffs located below stable dune ridges of the upper foredunes, growing at the cliff edges overhanging the ocean, alongside of *Antennaria monocephala* DC., *Saxifraga canadensis* Mill., *Elymus mollis* Pilg., and *Fritillaria camschatcensis* (L.) Ker-Gawl. This plant community is described as physiognomic Group II, a beach meadow, in relevé group (or community type) 4, namely *Elymus mollis - Senecio pseudo-arnica* Less. (Talbot & Talbot, 1994). The Attu-4 population was growing at the end of the U.S. military aircraft runway of Marston matting (constructed during WWII) (Mathis, 1943) on outcroppings on the hill growing through *Sphagnum* mosses along with *Lupinus nootkatensis* Donn ex Sims. This plant community is classified as Group III, a meadow on a colluvial slope (somewhat setback from the effects of the ocean), relevé group 5, namely *Lathyrus maritimus* Willd.-*Elymus mollis* (Talbot & Talbot, 1994). Plants growing in and adjacent to the Attu-4 population included *Elymus mollis, Viola langsdorffii* Fisch. Ex Gingins, *Achillea millefolium* L., and *Antennaria*

monocephala (DC.) Greene. Attu-5 to -9 populations were growing on cliff edges or in meadows in the same plant communities as Attu-1 to -3. The Attu-8 and -9 sites also had *Geranium erianthum* and *Veratrum album* L. growing in their plant communities. The soil was sparse and most rhizomes were hanging dry in the air with the shoot tips exposed. The Attu-10 site was on a heath-mountain ridge above the cliff line; Attu-11 population was approximately 100 m up the ridge from Attu-10, growing in the thick *Sphagnum* moss. Both Attu-10 and -11 populations were growing in a high meadow cliff edge community, classified as Group III, a sloped meadow, relevé group 3, namely *Artemisia tilesii-Veratrum album* (Talbot & Talbot, 1994). Attu-12, -13 and -14 populations grew on rocky cliffs whereas Attu-15 and -16 sites were top of sharp peaked cliffs at a distance between two sites of ~350 m. Attu-17 site was seaside by roadway on small rocks. Attu-18 site was in sight of Attu-13 and -14 locations, the sparse *C. arcticum* subsp. *arcticum* population was growing with *Sorbus sambucifolia* (Cham. & Schltdl.) M. Roem. and *Rhododendron kamtchaticum* Pall., classified as Group IV, relevé group 1, namely the *Vaccinium uliginosum* L.-*Empetrum nigrum* L. community (Talbot & Talbot, 1994). The Attu-19 site was in grassy, moss-covered hills whereas the Attu-20 population was along a roadside, growing in *Sphagnum* mosses, lichens, *Elymus mollis*, *Iris setosa* Pall. ex Link and *Geranium erianthum*. Attu-21 was an isolated plant, growing in a wetland, in standing water. Populations Attu-19 to -21 were all in the same plant community as Attu-4.

Plant material sampling— In 2017 and 2018, rhizome samples of 225 individuals of *Chrysanthemum arcticum* were collected from the nine populations and 326 individuals of *Chrysanthemum arcticum* subsp. *arcticum* were collected from the twenty-one populations (Figs. 2-3; Table 1). Leaves from these samples were used in the molecular analysis. An outgroup of three chrysanthemum clonal individuals, listed as *C. arcticum* 'JH-173-82' (PI 479351; collected by Fred Meyer, 1982, Kujiranohama, Hamanaka-machi, Akkeshi-gun, Hokkaidô, Japan; GPS coordinates: 42º 58"59' N lat. or 42.98333333 N lat., 145º 0" 0' E long. or 145.00000000 E long.), 'Roseum' (PI 502259; from H. Klose, 1985, Stauden Gaertnerei, Germany) and 'Schwefelglanz' (PI 502260; from H. Klose, 1985, Stauden Gaertnerei, Germany), were obtained from the U.S. Department of Agriculture Germplasm Resources Information Network (USDA-GRIN), and were used for wide genotypic comparisons. Since they were the first extant specimens obtained prior to plant collections, we also did multiple extractions to serve as biological replications to determine the variation among identical samples (clones). This is an additional comparison with the DArTseqLD technical replication error rate (inconsistency among replications) which is ~1% (Kilian, DArTseq, 2020, personal communication).

DNA extraction and DArTseqLD genotyping— All samples were young and healthy leaves which were stored in small coin envelopes (#3 Coin Envelopes, UNV 35301, Essendant Co., IL) at -80°C. Genomic DNA was extracted using the DNA Extraction Kit (96 well-plate SYNERGY™ Plant DNA Extraction Kit, SYNP 02-96-03, Lebanon, NJ). The weight of 25mg/leaf for each

sample was used for DNA extraction. A few modifications were made to the original protocol (https://opsdiagnostics.com/applications/nucleicacids/96wellSynergy-AppNote.html) as described by Noyszewski et al. as follows (2019). During the whole process of DNA purification, samples were kept on dry ice. A 400 µl of Plant Homogenization Buffer was added into each well. DNA was eluted by adding 50 µl of distilled, deionized water (which was autoclaved at 15 minutes for sterilization; steam autoclave (Amsco® Renaissance Remanufactured 3013 Prevac Steam Sterilizer, STERIS, Mentor, OH) and stored at room temperature. To optimize and utilize the DNA samples prior to SNP genotyping, the following procedures were used to determine the quality and quantity of DNA. First, Nanodrop 2000c (NanoDrop 2000 Spectrophotometer, Thermo Scientific™, Wilmington, DE) was used to analyze ultraviolet (UV) to quantify DNA and protein content, based on spectral wavelengths (Appendix 1, standard operating procedure or SOP for Operation of NanoDrop 2000 Spectrophotometer). The goal was to achieve a minimum concentration of 20 $ng \cdot \mu L^{-1}$ to optimize genotyping. Unqualified samples (DNA concentration <20 $ng \cdot \mu L^{-1}$) were re-extracted. Samples of acceptable quality/quantity of DNA were sent to Diversity Arrays Technology Pty Ltd., Yarralumla, Australia, for DArTseqLD™ genotyping (http://www. diversityarrays.com/dart-application-dartseq) using restriction enzyme combinations. In the case of *Chrysanthemum arcticum*, the enzyme combination was PstI-AseI. DArTseqLD generates two types of data: SilicoDArTs

(presence/absence markers) and SNPs (single nucleotide polymorphism) in fragments.

Genetic diversity and population structure statistical analyses— Three taxonomic groupings were established for statistical analysis in our study: Group one: species, subspecies; Group two: *C. arcticum;* Group three: *C. arcticum* subsp. *arcticum*. This allowed for analysis of species interrelationships, based on SNP data analyzed for genetic diversity (Principal Component Analysis, PCoA) and genetic STRUCTURE using R studio (Version 1.2.5033) and GenAlEx 6.5 (Peakall & Smouse, 2012) using Microsoft Excel.

The analytical package *dartR* in R studio was applied to analyze SNP data sent back from DArTseqLD™ genotyping and to enhance output diagnostics (Gruber et al., 2017). We prepared two sets of metafiles (using the original data file, *SNP_singlerow.csv*) and analyzed the database for each of the three Groups (one, two, three; see above). The Genlight Object file was created by assigning the original single row file with metadata (*pop, IndNames, Coord*); initially, in which *pop* metric was provided as the Alaska mainland (*C. arcticum*) and Attu Island (*C. arcticum* subsp. *arcticum*) for Group one analysis, followed by Groups two and three analyses. For Group two, the *pop* metric assigned was for all nine *C. arcticum* populations sites (Old Valdez-1, -2, -3 and -4; Anchor Point-; Kenai-1, -2, and -3; Ninilchik). In Group three, new metafiles were reassigned in *pop* metric into 21 collection sites belonging to Attu Island: Attu-1 to -21.

The percentage of missing SNP data in both *C. arcticum* and *C. arcticum* subsp. *arcticum* populations were calculated and presented as the *call rate* (the

proportion with non-missing scores). For the population STRUCTURE analysis, the loci and individuals with significant missing data need to be discarded. Thus, the call rate for each locus and individual below a specified threshold would be removed to make sure only high-quality loci (with few missing SNP data) and with a consistent quality are retained (Gan et al., 2018). We applied the following selection criteria in both the original and new Groups two and three metafiles: SNP markers with ≥95% non-missing data were selected. Subsequently, we examined the graphic plot of missing SNP data for all individuals to determine the mean threshold of SNP missing data wherein 78% of the individuals with non-missing data would remain.

The genetic similarity of individuals within the populations were visualized using a Principal Coordinates Analysis (PCoA) (Gruber et al., 2017). Packages of ADE4 (Dray & Dufour, 2007) and ADEGENET (Jombart, 2011) were pre-loaded for PCoA in *dartR*. The individuals were plotted in 2-D space defined by loci (attributes) with the position along each locus axis determined by individual (0 for homozygous reference SNP, 2 for homozygous alternate SNP, and 1 for the heterozygous state). In the PCoA plot, the PCoA-1 axis represents the most variation; PCoA-2 axis is orthogonal to PCoA-1 axis and indicates the most residual variation. Individuals from the same population will be formed as clusters by 95% inertia ellipses (default parameter in package adegenet and ade4), aiming to present the relationship between different subpopulations within the group. This separation will be compared and confirmed by other genetic population analysis.

Genetic STRUCTURE of the populations was evaluated by the Bayesian clustering method implemented with STRUCTURE v.2.3.4. individuals were assigned into a K number of clusters using the Admixture Ancestry Model with independent allele frequencies (Sun et al., 2018). For each Group, the program was set at a Length of Burnin Period of 100,000, with 100,000 Markov chain Monte Carlo (MCMC) replications after Burn-in. The STRUCTURE analysis simulated three replications with the values of subgroups (K) from 1 to 40. The estimated likelihood value of data [lnP(D)] and the ad hoc statistics DeltaK (ΔK) from the STRUCTURE output was plotted as x- and y- axes in the Microsoft Excel (Salem & Sallam, 2016). According to Evanno et al. (Evanno et al., 2005), the maximized log value of the plot or peak(s) illustrated the best K value.

Discriminant Analysis of Principal Components (DAPC) is a relatively new multivariate method for the analysis of the genetic structure of populations, which combines a PCA (Principal Component Analysis) with a DA (Discriminant Analysis) (Grewe et al., 2018; Jombart & Collins, 2015). This approach can be used to estimate the number of clusters of individuals using the Bayesian Information Criterion (BIC), which help to unravel possibly complex structures existing among clusters, while being several orders of magnitude faster than existing Bayesian clustering methods. The first two principal components of the DAPC were plotted to evaluate relationships among clusters (Jombart et al., 2010). Since evaluating solutions for 100 principal components (default retained PCs) is not useful and computer-intensive, we used the function optim.α.score to

approximate the optimal number of PCs to retain for three groups analyses specifically, as Group one, Group two and Group three.

Analysis of Molecular Variance (AMOVA) — The partitioning of variation at different levels in species and subspecies was calculated by the Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992)) in GenAlEx equipped in Microsoft Excel (Peakall & Smouse, 2012). The input data set parallel Groups one, two and three. In Group three, the Attu-21 population which consisted of one individual was eliminated. For Group two, we applied two AMOVA analyses based on genetic and geographic perspectives: one for two genetic clusters (k=2 from STRUCTURE 2.3.4) and nine populations; one set for three geographical regions (collection sites) and nine populations (Table 4). For Group three, similar pre-set input data, we applied three AMOVA analyses: one for two genetic clusters (k=2 from STRUCTURE 2.3.4) and 20 populations, set as hierarchical analysis for two geographical regions and 20 populations; global analysis for 20 populations (Hou & Lou, 2011). With 999 permutations for both the total data option and pairwise analysis, the alleles were conducted in GenAlEx to estimate genetic variation and the percentage of polymorphism (%P) among regions, among and within populations. For Φ (Phi), in the H_o hypothesis: There is no genetic difference among groups or regions ($\Phi_{RT}=0$), genetic differences within groups or regions and among populations ($\Phi_{PR}=0$), or among individuals ($\Phi_{PT}=0$) within populations.

Unweighted Pair Group Method (UPGMA) — The SNP markers for *Chrysanthemum arcticum* and subspecies *arcticum* were used to calculate the

genetic diversity by using the Jaccard similarity index and transformed into distance matrix by the function gl.pop.dist(gl, method=”jaccard”), wrapped in dartR package in R studio (Baloch et al., 2017). Pairwise genetic distances among the populations were obtained and input in MEGA X (Stecher et al., 2020) to construct dendrograms by the unweighted pair group method (UPGMA) (Nei, 1972) with 1,000 bootstrap replicates (Kumar et al., 2018).

Results

Molecular markers and genotyping

After filtering by DArTseqLD, a total of 7,443 polymorphic DArTseqLD SNP markers were generated from genotyping the nine *C. arcticum* and 21 *C.a.* subsp. *arcticum* populations. The call rate of SNP markers, i.e. the percentage of missing SNP data in both *C. arcticum* and *C. arcticum* subsp. *arcticum* populations ranged from 0.2 to 1.0 with a mean of 0.82. Missing SNP data for all individuals included in this study were graphically plotted and a mean threshold (1139.73 missing SNPs/individual; Fig. 5) was implemented, resulting in the selection of 78% of the individuals with >95% SNPs or non-missing data. After filtering with this threshold selection criterion, there remained 211 individuals and 1,168 loci in Group two for *C. arcticum* and 313 individuals with 1,752 loci in Group three for *C. a.* subsp. *arcticum*. Collectively, all samples were categorized in Group one, with 1,939 SNP markers.

The outgroup of three *C. arcticum* clonal individuals, 'JH-173-82', 'Roseum', and 'Schwefelglanz' from the USDA-GRIN system, were grown from cuttings (clones). Though 'JH-173-82', 'Roseum', and 'Schwefelglanz' were collected from fresh leaf samples (similar to the wild populations), the number of missing SNPs among DNA extraction replications varied significantly higher initially in the first extractions than in the latter (Table 4). Thus, the insufficient numbers of usable SNPS below the threshold led these samples to all being filtered out in the analyses and the distinguished missing SNPs from fresh samples brought their taxonomic identification into question. The outgroup of

three clonal individuals were extracted in two separate replications from the exact same leaf samples and genotyped repeatedly, revealing an error rate of DArTseqLD of 2%, 2%, and 7% for 'JH-173-82', 'Roseum', and 'Schwefelglanz', respectively (Table 4). Thus, if clones were present in any of the populations sampled, they could have <2-7% SNP variation. GenALEX results showed no evidence of matching multilocus individuals among *C. arcticum* and *C. a.* subsp. *arcticum* populations, so no clones were apparent. Phenotypic grow outs and taxonomic research on these three accessions showed that both 'Roseum' and 'Schwefelglanz' were not *C. arcticum* but were, instead, *C. zawadskii* Herbich (Anderson N. O., 2019, unpublished data). Thus, only 'JH-173-82' could be classified as *C. arcticum* whilst the other two individuals would be a more distantly related species.

Genetic diversity

Cluster analysis. For PCoA analysis, R studio automatically eliminated four populations of *C. arcticum* subsp. *arcticum* from Attu Island, specifically Attu-2, Attu-15, Attu-17 and Attu-21, since all of these populations had n<3 individuals/population. However, for all subsequent genetic cluster analyses it should be noted that, with one exception, these populations were not excluded (DAPC, STRUCTURE, AMOVA). The only exception was Attu-21 PCoA with n=1 individual; this was excluded from AMOVAs. Analysis for Group one revealed that there were two distinct clusters (Groups two and three) that completely separated the two species with one of the clusters (*C. arcticum*) having two

separate groupings (Fig. 6). Group two contained 211 *C. arcticum* individuals from the Alaska mainland; Group three contained 314 *C. a.* subsp. *arcticum* individuals from Attu Island. The first coordinate (Fig. 6) described a high amount (42.3%) of the total variation of the SNP data (Group one), specifically the species (*C. arcticum*) and subspecies (*C. a.* subsp. *arcticum*); the second coordinate described 4.9% of the total variation from the subpopulation in Group two (*C. arcticum*) from the Alaska mainland.

The PCoA analyses of the Alaska mainland and Attu Island populations showed that the individuals with close geographic distance tended to cluster closely (Fig. 7 & 8) with overlapping distributions. *C. arcticum* populations from Group two (Fig. 7) had the first coordinate with 30.7% while the second coordinate had 13.4% of the total variation, and the populations were distinguishable as three groups. Group A contained four populations from the Old Valdez sites (Old Valdez-1, -2, -3, -4); Group B contained three populations collected from the Kenai sites (Kenai -1, -2, -3); Group C contained the other two populations from Anchor Point (Anchor Point -1) and Ninilchik (Ninilchik -1) (Fig. 7).

In contrast, PCoA analysis of the Attu Island population showed two distinctly separate groups (i, ii; Fig. 8). However, Group ii was geographically more widely dispersed than Group i. The PCoA analysis showed the first coordinate with 6.5 % and the second coordinate with 3.6% of the total variation (Fig. 8).

Discriminant Analysis of Principal Components (DAPC). Consistent with the PCoA results, the DAPC analysis for Group one was clearly separated as two subgroups in distant genomic space with no admixture: the Alaska mainland and Attu Island subgroups (Fig. 9). However, the Attu Island subgroup occurred as two relatively broad and flat curves with a narrow space between them. In the DAPC analysis of Group two, 15 principal components (PC) (Fig. 10) and three discriminant functions were retained, which revealed four clusters (Anchor Point-1 and Ninilchik population were separated in DAPC) within the species instead of three clusters in the PCoA analysis (Fig. 7). For Group three, the DAPC analysis (12 PCs and four DA retained) of the first two principal components distinguished the two main groups (Groups i, ii) with numerous clusters in Group ii (Fig. 11). These differences in Group ii for the PCoA analysis are less definitive (Fig. 8). Group i (Attu-1, -3 populations) showed the most distinct separation from Group ii, which matched the PCoA analysis (Fig. 8). Group ii (Attu-4 to -14, -16, -18 to -20) contained the most populations and individuals (Fig. 11) and in only one instance were geographically sympatric (adjacent) populations clustered strictly together (populations Attu-8 and -9). The Attu-4 population also formed a distinct cluster from the other populations in Group ii.

Genetic structure

For Group one, STRUCTURE 2.3.4 calculated that the best $\Delta K = 2$ (Fig. 12), which matches the distinct taxonomic differentiation between the main species (*C. a.*) and subspecies (*C. a.* subsp. *arcticum*). STRUCTURE analysis of

Group two also resulted Δ K = 2 (Fig. 13). These two subgroups correspond to the Old Valdez populations collectively and other the populations on mainland Alaska, again corresponding to the geographic separation of the collection sites. However, for STRUCTURE analysis of Group three, while two subgroups had an optimal estimation ofΔK =2 (Fig. 14A), the difference among populations was less definitive. Using the q-matrix obtained by STRUCTURE, we performed an ANOVA analysis for Group three, defined these into Group-1 Group-1(a) (Attu-1, -2, -3), Group-1(b) (Attu-4 to -7, -10 to -12, -15 to -21), Group-2(a) (Attu-8, -9), and Group-2(b) (Attu-13, -14) (Fig. 14). Admixture of individuals appeared in the population Attu-4 to Attu-7 and Attu-10 to Attu-21. Inconsistent with the PCoA analysis and DAPC analysis, the groups divided in the STRUCTURE analysis did not completely correspond to the geographic distribution.

Analysis of Molecular Variance (AMOVA).

The AMOVA for Δ K = 2 or two genetic clusters from STRUCTURE in Group two (*C. arcticum*) for each of the partitioned molecular variations (among groups, among populations within groups, and within populations) were all very highly significant different ($p \leq 0.001$, Table 2 & 3). Among group variation accounted for 35%, among populations within groups had 13%, while within populations was the largest accounting for 52% of the genetic variation (Table 2). However, when the populations were hierarchically assigned into clusters of the three geographical regions (Old Valdez; Kenai; Anchor Point and Ninilchik), AMOVA indicated that there was greater genetic variation among regions (40%)

and significantly less genetic variation among populations within the regions (5%) than within populations (55%; Table 2). Thus, the groups divided by geographic distribution may provide a more effective foundation to analyze the relationship among populations within the regions and individuals within the populations. The high global Φ_{PT} = 0.453 ($p \leq 0.001$) within populations indicated significant genetic variability. Global AMOVA (all nine populations) showed 37% among populations and 61% within populations and a high Φ_{PT} = 0.389 ($p \leq 0.001$; Table 2). With the aim to verify the specific genetic diversity among populations within the Old Valdez and Kenai collection sites, additional AMOVAs were performed. AMOVA showed only 1% of the diversity was distributed among populations for both Old Valdez and Kenai sites (Table 2), which indicated that 99% of the genetic variation occurred within populations at Φ_{PT} = 0.001 ($p \leq 0.001$) and Φ_{PT} = 0.015 ($p \leq 0.001$; Table 2) for Kenai and Old Valdez populations, respectively.

For the 313 individuals of *C. arcticum* subsp. *arcticum* collected from Attu Island, the SNP dataset of this subspecies Δ K = 2 (two genetic clusters) showed significantly higher levels of genetic variation within populations (82%; Table 3) than among groups (5%) and among populations within groups (14%). As noted earlier, one Attu Island population (No. 21) was dropped due to its small population size of one founding individual. It should also be noted that these populations were in significantly closer geographic proximity than the majority of *C. arcticum* populations (our minimum population distance was 100m), with many *C. a.* subsp. *arcticum* populations being sympatric and capable of gene exchange (Fig. 4). A similarly high within population level (81%) was found for

the hierarchical two geographic regions group and the exact same level (14%) for among populations within regions (Table 3). Slightly higher within population variation (84%; Table 3) occurred in the global - 20 sites and eastern shoreline of Massacre Bay group while 86% within population variation was found in the Western shoreline of Massacre Bay (Cosco Cove). Moreover, the relatively low global Φ_{PT} = 0.185 ($p \leq 0.001$; Table 3) for *C. a.* subsp. *arcticum* versus *C. arcticum* (Φ_{PT}= 0.453; Table 2) was found.

Unweighted Pair Group Method (UPGMA).

The phylogenetic tree with genetic distances among *C. arcticum* populations from mainland Alaska showed two distinct groupings of the four Old Valdez, Alaska populations separating from all others at genetic distance >0.2 (Fig. 15), similar to the PCoA analysis (Fig. 7). Geographic distances explained much of the genetic distances with sympatric populations, e.g. Old Valdez 1 and 2, Kenai 1 and 2, being extremely close (genetic distance = 0.05; Fig. 15). Anchor Point and Ninilchik populations had a genetic distance = 0.17 (Fig. 15), significantly greater than either set of sympatric populations from different locations. In contrast, the phylogenetic tree of 21 *C. a.* subsp. *arcticum* populations from Attu Island, Alaska, while in closer proximity than among the major locations of *C. arcticum* populations, were not all sympatric (Fig. 16). For example, Attu-1 and -2 populations and -8 and -9 populations were each sympatric (<100 m apart from each other, respectively; Fig. 4) on separate branches of the tree (Fig. 16). Geographic distance did not always correlate with

genetic distance as Attu-4, positioned in between Attu-5 and Attu-6 populations (Fig. 4) and >1,000 m away from Attu-5, yet these two populations were on separate branches whilst Attu-6 and -7 populations were more closely aligned with Attu-5 than Attu-4 (Fig. 16). Attu-21 was distinct from all other Attu populations although this was most likely due to its small population size of one founding individual.

Discussion

The DArTseqLD GBS approach (Kilian et al., 2012, 2014) was used for examining genetic diversity and genetic structure within and among species and populations of *C. arcticum* and *C. a.* subsp. *arcticum*. This method was applied in genetic diversity analyses because of its efficient combination of marker discovery and genotyping in low cost of genome-wide scans and generating a larger number of genome-wide SNP data without prior SNP markers discovery for non-model plants (Dossa et al., 2016; Nguyen & Lim, 2019). In our research, 7,334 SNP markers of extant populations of *C. arcticum* and *C. a.* subsp. *arcticum* individuals had a mean call rate of 0.82 (Fig. 5), which indicated that high quality SNP markers could be generated which exhibited high levels of polymorphism (Gan et al., 2018; Noyszewski et al., 2019). After filterings, a total of 524 individuals and 1,939 SNP markers were determined to be highly informative for genetic structure analyses (Figs 12-14). Comparative studies using DArTseq (after filtering) have reported as few as 8,514 SNPs in pea, *Pisum fulvum* Sm. (Barilli et al., 2018), 9,300 SNPs in *Aegilops speltoides* Tausch. to 20,288 SNPs in *A. tauschiii* Coss. (Edet et al., 2018), 30,000 SNPs in *Citrus sinensis* (L.) Osbeck mapping populations (Curtolo et al., 2017), to 49,911 SNPs in corn, *Zea mays* L. (Tomkowiak et al., 2019). Other well-studied species in the Asteraceae have similar or larger numbers of SNPs than we found for *C. arcticum* and *C.a.* subsp. *arcticum*, depending on low vs. high density genotyping by sequencing (GBS), including domesticated sunflower, *Helianthus annuus* L.*,* with a set of 20,502 SNP markers (Bachlava et al., 2012; Livaja et al., 2016); a

vernal pool species, *Lasthenia fremontii* (Torr. ex Gray) Greene had 3,918 candidate SNPs (Torres-Martínez, 2016); 8,470 SNPs were found in goldenrod, *Solidago* spp. (Beck & Semple, 2015); globe artichoke (*Cynara cardunculus* var. *scolymus*) had ~34,000 SNPs (Scaglione et al., 2012), whereas 93,558 SNP markers were found in guayule, *Parthenium argentatum* A. Gray (Ilut et al., 2015).

As would be expected, the number of SNPs detected in extant *C. arcticum* and *C. a.* subsp. *arcticum* using DArTseqLD are lower than other chrysanthemum studies using high density (HD) GBS where 480,592 SNPs were found in 199 cultivated chrysanthemum individuals (Chong et al., 2017) and 183,130 SNPs in a hexaploid chrysanthemum species panel (van Geest et al., 2017). Cultivated chrysanthemum (*C.* x*grandiflorum, C.* x*hybridum*), as an outcrossing hexaploid (2*n* = 6*x* = 54), has a large genome, and is a segmental allopolyploid (Klie et al., 2014; Nguyen & Lim, 2019). In the classic diploid chrysanthemum model plant, *C. seticuspe,* assembled sequences covering 89.0% of the 3.06 Gb genome had 27,855 SNPs identified from six cultivars (Hirakawa et al., 2019). The differences observed in this study with other chrysanthemums are most likely due to the use of low density DArTseq since high density (HD) generated for genome sequencing produces >100,000 SNPs (Kilian et al., 2014). Likewise, *C. arcticum* most likely has a smaller genome size than *C.* x*grandiflorum, C.* x*hybridum* since it is reportedly a diploid (2*n*=2*x*=18) (Nishikawa & Kobayashi, 1989), although specimens from eastern Russia were potentially octoploid (2*n* = 8*x* = 72?) (Tolmatchew, 1987). If *C. arcticum* is diploid

then a reduced number of SNPs compared with the cultivated allohexaploid species is reasonable and its genome size may or may not be similar to *C. seticuspe* (although both this and ploidy need to be determined for the *C. arcticum* species complex across all of the sampled populations). Potentially, future generation of DArTseqHD SNPs may match those of *C. seticuspe.* Given the wide range of distribution along with its perenniality, the ploidy range may surpass the reported diploid level (Nishikawa & Kobayashi, 1989). *Chrysanthemum arcticum* and *C. a.* subsp. *arcticum* also may not possess high levels of heterozygosity since, as a diploid species, it may parallel other diploid *Chrysanthemum* in being self-compatible (*C. seticuspe, C. coronarium, C. segetum, C. carinatum*), rather than outcrossing and self-incompatible *C.* x*grandiflorum* and *C.* x*hybridum* (Zagorski et al., 1983). Future research will be devoted to these questions.

Heterozygosity in cultivated chrysanthemum germplasm may challenge the use of genome information as allelic expression bias is specifically common in allopolyploids (Wu et al., 2018) and high heterozygosity may result in false polymorphism calls if the gene homologues are assembled together in one contig, otherwise failing to detect polymorphisms if alleles are assembled into different contigs (Shahin et al., 2012). If wild *C. arcticum, C. a.* subsp. *arcticum,* as well as *C. a.* subsp. *polaré* populations are, indeed, diploid, this would provide a more straightforward informative SNP marker pool for genetic structure analyses. Additionally, it may be worthwhile to sequence the genome of this species to compare with the sequenced genome of *C. setiscuspe* (Hirakawa et

al., 2019). Meanwhile, as an important genetic resource, the germplasm of wild *C. arcticum, C. a.* subsp. *arcticum* and *C. a.* subsp. *polaré* could be used to utilize and expand the chrysanthemum genomic analyses to utilize marker assisted selection of traits, such as salt tolerance, from the *C. arcticum* species complex. Future research will be devoted to these questions.

The genus *Chrysanthemum* consists of three sections: *Chrysanthemum*, *Ajania* and *Arctanthemum* with flavonoids from the leaves in the section *Arctanthemum* distinguishable from the other sections (Uehara et al., 2017; Zhao et al., 2010). *Chrysanthemum arcticum* and *C. a.* subsp. *arcticum* are from the section *Arctanthemum* (Hultén, 1960, 1968). Morphological and phenotypic distinctions have been considered insufficient to classify many species in the *Chrysanthemum* genus. As for *C. arcticum* and *C. arcticum* subsp. *arcticum*, the latter species had been considered to be synonymous (Kitamura, 1940) and then treated as two distinct species in different genera respectively, *Dendranthema arcticum* and *Arctanthemum arcticum* (Bremer & Humphries, 1993; Tzvelv, 1985; Uehara et al., 2017). Based on previous phylogenetic analysis, *C. arcticum* was closely aligned in the *Chrysanthemum-Ajania* complex. Previous studies tended to focus on the major taxonomic classification on the whole genus *Chrysanthemum* and related cultivars from Asia, while lacking records on section *Arctanthemum* mostly from E. Asia and N. America. As a reference, our study provides genetic data of *Chrysanthemum arcticum* and *C. arcticum* subsp. *arcticum* from section *Arctanthemum* which confirms their taxonomic separation and differentiation.

In the present study, we integrated conventional genetic structure analysis techniques for 1,939 SNP markers for 524 individuals (Group one) to confirm taxonomic classifications, determine genetic differences and potential traits for breeding purposes among *C. arcticum* and *C. a.* subsp. *arcticum*. It was found in the genetic cluster analyses of Group A for both PCoA (Fig. 6), STRUCTURE (Fig. 12) and DAPC results (Fig. 10), *C. arcticum* and *C. a.* subsp. *arcticum* were significantly and distinctly separated as taxa. Likewise, the PCoA analysis of Group A showed very high levels of genetic variation (47.2%) accounted for by the first two coordinates (42.3% and 4.9% for PCoA1 and PCoA2, respectively; Fig. 6). This genetic distinction agrees with the taxonomic classifications and is significant, particularly given the overlap in distribution of these two species. Based on our extensive extant population datasets, the differences in genetic variation among *C. arcticum* and *C. a.* subsp. *arcticum* may be increased due to the decreasing gene migration caused by the restrictive geographical isolation imposed by the rugged Alaskan terrain. Future research will examine whether the diagnostic morphological traits separating these two species, in coordination with SNP markers, are both corollary taxonomic (morphological) and genetic distinguishing characteristics.

Genetic variance within and among populations, and gene migration between populations or subpopulations are important parameters to provide understanding of population dynamics (Hartl, 2007). The different approaches used to analyze the genetic structure of the wild population collections in this study provided complementary information from comprehensive perspectives.

Our genetic cluster analyses showed a good consistency between the PCoA (Fig. 7) and DAPC (Fig. 10) results on clustering *C. arcticum* individuals from four major collection sites on the Alaska mainland, Old Valdez, Kenai, Anchor Point and Ninilchik, consistent with the regional distribution of the collections, suggesting that geographical isolation might be the major reason leading to the genetic variation among populations within *C. arcticum*. The phylogenetic tree (Fig. 15) also reconfirmed these clusterings. Nonetheless, in the DAPC analysis the reduction of genetic information to interindividual distance caused a more detailed clustering separation between the Anchor Point and Ninilchik populations compared to PCoA analysis. However, the STRUCTURE analysis (Fig. 13) produced an alternative result, presenting two subgroups of *C. arcticum* (Fig. 13b; Old Valdez populations and other populations), which indicated the Old Valdez populations separated from other populations significantly. Overall, in agreement with the geographical distribution, the Old Valdez population was located the furthest away from all other populations and presented the majority of genetic variation within *C. arcticum* populations correspondingly. Meanwhile, more closely located populations tend to be more genetically similar to another (Wright, 1943), which indicated that shared gene flow among the Kenai *C. arcticum* populations (Group ii, Fig. 7), the Anchor Point and Ninilchik populations (Group iii, Fig. 7), and the Old Valdez populations (Group i, Fig. 7). In contrast to *C. a.* subsp. *arcticum* populations which were all in closer proximity, the higher genetic variation among groups and distinct separation among populations is most likely due to their geographic isolation and reproductive isolation due to the

potential existence of self-incompatibility (although this awaits discovery) and lack of pollinators traveling among populations.

According to the different genetic clusters results, we applied two hierarchical AMOVA analyses, two genetic clusters from STRUCTURE (Δ K = 2) and three geographical regions (Table 2). Both of these analyses showed that most of the genetic variation occurred within populations (52% - 58%, respectively, Table 2), which indicated that wild *C. arcticum* populations on the Alaska mainland in this study had high genetic diversity. Genetic diversity among groups was greater for the hierarchical three geographical regions than the two genetic clusters. 40% of the genetic variation was among regions in the hierarchical three geographical regions AMOVA (Table 2). Additionally, the two geographic areas with populations sympatric or nearly so (Kenai and Old Valdez) had the significantly highest levels of genetic variation within populations, i.e. 99% for both the three Kenai (Φ_{PT} =0.011; $p \leq 0.001$) and the four Old Valdez populations (Φ_{PT} =0.015; $p \leq 0.001$; Table 2). Both of these population groupings also had the lowest level of genetic variation among populations (1%; Table 2). Clearly, close geographical proximity (sympatry) among populations allows for gene flow in *C. arcticum*, although specific distances limited gene exchange. For example, the phylogenetic tree had Old Valdez-1 and -2 populations in the same genetic grouping whilst -3 and -4 were successively more distantly related (Fig. 15). As noted earlier, the Old Valdez #1 and #2 populations were across the road from each other, separated by 20 meters (Table 1), and are potentially related enough to potentially be the same population. The Old Valdez-3 site was due west of the

Old Valdez-1 site, separated by ~50 m whereas Old Valdez-4 was ~4.023 km from the Old Valdez-1 site and geographically isolated from Old Valdez-1 to -3 populations. Thus, the distance of ~50 m to ~4.023 km was significantly far enough away to reduce genetic similarity among the populations (Fig. 15). It was observed that bees (possibly *Bombus distinguendus;* Schweitzer, et al., 2012) existed on Attu Island, however *C. arcticum* subsp. *arcticum* were not in flower at the time of collection. The exact insect pollinators of this species and the distances traveled for pollen and nectar rewards, allowing for gene exchange, awaits determination.

Chrysanthemum arcticum. subsp. *arcticum* populations didn't present the same distinct genetic clustering as was found for *C. arcticum,* although both species always had significantly greater within population genetic variance regardless of the AMOVA group runs (Tables 2, 3). As an analog to *C. arcticum*, we expected that *C. a.* subsp. *arcticum* population genetic diversity would be related to the collection sites, based on distribution in the eastern and western shorelines of Massacre Bay, Attu Island (Fig. 4a). However, in contrast with the 35-40% genetic variation among groups found for *C. arcticum* (two genetic clusters Δ K = 2, hierarchical three geographic regions, respectively; Table 2), *C. a.* subsp. *arcticum* populations showed significantly less genetic diversity of 5% (Φ_{RT} =0.045; $p \leq 0.001$; Table 3) and 4% (Φ_{RT} =0.044; $p \leq 0.001$) for AMOVA of two genetic clustersΔ K = 2 and hierarchical three geographic regions, respectively (Table 3). The global - 20 sites group AMOVA among population variation was also higher (15%; Φ_{PT} =0.164; $p \leq 0.001$; Table 3). As confirmed by the global

AMOVA analyses for 20 sites, genetic diversity among *C. a.* subsp. *arcticum* populations (16%; Table 3) were lower than *C. arcticum* (39%; Table 2). Cumulatively, this indicates that the shared gene flow among collection sites might be more frequent and less limited by terrain restrictions since the island is treeless with predominantly meadows (in valleys, hollows among hills, plateaus or ridges) and heaths (found on more exposed locations) (Hultén, 1960) separating any of the populations. No mountains directly descended to either shorelines of Massacre Bay.

For *C. a.* subsp. *arcticum*, STRUCTURE analysis detected global clusters of diversity and resulted in two mixed subgroups (Δ K = 2; Fig. 14a) from 21 populations in the SNPs dataset on the Attu island: Group-1(a) contained three sympatric populations <50 m apart (Attu-1 to Attu-3; Fig. 4c); Group-1(b) had a mixture of sympatric and non-sympatric populations, i.e. sympatric Attu-4 through Attu-7 populations (Fig. 4c), as well non-sympatric Attu-10 through Attu-12 and Attu-15 to Attu-21 populations (Figs 4b, 14b). Group-2(a) was restricted to sympatric but isolated Attu-8 and Attu-9 populations that grew on adjacent shoreline rocky bluffs (Figs 4b, 14b); Group-2(b) included populations Attu-13 and Attu-14 which were also sympatric and restricted to a small peninsula extending into Casco Cove (Fig. 4b). The phylogenetic tree also confirmed these findings (Fig. 16). Oddly enough, however, the sympatric Attu-15 population (Fig. 4b) was not in this group and was significantly genetically distant (Fig. 16). Based on Wright (1943), more closely located populations tend to be more genetically similar to another although Attu-15 provides an example exception. The higher

genetic diversity among individuals (84%, Φ_{PT} =0.164; $p \leq 0.001$; Table 3) in the global - 20 sites AMOVA, presumes an admixture genetic structure among *C. a.* subsp. *arcticum* populations. However, it was astonishing that the Attu-8, -9 and Attu-13, -14 populations from Group-2 in STRUCTURE analysis were inconsistent with the geographic distribution since they were surrounded by other collection sites from Group-1. Either these populations are from distinctly different ancestral genetic sources or have evolved to be significantly different based on their geographic isolation on shoreline bluffs (Attu-8, -9) or peninsular extension into Casco Cove (Attu-13, -14). Equally possible is the evolution of ploidy differences among these populations causing the genetic distinctions since diploid to highly polyploid individuals of *C. arcticum* have been found (Rice et al., 2015).

According to the STRUCTURE q-matrix and bar plot, only Group-1a (Attu-1 to -3) and Group-2a (Attu-8 and -9) were clearly assigned to subgroup-1 and subgroup-2, respectively, as other populations appeared to be admixed derived from both subpopulation-1 or -2. Interestingly, these subgroups were not presented with landscape-level genetic diversity based on sampling locations on Attu island; the same was true for *C. arcticum* on mainland Alaska. In addition, PCoA analyses of *C. a.* subsp. *arcticum* provided an alternative clustering with Group-i (Attu-1 and -3) significantly distinguished from other populations (Fig. 8), most likely due to their sympatry and geographical isolation on the peninsula. For DAPC analysis, *C. a.* subsp. *arcticum* populations showed consistency with PCoA analysis (Fig. 11) excluding Attu-1, -2 and -3 populations from the

remainder. In contrast to PCoA analysis, however, DAPC provided detailed variation and relatedness among populations (Fig. 11) which differed slightly from STRUCTURE: Group-ii subdivided into subgroups including Attu-4 through -7; Attu-10 -12, -15 through -21; Attu-13 to -14 and Attu-8 to -9. Given the various algorithms inherent within each data analysis approach, each method can provide common or unique insights into the genetic variation of both species. We aim to test the potential relationships with future interpopulation crossings to determine the fluidity or restrictive gene flow potential, based on these analyses. Mating system and mating patterns are important factors, since they are related to the effectiveness of populations outcrossing and structuring genetic diversity (Brown, 1989; Hamrick et al., 1979). However, it is limited known of subspecies, likewise, pollinator activity is unknown for the species although during the collection trip only flies were noted visiting the flowers; no bees were observed throughout the eastern end of Attu Island.

Overall, *C. a.* subsp. *arcticum* had its major genetic diversity among individuals within populations (Table 3), indicating the relatively high frequency of shared gene flow on the global - 20 sites populational scale may benefit increasing effective population size and facilitating exchange of alleles. We do not know whether this gene flower within populations is true for other populations of the subspecies on additional Aleutian Islands or not. Other researchers have also studied the subspecies (Nishikawa & Kobayashi, 1989; Ohashi & Yonekura, 2004; Uehara et al., 2017) although our research is the most extensive to date for this subspecies. We aim to expand our collection of *C. a.* subsp. *arcticum*

populations include herbaria sampling across the range for this subspecies as well as *C. arcticum* and *C. a.* subsp. *polaré*, aiming to see the relatedness and difference between extant populations from different continents to compare with historic (herbaria) individuals.

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List of tables

Table 1. Chapter 2 Population site code locations of the nine *Chrysanthemum arcticum* (mainland Alaska) and 21 *Chrysanthemum arcticum* subsp. *arcticum* (Attu Island) populations collected, the number of individuals (N), and global positioning system (GPS) locations (latitude, longitude) for the center of each population.

Population Site Code	Latitude	Longitude	Population size
Chrysanthemum arcticum			
Old Valdez-1	61° 6' 52.1964"N	-146° 16' 0.5550"W	37
Old Valdez-2	61° 6' 52.7070"N	-146° 16' 1.3650"W	18
Old Valdez-3	61° 6' 47.6418"N	-146° 16' 0.7284"W	23
Old Valdez-4	61° 7' 59.7354"N	-146° 17' 42.4062"W	15
Kenai-1	60° 31' 31.4898"N	-151° 12' 34.6782"W	32
Kenai-2	60° 31' 31.6590"N	-151° 12' 35.7006"W	22
Kenai-3	60° 31' 31.6590"N	-151° 12' 44.3880"W	25
Anchor Point-1	59° 46' 25.7484"N	-151° 51' 57.5748"W	40
Ninilchik	60° 1' 45.1128"N	-151° 42' 13.4280"W	13
Total			225
Chrysanthemum arcticum subsp. *arcticum*			
Attu-1	52° 48'41.7780"N	173° 18'2.8440"E	24
Attu-2	52° 48'42.2136"N	173° 18'1.8450"E	2
Attu-3	52° 48'42.2136"N	173° 18'4.0212"E	7
Attu-4	52° 49'45.7176"N	173° 17'25.1916"E	36
Attu-5	52° 49'56.5098"N	173° 18'42.3984"E	6
Attu-6	52° 50'22.7256"N	173° 15'44.0856"E	5
Attu-7	52° 50'45.9306"N	173° 15'5.9358"E	8
Attu-8	52° 48'19.0326"N	173° 9'56.0124"E	42
Attu-9	52° 48'21.4704"N	173° 9'56.8830"E	45
Attu-10	52° 48'38.5236"N	173° 9'37.2702"E	10
Attu-11	52° 48'44.9784"N	173° 9'30.0240E	6
Attu-12	52° 48'10.4862"N	173° 10'4.8174"E	21
Attu-13	52° 48'15.9150"N	173° 10'22.9908"E	32
Attu-14	52° 48'15.7926"N	173° 10'23.6598"E	26
Attu-15	52° 48'59.7888"N	173° 9'26.7372"E	3
Attu-16	52° 49'2.7192"N	173° 9'23.2698"E	4
Attu-17	52° 48'51.1776"N	173° 9'35.1684"E	2
Attu-18	52° 48'9.9246"N	173° 10'12.9756"E	18
Attu-19	52° 47'51.9210"N	173° 10'17.8998"E	14
Attu-20	52° 47'47.1156"N	173° 10'15.2322"E	14
Attu-21	52° 48'21.9018"N	173° 9'34.7394"E	1
Total			326

Table 2. Chapter 2 Analysis of molecular variance (AMOVA) for groups, partitioning, degrees of freedom (df), sums of squares (SS), mean squares (MS), estimated variation (Est. Var.), percent (%) variation, Φ and P-values of the nine *C. arcticum* populations from the Alaska mainland.

Group	Partitioning	df	SS	MS	Est. Var	%	Φ, *P*-Values
Two genetic clusters[a] (K = 2)	Among groups	1	4517.575	4517.575	39.788	35%	Φ_{RT}=0.350***
	Among populations within groups	7	2836.852	405.265	15.242	13%	Φ_{PR}=0.207***
	Within populations	202	11826.555	58.547	58.547	52%	Φ_{PT}=0.485***
	Total	210	19180.981		113.577	100%	
Hierarchical - Three geographical regions[b]	Among regions	2	6280.792	3140.396	42.971	40%	Φ_{RT}=0.402***
	Among populations within regions	6	1073.635	178.939	5.507	5%	Φ_{PR}=0.086***
	Within populations	202	11826.555	58.547	58.547	55%	Φ_{PT}=0.453***
	Total	210	19180.981		107.025	100%	
Global - nine sites	Among populations	8	7354.4	919.3	37.3	39%	ΦPT=0.389***
	Within populations	202	11826.6	58.6	58.6	61%	
	Total	210	19180.9		95.8	100%	
Kenai site	Among populations	2	155.971	77.986	0.674	1%	Φ_{PT}=0.011***
	Within populations	74	4507.522	60.912	60.912	99%	
	Total	76	4663.494		61.586	100%	
Old Valdez site	Among populations	3	235.271	78.424	0.892	1%	Φ_{PT}=0.015***
	Within populations	83	4964.361	59.812	59.812	99%	
	Total	86	5199.632		60.703	100%	

a Group – 1: Old Valdez sites; Group – 2: Anchor Point, Ninilchik, Kenai, based on the results of STRUCTURE.
b Region – 1: Kenai; Region – 2: Old Valdez; Region – 3: Anchor Point & Ninilchik

Table 3. Chapter 2 Analysis of molecular variance (AMOVA) for groups, partitioning, degrees of freedom (df), sums of squares (SS), mean squares (MS), estimated variation (Est. Var.), percent (%) variation, Φ and P-values of the 20 *C. a.* subsp. *arcticum* populations* from Attu Island.

Group	Partitioning	df	SS	MS	Est. Var	%	Φ, *P*-Values
Two genetic clusters[a]	Among groups	1	2343.065	2343.065	9.099	5%	Φ_{RT}=0.045***
	Among populations within groups	18	10020.540	556.697	27.392	14%	Φ_{PR}=0.143***
	Within populations	292	48119.600	164.793	164.793	82%	Φ_{PT}=0.181***
	Total	311	60483.205		201.284	100%	
Hierarchical - Two geographical regions[b]	Among regions	1	1902.685	1902.685	8.936	4%	Φ_{RT}=0.044***
	Among populations within regions	18	10460.920	581.162	28.545	14%	Φ_{PR}=0.148***
	Within populations	292	48119.600	164.793	164.793	81%	Φ_{PT}=0.185***
	Total	311	60483.205		202.274	100%	
Global – twenty sites	Among populations	19	12363.605	650.716	32.327	16%	Φ_{PT}=0.164***
	Within populations	292	48119.600	164.793	164.793	84%	
	Total	311	60483.205		197.120	100%	
Eastern sites[c]	Among populations	6	2883.823	480.637	30.921	16%	Φ_{PT}=0.015***
	Within populations	75	12536.579	167.154	167.154	84%	
	Total	81	15420.402		198.075	100%	
Western sites[d]	Among Pops	12	7577.096	631.425	27.806	14%	Φ_{PT}=0.145***
	Within Pops	217	35583.021	163.977	163.977	86%	
	Total	229	43160.117		191.783	100%	

* Population size for AMOVAs needs to be >1 individual. Population Attu-21 was eliminated as the number of individuals was n=1.
a Group-1: Attu-8, Attu-9, Attu-13 and Attu-14, four populations. Group-2: Attu-1 to Attu-7, Attu-10 to Attu-12, and Attu-15 to Attu-20, seventeen populations, based on the results of STRUCTURE.
b Twenty collection sites on Attu Island could be divided by geographic distribution as eastern sites (seven sites) and western sites (thirteen sites).
c Eastern shoreline sites of Massacre Bay include Attu-1 to Attu-7 populations.
d Western sites of Massacre Bay (Cosco Cove) include Attu-8 to Attu-20 populations.

Table 4. Chapter 2 Three outgroup clonal individuals *C. a.* subsp. *arcticum* JH-173-82*, C. zawadskii* 'Roseum', and 'Schwefelglanz' from the USDA-GRIN system with repeated genotyping, denoting the number of SNP marker matches and rate of variation among the two extraction replications performed (calculated as a ratio with the no. of total SNP markers). See text regarding the actual species designation of these misidentified individuals in the USDA-GRIN database.

Genotypes (Names)	JH-173-82	'Roseum'	'Schwefelglanz'
No. of matching SNP markers	7290	7291	6953
No. of total SNP markers		7443	
Rate of variation among the two extraction replications	2.00%	2.00%	7.00%

List of figures

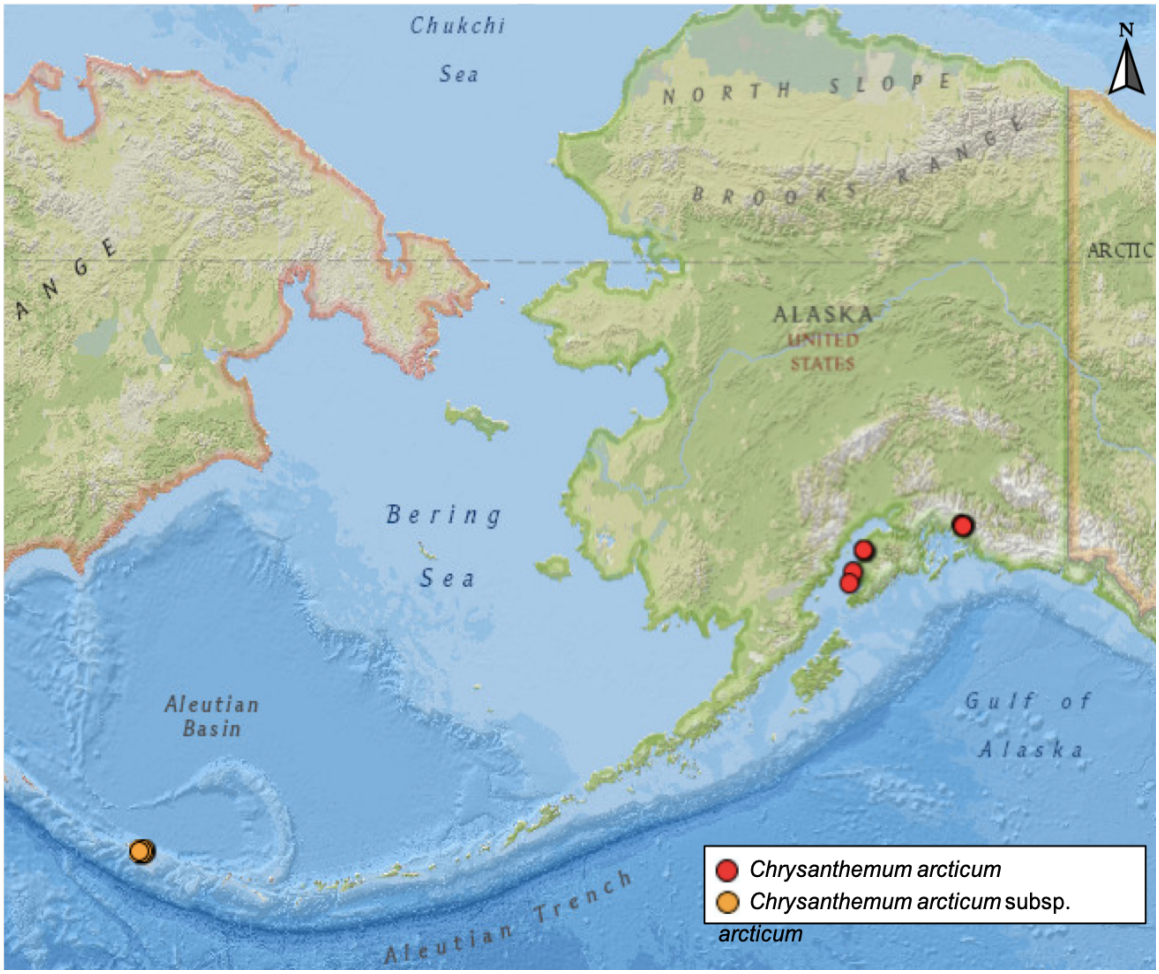

Figure 2. Chapter 2 Map of collection sites for *Chrysanthemum arcticum* on the mainland State of Alaska and its subspecies, C.a. subsp. arcticum, on the western-most Aleutian Island, Attu Island. Each circle represents one collection site, although several circles overlap due to close proximity in geographical locations (GPS visualizer: http://www.gpsvisualizer.com/map_input?form=google).

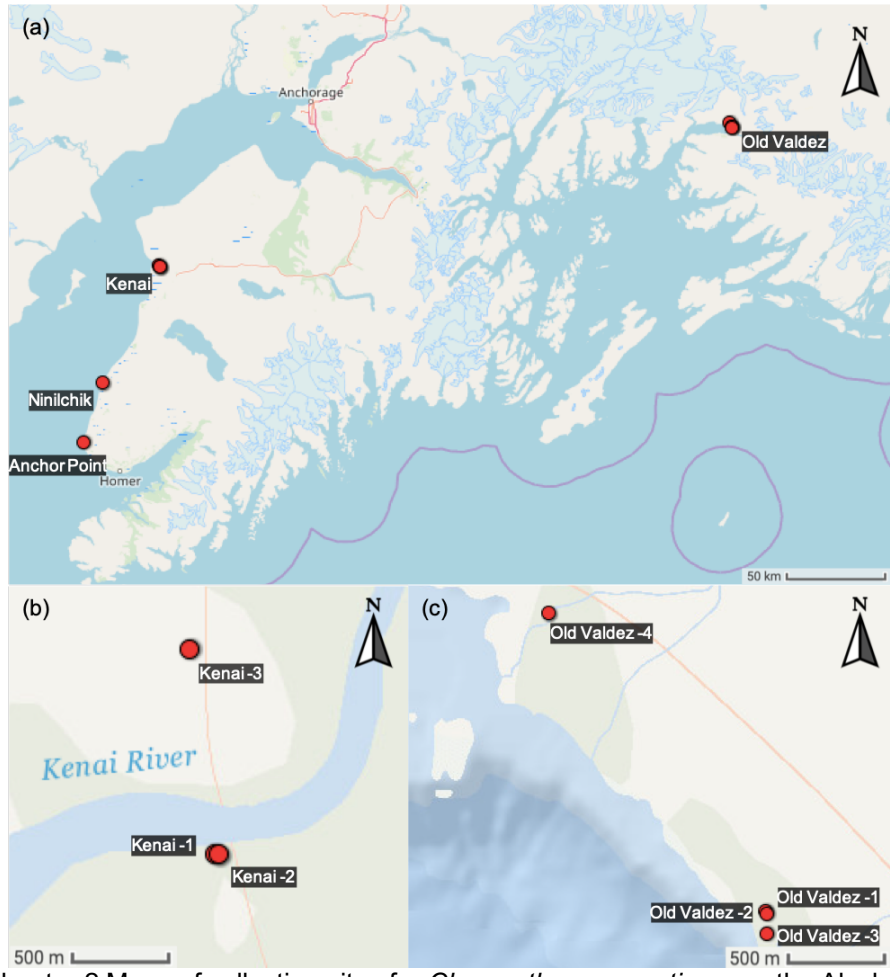

Figure 3. Chapter 2 Maps of collection sites for *Chrysanthemum arcticum* on the Alaska mainland: (a) A total of nine collection sites were located along the Gulf of Alaska (a portion of the Pacific Ocean) coastline of the southeast Alaska mainland (Anchor Point, Ninilchik, and Kenai sites) or the Prince William Sound inlet of the Gulf of Alaska (Old Valdez sites) and represented diverse geographical locations for the maritime species; (b) Three Kenai populations, slightly inland from the Pacific Ocean with saltwater backwash occurring in this segment of the Kenai River; (c) Four Old Valdez populations located in Prince William Sound (GPS visualizer: http://www.gpsvisualizer.com/map_input?form=google).

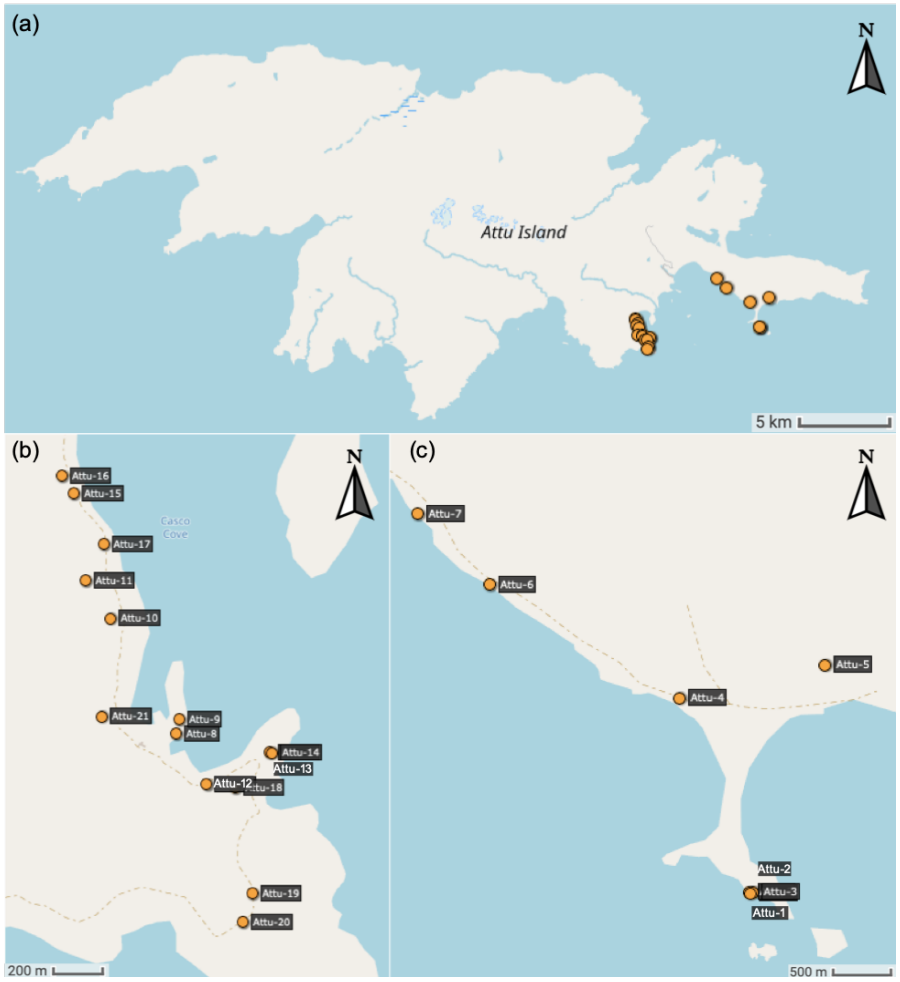

Figure 4. Chapter 2 Maps of collection sites for *C. a.* subsp. *arcticum* from Attu Island: (a) 21 sites located along the southeast coastline of Attu Island which were the only ones accessible by boat and skiff to the shoreline in Massacre Bay (the only sites allowed by the USFWS collection permit); (b) Detailed sampling of Attu populations Nos. 8-21, located along the southwestern side of Massacre Bay, known as Casco Cove; c) Sampling locations for Attu populations Nos. 4-7, on the northeastern edge of Massacre Bay along cliffs along the bay, and Attu populations Nos. 1-3 located adjacent to the WWII landing strip of Marston matting (Attu-3) or on adjacent sea cliffs (Attu-1, -2) (GPS visualizer: http://www.gpsvisualizer.com/map_input?form=google).

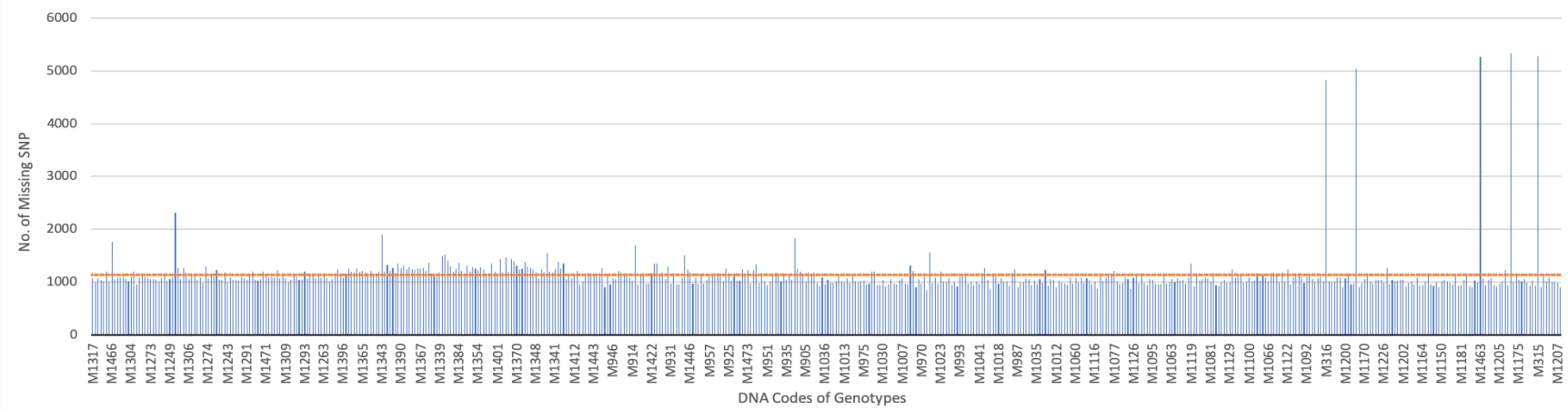

Figure 5. Chapter 2 Frequency plot of missing SNPs for each extant *C. arcticum* and *C. a.* subsp. *arcticum* individual analyzed by DArTseqLD in this study. The horizontal bar graphically represents the mean threshold of missing SNP data (1,139.73 SNPs/individual) wherein 78% of the individuals with >95% non-missing SNP data remained for the analyses.

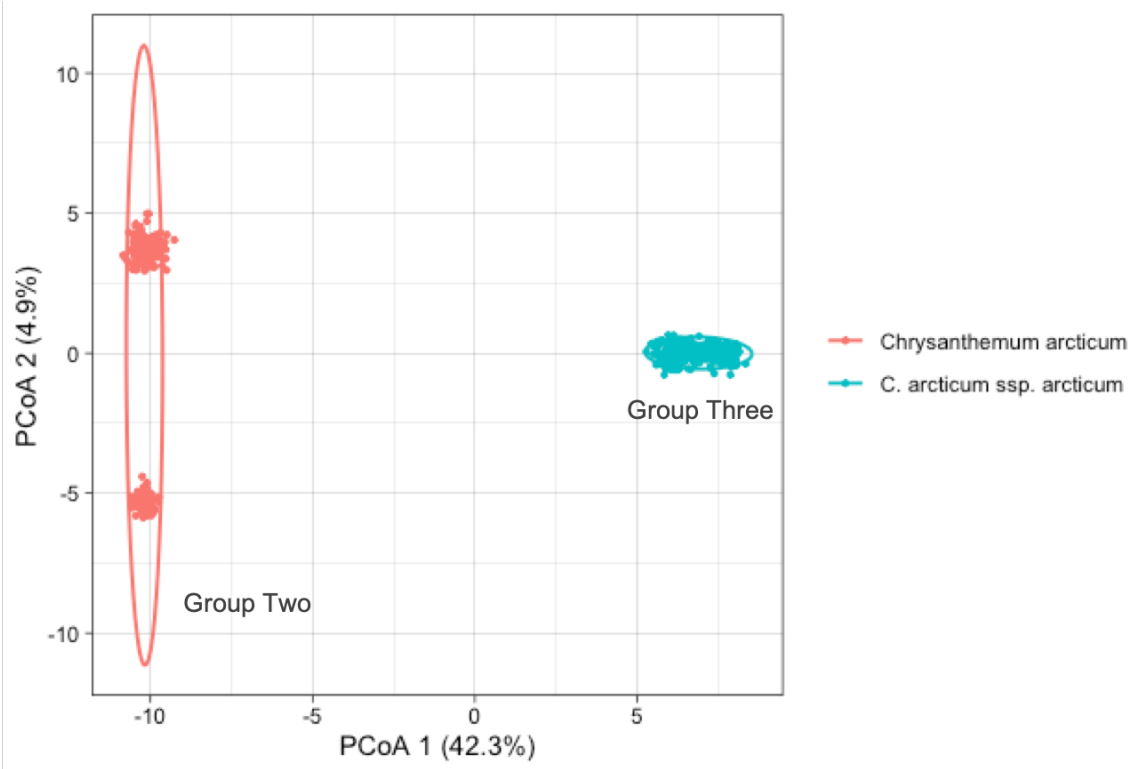

Figure 6. Chapter 2 Principal coordinates analysis (PCoA) plot for the first two principal coordinates (PCoA 1, PCoA 2) of n=524 sample SNPs, including n=211 *C. arcticum* from mainland Alaska (Group two; red filled circles) and n=313 *C. a.* subsp. *arcticum* from Attu Island (Group three; aqua-colored filled circles).

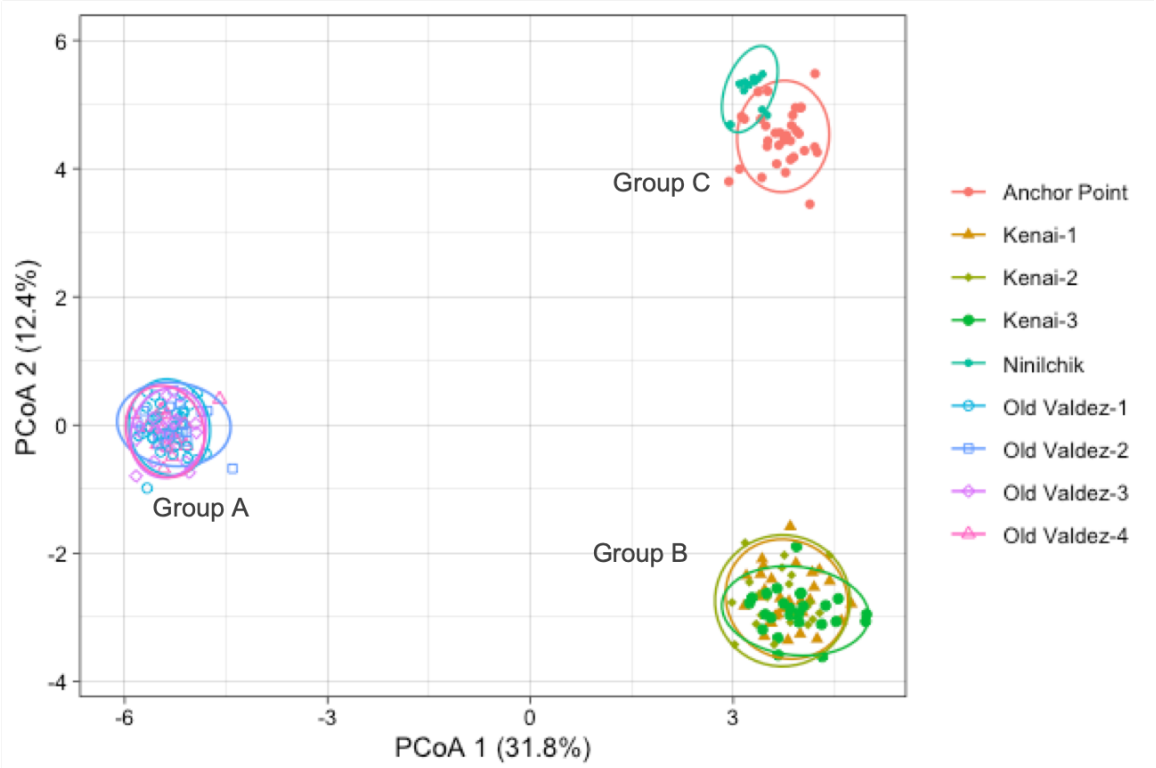

Figure 7. Chapter 2 Principal coordinates analysis (PCoA) plot for the first two principal coordinates (PCoA 1, PCoA 2) of Group two's n=211 individuals sample SNPs of nine *C. arcticum* populations from the Alaska mainland.

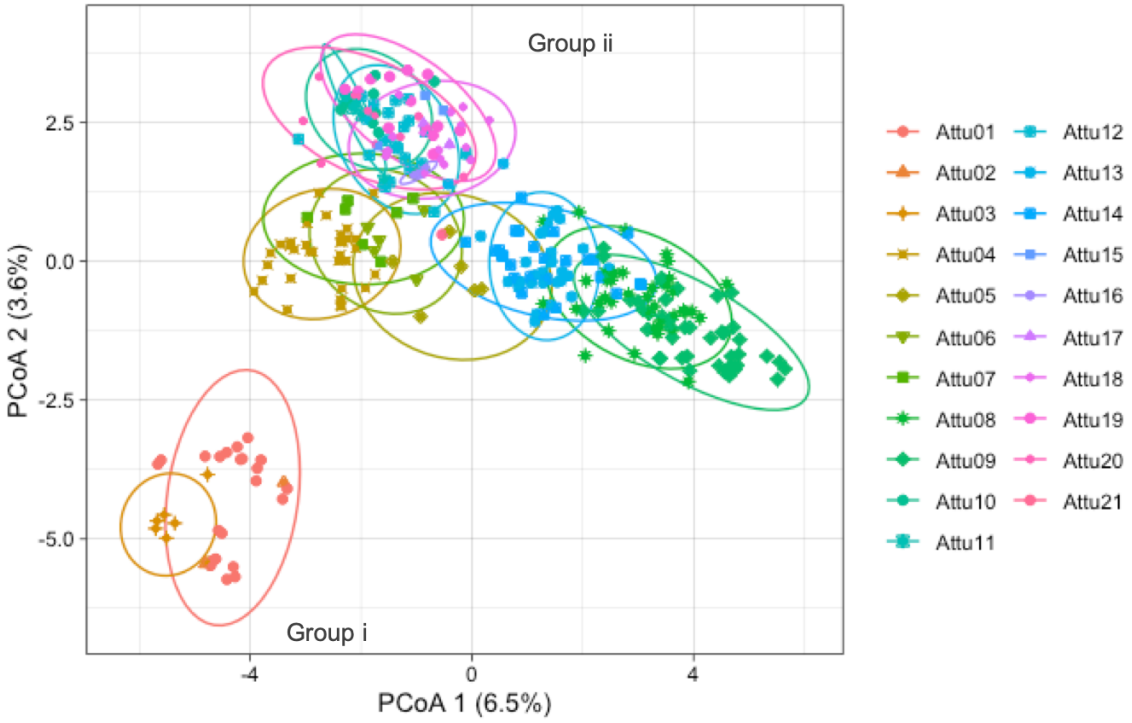

Figure 8. Chapter 2 Principal coordinates analysis (PCoA) plot for the first two coordinates (PCoA 1, PCoA 2) of Group three's n=313 individual sample SNPs of 21 *C. a* subsp. *arcticum* populations located on Attu Island, Alaska.

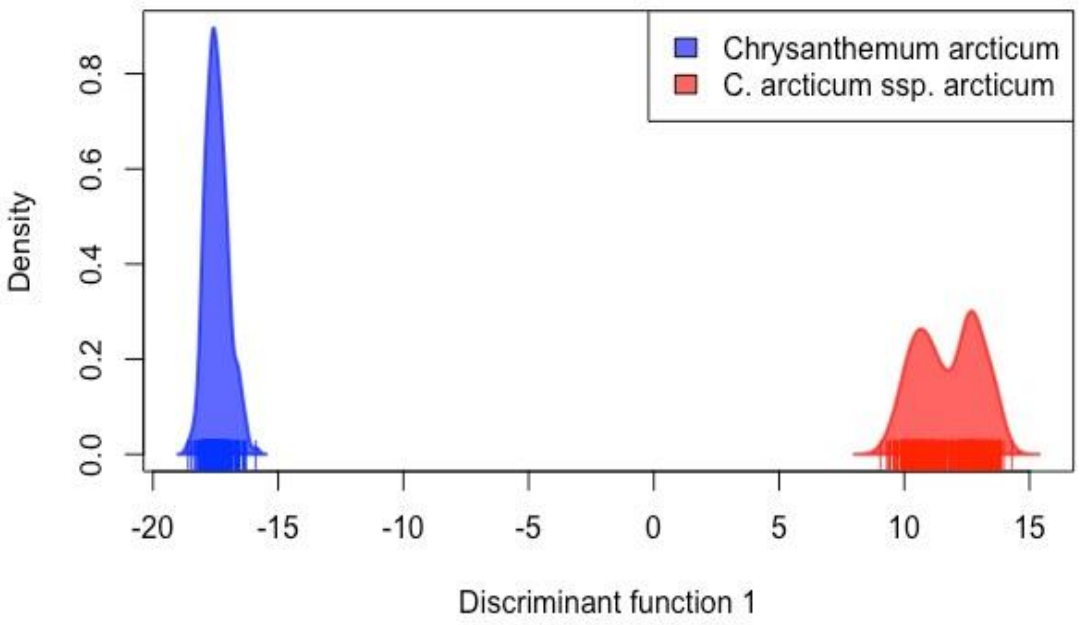

Figure 9. Chapter 2 Scatter plot of discriminant function 1 and density of n=524 individuals from 20 populations of *C. arcticum* (blue) and *C. a.* subsp. *arcticum* (red), statistically divided into two groups, based on Discriminant Principle Component Analysis (DAPC). Note: One population, the Attu-21 population, was dropped from the analysis due to low sample size (n=1).

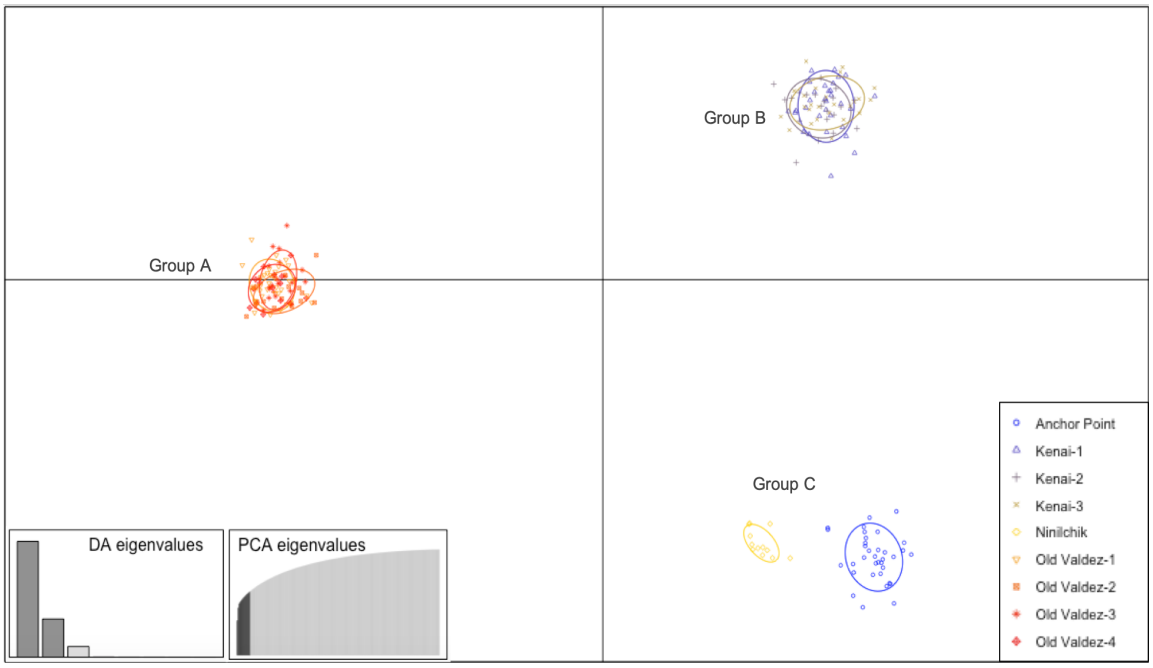

Figure 10. Chapter 2 Scatter plot of discriminant function 1 and density of n=211 individuals from nine populations of *C. arcticum,* statistically divided into three groups (A, B, C), based on Discriminant Principle Component Analysis (DAPC). The DA and PCA eigenvalues are also plotted herein (see inset).

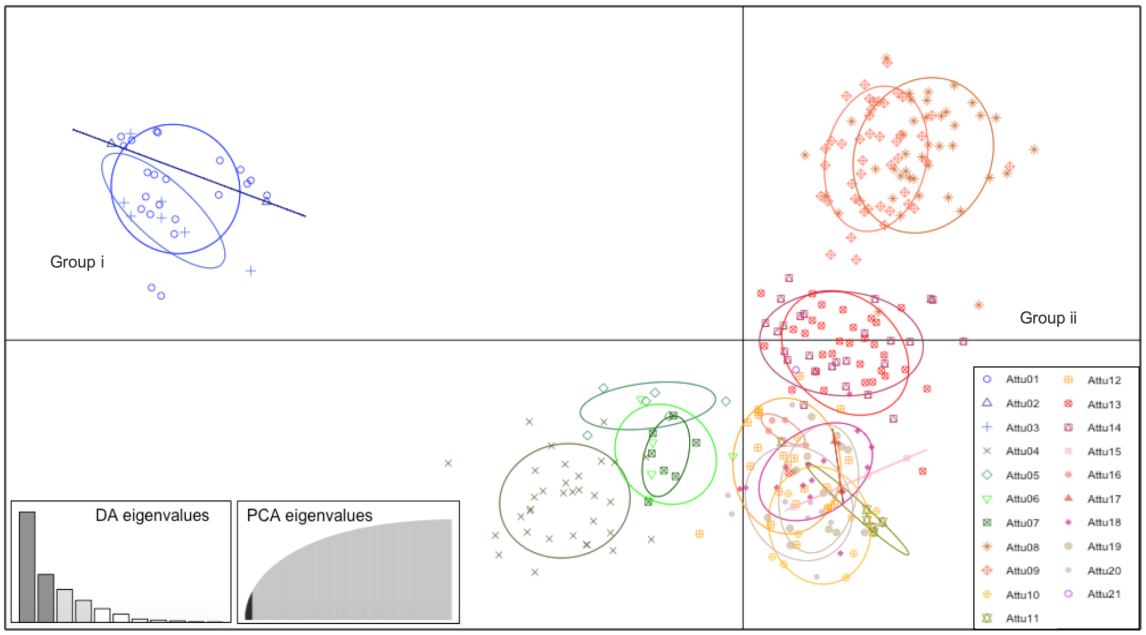

Figure 11. Chapter 2 Scatter plot of discriminant function 1 and density of n=313 individuals from *C. a.* subsp. *arcticum* (groups I, ii), based on Discriminant Principle Component Analysis (DAPC). The DA and PCA eigenvalues are also plotted herein (see inset).

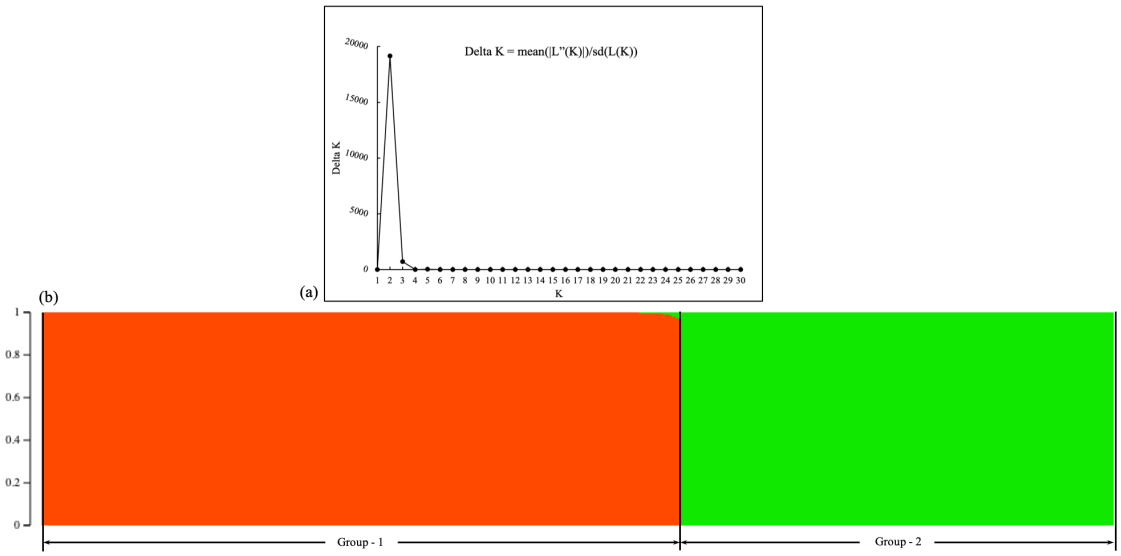

Figure 12. Chapter 2 Bayesian clustering in STRUCTURE 2.3.4 analysis (Pritchard et al., 2000) for the n=211 individuals of *Chrysanthemum arcticum* and the n=313 individuals of *C. arcticum* subsp. *arcticum*: (a) The distribution of subgroups K from one to 30, as calculated by STRUCTURE 2.3.4, occurred with the peak at Δ K=2, indicating the best number of genetic clusters is two; (b) The STRUCTURE bar plot wherein Group-1 (red) includes the 21 populations of *C. a.* subsp. *arcticum* from Attu Island whilst Group-2 includes the nine populations of *C. arcticum* from the Alaska mainland. With the exception of a few individuals, there are no shared SNPs among the species.

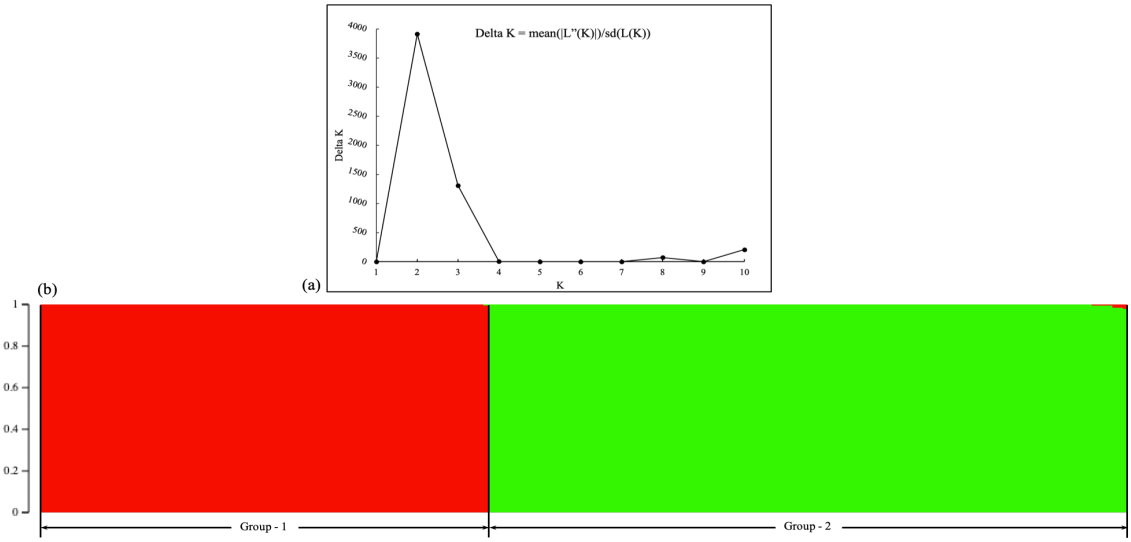

Figure 13. Chapter 2 Bayesian clustering in STRUCTURE 2.3.4 analysis (Pritchard et al., 2000) for the n=211 individuals of *C.arcticum*: (a) The distribution of subgroups K from one to 10, as calculated by STRUCTURE 2.3.4, occurred with the peak at Δ K=2 and a slight shoulder at Δ K=3, indicating the best number of genetic clusters is two; (b) The STRUCTURE bar plot wherein Group-1 includes the four populations from Old Valdez (Old Valdez-1 to Old Valdez-4) and Group-2 includes the five populations from Kenai (Kenai-1, -2, -3), Anchor Point and Ninilchik. With the exception of a few individuals, there are no shared SNPs among the species' populations.

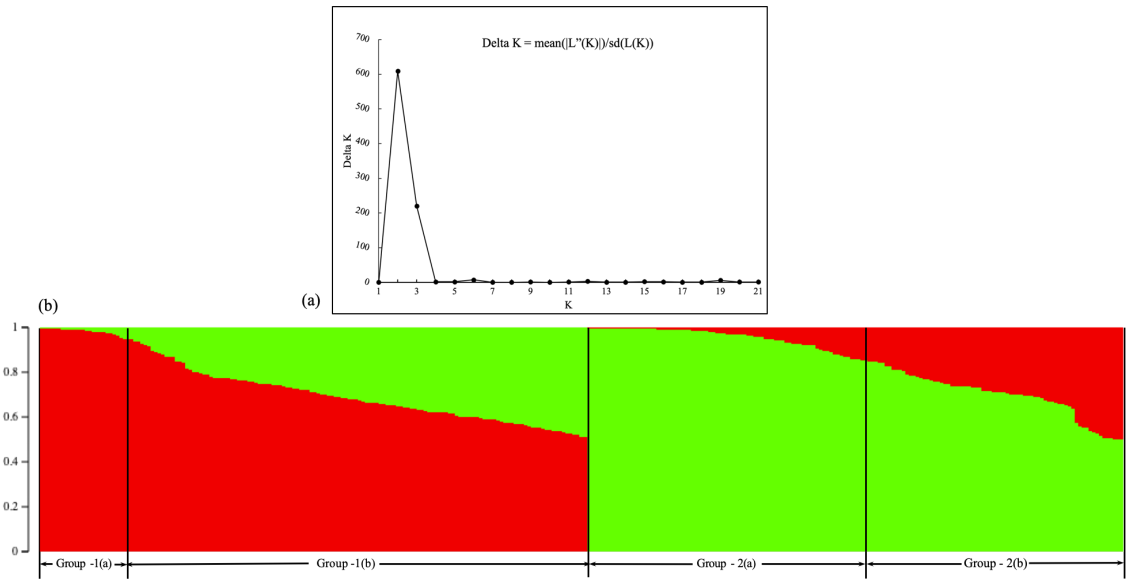

Figure 14. Chapter 2 Bayesian clustering in STRUCTURE 2.3.4 analysis (Pritchard et al., 2000) for the n=313 individuals of *C. a*. subsp. *arcticum*: (a) The distribution of subgroups K from one to 21, as calculated by STRUCTURE 2.3.4, occurred with the peak at Δ K=2, with a slight shoulder at Δ K=3, indicating the best number of genetic clusters is two; (b) The STRUCTURE bar plot wherein Group-1 includes Group-1(a) and Group-1(b). Group-1(a) contains the Attu-1 to Attu-3 populations; Group-1(b) consists of the Attu-4 through Attu-7, Attu-10 through Attu-12, and Attu-15 through Attu-21 populations; Group-2 includes Group-2(a) and Group-2(b) such that Group-2(a) includes the Attu-8 and Attu-9 populations whereas Group-2(b) consists of Attu-13 and Attu-14 populations. There is significantly greater shared SNPs within and among populations *of C. a.* subsp. *arcticum*, than found in *C. arcticum* populations (*cf*. Fig. 13).

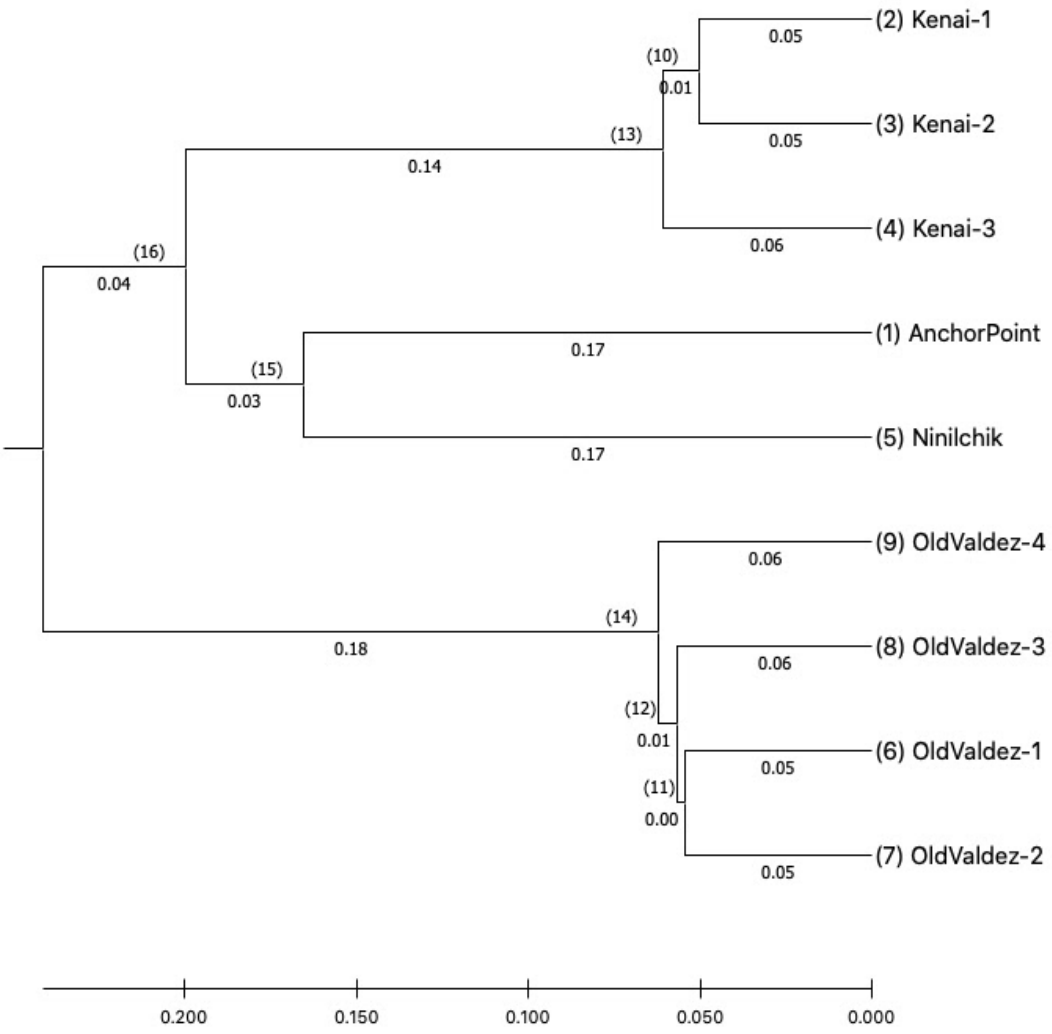

Figure 15. Chapter 2 Phylogenetic tree representing genetic distances among *C. arcticum* populations from Old Valdez (n=4), Ninilchik (n=1), Anchor Point (n=1), and Kenai (n=3), Alaska, derived from single nucleotide polymorphisms (SNPs), based on Jaccard genetic distance by function gl.dist.pop of package dartR in R studio (Baloch et al., 2017). Pairwise genetic distances among the populations were obtained and input in MEGA X (Stecher et al., 2020) to construct dendrograms by the unweighted pair group method (UPGMA) (Nei, 1972) with 1,000 bootstrap replicates (Kumar et al., 2018). Note: Population identification numbers used in this study are in parenthesis prior to each population's name.

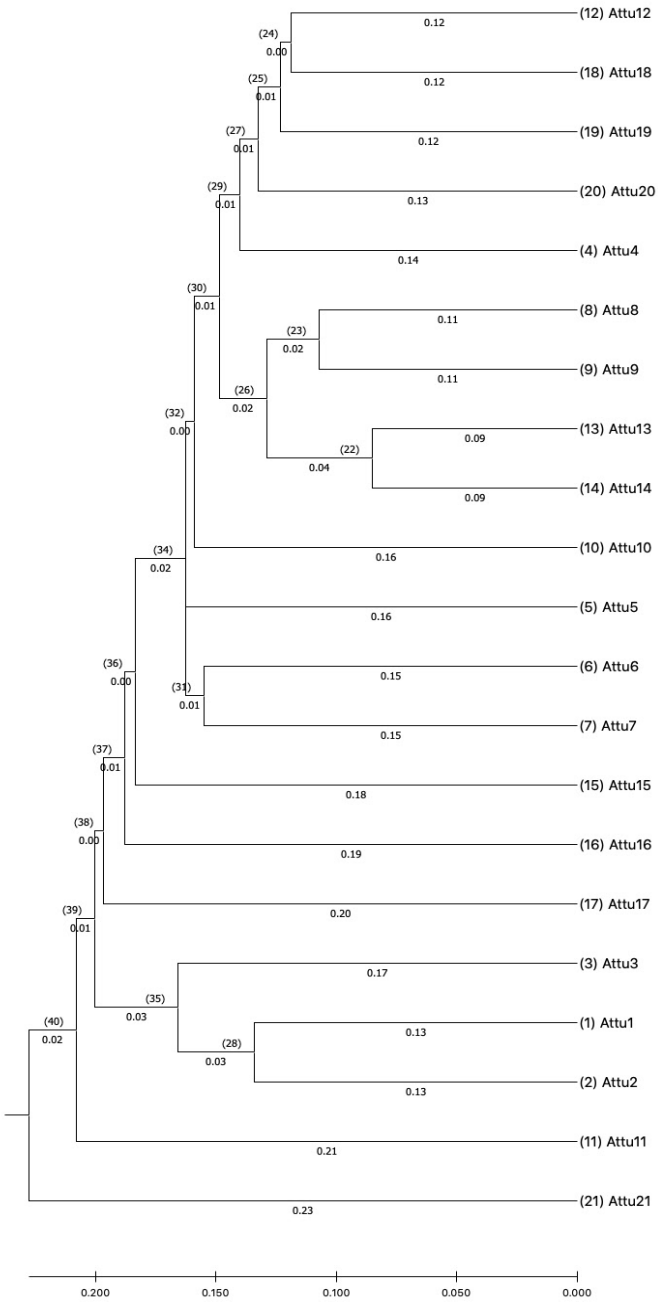

Figure 16. Chapter 2 Phylogenetic tree representing genetic distances among 21 *C. a.* subsp. *arcticum* populations from the eastern end of Attu Island (Aleutian Island Chain), Alaska, derived from single nucleotide polymorphisms (SNPs), based on using the Jaccard genetic distance by function gl.dist.pop of package dartR in R studio (Baloch et al., 2017). Pairwise genetic distances among the populations were obtained and input in MEGA X (Stecher et al., 2020) to construct dendrograms by the unweighted pair group method (UPGMA) (Nei, 1972) with 1,000 bootstrap replicates (Kumar et al., 2018). Note: Population identification numbers used in this study are in parenthesis prior to each population's name.

Chapter 3 Phenotypic differences among and within extant populations of *Chrysanthemum arcticum* L. and *C. a.* subsp. *arcticum*

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Abstract

Chrysanthemum arcticum L., Arctic daisy, (=*Arctanthemum arcticum*; =*Dendranthema arcticum*) and its two subspecies (*C. arcticum* L. subsp. *arcticum*, *C. arcticum* L. subsp. *polaré* Hultén), collectively the *C. arcticum* species complex, are the only chrysanthemum species native to North America. A study on the species' variation in morphological and diagnostic traits is of importance to link morphological traits with the previously described single nucleotide polymorphism (SNP) markers, particularly when the genomes are sequenced. The purpose of this study was to establish phenotypic differences among genotypic clones from wild *C. arcticum* and *C. a.* subsp. *arcticum* populations from the State of Alaska, when grown in a uniform environment for two years (2018-2019), for potential linkages with our SNP library of the two species. At the time of collection in the wild, 0% of the *C. a.* subsp. *arcticum* individuals were in flower due to collection in May-June whereas 100% of the *C. arcticum* were in flower during July. Rhizomes were collected from all individuals, along with flowering stems for each *C. arcticum* individual. In this study, 16 quantitative morphological traits and 5 qualitative morphological traits were investigated for 255 individuals from *C. arcticum* nine populations, and 326 individuals from 21 *C. a.* subsp. *arcticum* populations. While 100% of the *C. a.*

subsp. *arcticum* individuals flowered under long days in both 2018 and 2019, 0% of the *C. arcticum* individuals flowered in 2018 while only 2.7% flowered in 2019. This posed difficulty in assessing inflorescence-based, morphological traits for this species. Consequently, flowering data was used from the wild for *C. arcticum* whereas greenhouse flowering data was used for all *C. a.* subsp. *arcticum* individuals. Two distinct clusters, distributed by taxonomic classification, were detected by Principal component analysis (PCA) for 551 individuals from *C. arcticum* and *C. a.* subsp. *arcticum.* Pearson's correlation coefficient analysis indicated a positive and significant correlation between plant height, flower fresh and dry weights. The flower fresh weight showed the highest positive correlation (r=0.997**) with Δflower weight (flower fresh weight - dry weight); while the inflorescence length showed the highest negative correlation (r= -0.604**) with the number of leaves. Soil samples revealed extremely high levels of Na, along with heavy metals in the soils of where all populations were collected. Thus, the species are salt-tolerant. Univariate ANOVAs revealed consistent results, similar to genetic structure analysis for variation among populations within *C. arcticum* species. *Chrysanthemum arcticum* Old Valdez-1 and Kenai-2 populations showed a significant variation in the majority of traits, such as plant height, inflorescence length, number of leaves, and flower diameter. In contrast, *C. a.* subsp. *arcticum* populations didn't present a consistent tendency, clustering for different dependent variables. Interestingly, similar indistinct clustering were revealed in a genetic cluster analysis for *C. a.* subsp. *arcticum,* which solidified

the likelihood of a higher frequency of gene flow among Attu island collection sites.

Key Words: chrysanthemum, *Chrysanthemum arcticum,* plant morphology, population structure, salt tolerance, Principal component analysis (PCA), ANOVA

Introduction

Cultivated chrysanthemums (*Chrysanthemum* x*grandiflorum* Tzvelv. and *C.* x*hybridum* Anderson) (Asteraceae) are one of the most important and popular ornamental crops (Anderson, 2006; Anderson et al., 2008), ranking second worldwide in the commercial floriculture market after roses, *Rosa* x*hybrida* (Xia et al., 2006). The demand of chrysanthemum production is increasing for comprehensive ornamental and commercial use, encompassing cut flowers, garden herbaceous perennials, potted and ground-cover types (Zhang et al., 2011). Accordingly, worldwide breeders focused their efforts on studying and improving chrysanthemum ornamental traits for improved inflorescence substance, floret color, leaf structure, and drought tolerance (F. Chen et al., 2003; Li et al., 2018; Noda et al., 2017; Su et al., 2017; Zhang et al., 2012). Though the morphological characteristics within *Chrysanthemum* taxa have been identified, most of which were focused on cultivars and limited wild species within Eurasia, including *C. indicum* and *C. zawadskii*. (Dowrick & El-Bayoumi, 1966; Kim et al., 2003; Koyama, 1995; Zhao et al., 2009).

The genus *Chrysanthemum* (Asteraceae) consists of a range of species, 40 (Liu et al., 2012), 75 (Ohashi & Yonekura, 2004) or more (Anderson, 1987),

depending on whether taxonomists lump or split members of the Chrysanthemum complex. Species are classified into three sections of the genus: *Chrysanthemum, Ajania* and *Arctanthemum*. The genus was once changed to *Dendranthema* instead of *Chrysanthemum*, based on genetic and sectional perspectives (Anderson, 1987; Anderson et al., 1988b; Iwatsuki et al., 1995; Koyama, 1995; Tzvelv, 1985), although currently most species were reclassified as *Chrysanthemum*. Members of the *Chrysanthemum arcticum* L. complex (*C. arcticum, C. a.* subsp. *arcticum, C. a*. subsp. *polaré*) were initially considered as infraspecific taxa in the section *Chrysanthemum* (Kitamura, 1940). Subsequently, Bremer and Humphries (1993) recognized the genus *Arctanthemum* and the name was changed to *Arctanthemum arcticum* L. Currently, the names have been changed back to *Chrysanthemum* and assigned into the generic section *Arctanthemum* (Ohashi & Yonekura, 2004). Thus, the species are now referred to as *C. arcticum, C. arcticum* subsp. *arcticum*, and *C. arcticum* subsp. *polaré*.

Chrysanthemum arcticum L., Arctic daisy (=*Arctanthemum arcticum*; =*Dendranthema arcticum*) and its two subspecies (*C. arcticum* subsp. *arcticum*, *C. arcticum* subsp. *polaré* Hultén), hereafter collectively termed the "*Chrysanthemum arcticum* species complex", are the only chrysanthemum species native to North America (Hultén, 1968; Steller, 1993) with the center of origin and diversity in the State of Alaska (USA) and also distributed throughout much of the maritime coastlines of Canada. Both *C. arcticum* and *C. arcticum* subsp. *polaré* are only found in the N. American mainland (from Alaska south

and eastward in Canadian provinces), whereas *C. arcticum* subsp. *arcticum,* occurring both on the western coastal mainland as well as sporadically throughout the Aleutian Islands, has two remnant populations occurring in Eurasia adjacent to the western-most Aleutian Island (Attu Island) in the Kamchatka peninsula (Russian Federation) and Hokkaido, Japan.

Due to the taxonomic name changes and the unique position of this *C. arcticum* species complex as an evolutionary remnant from the Eurasian center of origin and diversity for the *Chrysanthemum* genus (Ohashi & Yonekura, 2004; Tzvelv, 1985; Uehara et al., 2017), comparative studies with other members of the genus are of great interest, particularly given the salt-tolerant nature of these N. American species. Taxa within the *Chrysanthemum arcticum* species complex share many phenotypic traits, although species-specific diagnostic traits (qualitative) in the dichotomous keys inherently differentiate them (Hultén, 1937, 1968). Leaves from both the species and subspecies are tripartite with primarily regularly toothed leaf margins whereas *C. arcticum* leaves tend to have a few more five-segmented leaves and a deep sinus. *Chrysanthemum a.* subsp. *arcticum* has leaves with a finely shallow sinus (Nishikawa & Kobayashi, 1989). The number of midveins in the ray floret petals also differs among the species and subspecies (Hultén, 1937, 1968). Some quantitative differentiation of the taxa within the *C. arcticum* species complex also distinguish them, e.g. *C. a.* subsp. *arcticum* flowering stems are 30-40 cm tall whereas *C. a.* subsp. *polaré* has the shortest stems of 6-20 cm (Iwatsuki et al., 1995; Johnson, 1987). Stem height for *C. arcticum* has not been reported (Hultén, 1937, 1968; Studebaker,

2010). In all instances, however, these quantitative measurements - which are highly affected by factors of plant growth (Anderson, 2006; Dole & Wilkins, 1999) - were not performed with individuals growing in a uniform environment.

Plant structure, flower and leaf architecture influence *C.* x*grandiflorum* and *C.* x*hybridum* selection and breeding for important phenotypic traits, including plant height, photoperiodic response and flower color/type (De Visser et al., 2006; Zhang et al., 2012). There are studies using multivariate analysis methods for identification within the species and populations via the morphological characteristics of the plant, including qualitative and quantitative data (Kim & Lee, 1995; Kim et al., 2014). As winter-hardy herbaceous perennials, members of the *Chrysanthemum arcticum* species complex possess advantageous phenotypic traits that do not occur in the common chrysanthemum cultivars, such as salt tolerance (growing only in coastal, maritime habitats) and a ground-cover plant habit. Unique phenotypic and genotypic features within the *C. arcticum* species complex may offer new options for transforming commercial, cultivated chrysanthemums.

We have characterized the genetic variation among extant, wild populations of *C. arcticum* and *C. a.* subsp. *arcticum,* based on 7,449 Single nucleotide polymorphism (SNP) markers from DArTseqLD (Liu, 2020). SNP data distinctly separated these two taxa, based on STRUCTURE 2.3.4, principal coordinate analysis (PCoA), discriminant analysis of principal components (DAPC), and unweighted pair group method with arithmetic mean (UPGMA) (Liu, 2020), thus providing unique SNP markers for these two species. Possible

linkage of these SNP markers with phenotypic (qualitative, quantitative) traits is of great interest, particularly for species-specific traits and those of commercial interest, such as salt tolerance. The objective of this study is to establish phenotypic differences among wild *C. arcticum* and *C. a.* subsp. *arcticum* individuals when grown in a uniform environment. Traits could be linked with these SNPs (Liu, 2020) for future breeding purposes. Populations (individuals) were evaluated for native soil type composition as well as % survival in cultivation. We used univariate and multivariate analysis to categorize species with representative populations (as identified with SNP data) based on the phenotypic traits (Harris and Harris, 1994; Kim et al., 2014; UPOV, 2011). Traits recorded included plant height, inflorescence length, number of leaves, internode length, leaf length, leaf width, petiole length, number of days to visible bud date, number of days to flowering, lamina length, inflorescence diameter, disc floret diameter, fresh weight, dry weight and Δflower weight. Soil samples were also sampled at the collection sites for analyses of sodium (Na) content, along with other nutrient levels. The null hypothesis tested for each phenotypic trait was: H_o: There is no difference in phenotypic variation of each phenotypic trait within and among extant populations of *C. arcticum* and *C. arcticum* subsp. *arcticum*.

Materials and methods

Study Sites. This study focused on extant *C. arcticum* collected by Dr. Neil Anderson (University of Minnesota) during 2017-2018 from the coastline of southwest Alaska mainland (59° 46'N to 61° 6'N, -146° 16'W to -151° 51'W) and *C. arcticum* subsp. *arcticum* collected from the coastline of the westernmost Aleutian Island, Attu Island (52° 48'N to 52° 50'N, 173° 9'E to 173° 18'E) (*cf.* Fig. 2, Liu, 2020). There were four collection sites on the Alaska mainland for nine extant *C. arcticum* populations (n=225 individuals in total; Table 1 *cf.* Fig. 3, Liu, 2020): Anchor Point (n=1 population), Kenai (n=3 populations), Ninilchik (n=1 population), and Old Valdez (n=4 populations) (*cf.* Fig. 3, Liu, 2020) and 21 collection sites on Attu island along the coastline for 21 extant *C. a.* subsp. *arcticum* populations, Attu-1 to Attu-21 (n=326 individuals in total; Table 1 *cf.* Fig. 4, Liu, 2020). All *C. arcticum* populations were in full flower at the time of collection (July, 2018) whereas all *C. a.* subsp. *arcticum* populations were only vegetative at the time of collection (May-June, 2018). Attu Island is the western-most Aleutian Island of North America (Heusser, 1990; Talbot & Talbot, 1994) and is generally classified as an Arctic (Hultén, 1960) or Hypoarctic zones (Yurtsev, 1994). The climate on Attu island is cool (3.8°C mean annual temperature) with 90% of the days having measurable precipitation (average rainfall=1,372mm/yr) (Leslie, 1989). Clones (ramets) of each ortet growing in the wild were collected for this study and were identical to those used to generate SNPs (Liu, 2020).

Germplasm. Where necessary, collection permits were issued for the collection and research of *C. arcticum* germplasm (USFWS No. 74500-17-018). In 2018, 225 individuals of *C. arcticum* were collected from the nine populations and 326 individuals of *C. a.* subsp. *arcticum* were collected from the 21 populations (Table 1 *cf.* Fig. 4, Liu, 2020). These plants were collected as rhizomes (*C. a.* subsp. *arcticum* individuals were vegetative whereas all *C. arcticum* were flowering and the complete flower stems were brought to the lab). In addition, bagged in resealable plastic bags (1.75 mil, 1 Quart Get Reddi® Reclosable Food Service Bags, https://www.usplastic.com/catalog/item.aspx?itemid=128308&catid=) and put on ice in a portable cooler. Samples were placed in a refrigerator (~3-5°C) until eventual transport to the lab at the University of Minnesota (within 2-3 weeks after collection). Rhizomes were subsequently transplanted and rooted in the mist house, with an intermittent mist system (10 minutes of frequency; reverse osmosis water). Since the *C. arcticum* individuals were harvested with the flowers, reproductive data (with the exception of the number of days to visible bud date and flowering) was collected from them prior to rooting. The flower stems were then removed and placed into floral preservative for seed ripening (for use in subsequent experiments). After rooting for 1-2 weeks, plants were moved to an environmentally controlled glass greenhouse with a 24.4±3.0/18.3±1.5°C day/night daily temperature regime and a 16 hr photoperiod (0600–2200 HR; long days). During the winter months, supplemental lighting was applied with 400 w high pressure sodium high intensity discharge (HPS-HID)

lamps, at a minimum of 150 µmol m^{-2} s^{-1} at plant level. The computerized greenhouse was in the St. Paul campus Plant Growth Facilities (University of Minnesota, St. Paul, MN). Fertigation water was applied twice daily, between 0700-0800 HR and 1600-1700 HR, using a constant liquid feed (CLF) of 125 ppm N supplied from a water-soluble 20N–4.4P–16.6K fertilizer (Scotts, Marysville, OH). Monthly rotational fungicide drenches were administered (*cf.* Liu, 2020).

Soil Sampling. Soil samples were collected from all mainland Alaska (Anchor Point-1, Kenai-1, Kenai-2, Ninilchik-1, Old Valdez-1, -2, -3, -4) populations for *C. arcticum* individuals and one *C. arcticum* subsp. *arcticum* sample was collected from Attu island, population 10 (weight limit restrictions limited sampling all of the 21 populations, due to the need to transport via boat on the Bering Sea). Soil samples from mainland Alaska and Attu Island were collected at the base of the first plant collected, with a 250g sample collected as topsoil subtending the existing plant material. Samples were returned to the lab in resealable plastic bags (1.75 mil, 1 Quart Get Reddi® Reclosable Food Service Bags, https://www.usplastic.com/catalog/item.aspx?itemid=128308&catid=) and kept at 3-5C until submitted for Spurway Greenhouse, Florist, & Nursery Crops testing at the Department of Soil, Water and Climate's University of Minnesota Soil Testing Laboratory (http://soiltest.cfans.umn.edu/) to determine nutrient and other factors of the native soil for species and subspecies. Soil samples were evaluated for NO_3^-N (mg/kg soil), SO_4^-S (mg/kg soil), Bray P (mg/kg soil), NH_4OAc-K (mg/kg soil), organic matter or LOI OM (%), water pH, 1:1 electrical conductivity or EC

(mmhos/cm), saturated paste extract EC (mmhos/cm), hot water boron (mg/kg soil), DTPA-Fe (mg/kg soil), DTPA Mn (mg/kg soil), DTPA Zn (mg/kg soil), DTPA Cu (mg/kg soil), exchangeable NH_4OAc-K (mg/kg soil), NH_4OAc-Ca (mg/kg soil), NH_4OAc-Mg (mg/kg soil), and NH_4OAc-Na (mg/kg soil). The NH_4OAc-Na (mg/kg soil) determined salt concentrations rather than just EC values since ECs represent dissolved solutes, including Na.

Measurement of Phenotypic Traits. The phenotypic (morphological) characteristics investigated were based on the Chrysanthemum Test Guidelines criteria set by the International Union for the Protection of New Varieties of Plants and Plant Identification Terminology (Harris and Harris, 1994; Kim et al., 2014; UPOV, 2011). To obtain comprehensive morphological traits datasets for *C. arcticum* populations, the same clones were grown in 2018 (from rooting onwards) through 2019 to create data sets. In 2018, only ramets of *C. arcticum* subsp. *arcticum* flowered (100%) which limited the data collection of flower data for *C. arcticum* (0% flowering). Thus, the experiment was continued into 2019 (after 6 weeks or 1000 hrs of cold at 3-5C; Dole and Wilkins, 1999) in the event that any of the *C. arcticum* clones would subsequently flower. In the event that these did not flower in 2019, most reproductive traits (with the exceptions of the number of days to visible bud date and flowering) were measured on the flowers collected originally on site (see above). In 2018, plant height (cm), inflorescence length (cm), number of leaves on the primary stem, internode length (cm), inflorescence diameter (cm), disc floret diameter (cm), petal length (cm), flower fresh weight (g), flower dry weight, water in fresh flower (Δflower weight (g) =

fresh flower weight - dry weight) were recorded. In 2019, we added leaf morphology, leaf length (cm), lamina length (cm), petiole length (cm), leaf width (cm), leaf margin, shape and color. Plant height was measured using a standard ruler (30 cm) placed vertically from the tallest point of the canopy of an inflorescence (if flowering) or from the tallest leaf (if nonflowering) to the soil line (base of the plant). Inflorescence length was measured from the bracts to the top of the plant (Z. Chen et al., 2019). The color of each leaf, flower ray floret (petals) and disc floret were determined using the Royal Horticultural Society (RHS) chart with the visual appearance under natural sunlight in the greenhouse. Leaf morphological data was recorded by removing a representative, fully matured leaf from each individual and taking a photo of each leaf sample which were subsequently measured in Image J software (Rueden et al., 2017). Leaf length (cm) was measured from the lamina tip to the base of the leaf where the leaf stem (petiole) ended at the node on the primary stem. Petiole length was measured from the base of the petiole (at the primary stem) to the lamina base; lamina length (cm) was obtained by subtracting leaf length from the petiole length; leaf width (cm) was measured from the widest lamina lobes. The number of leaves on the primary stem of each individual were counted and mean internode length (cm) was obtained by the following equation:

Mean internode length (cm) = plant height / leaf number

Leaf shapes were classified into five types: flabellate, hastate, pandurate, oblong or round (Harris and Harris, 1994). The Leaf margins were classified into four types: cleft, crenate, entire, lobed or tripartite.

All *C. arcticum* populations were flowering in the wild during the 2018 collecting trips, but most failed to flower as clones thereafter in the greenhouse (2018-9; Table 6) despite being under, presumably, the correct photoperiod of long days to induce flowering. For *C. arcticum* subsp. *arcticum,* visible bud and flowering dates were recorded. In 2018, the number of days to visible bud date (VBD) was counted from the day plants were rooted in the greenhouse whereas in 2019, it was the date they were taken from the cooler after a six-week cold treatment (3-5°C; Dole & Wilkins, 1999), to the day when terminal flower bud was visible. The number of days to flowering was counted from the same start date each year to the day the flower expanded to the widest diameter and was at anthesis (pollen shed).

Flower morphological traits were observed and recorded mainly during the mature flowering period (2018 data set for *C. arcticum* and 2019 data set for *C. a.* subsp. *arcticum*). Inflorescence diameter was measured by standard ruler from the widest points of the flower. Disc floret diameter was measured from the widest point of the yellow floret disc. The petal length (cm) was calculated by the formula:

Petal Length (cm) = (inflorescence diameter - disc diameter)/2

The first flower on each individual was cut and weighed for fresh weight (g), placed in a high temperature oven (76.67°C) (Hotpack, Philadelphia, PA) for 24 hours, and then weighed to obtain dry weights (g). To calculate the Δ flower weight or water content, the following equation was used:

Δ flower weight (g) = fresh weight (g) - dry weight (g)

Data analysis. We conducted a series of statistical analyses to evaluate the morphological characteristics and establish phenotypic relationships between species and subspecies and among populations. We used multivariate approaches to quantify the variance for each trait as well as qualify visible attributes (color) of *C. arcticum* and *C. a.* subsp. *arcticum*. We also used univariate and multivariate regression approaches based on previous studies (Lande & Arnold, 1983; Murren et al., 2020) to analyze the morphological characteristics among the nine extant *C. arcticum* populations and 21 extant *C. a.* subsp. *arcticum* populations, respectively. Three taxonomic groupings were established for the statistical analyses: Group one: species, subspecies; Group two: *C. arcticum;* Group three: *C. arcticum* subsp. *arcticum*. In our study, statistical analyses were conducted to detect the variation among the three taxonomic groupings: Groups one, two and three respectively, by considering corresponding quantitative morphological variables for individuals in each group. For Group one, the group label was set as *C. arcticum* and *C. a.* subsp. *arcticum*; 16 quantitative morphological variables were applied using PCA. For Group two, the group label was set at nine extant populations of *C. arcticum*; 16 quantitative morphological variables were analyzed with PCA. For Group three, PCA was used to detect the differences among the 21 populations of *C. a.* subsp. *arcticum* by analyzing 16 quantitative morphological variables. Collected quantitative morphological data were analyzed and performed using R studio (v. 1.3.959) and the Statistical Package for the Social Sciences (SPSS) software, v. 25.0 (IBM Corp., 2017).

Two multivariate analyses, principal component analysis (PCA) and Pearson's correlation were performed. The PCA is one of the most effective and frequently used multivariate statistical methods for investigating a large set containing individuals/entities of multiple inter-correlated variables (Lê et al., 2008; Zar, 1999). PCA reduces the dimensionality of a multivariable dataset to few new variables, termed principal components, which correspond to a linear combination of the original variable (Zar, 1999). Each principal component was reassigned a different portion of original variables, whereby PC1 would be considered as the greatest weight, PC2 would be the second, etc. (Murren et al., 2020). Principal components analyses for three groups (Group one, two, three) were conducted with R studio by using the FactoMineR (Lê et al., 2008) and factoextra R packages (Kassambara, 2017). The relatedness between morphological traits among populations for each group were assessed using Pearson's correlation coefficients and tested at $p \leq 0.05$ and $p \leq 0.01$ (Yang et al., 2020).

Univariate Analysis of Variance (ANOVA; general linear model) and descriptive statistics were conducted using SPSS to identify the discriminative descriptors and statistically differentiate among populations for quantitative phenotypic characteristics. Mean separations were conducted using 5% Tukey's Honestly Significant Difference (HSD) test at $\alpha=0.05$. The ANOVA analyses applied to *C. arcticum* and *C. a.* subsp. *arcticum* species, separately. While the *C. arcticum* dataset was combined by using both data from year 2018 and 2019, *C. a.* subsp. *arcticum* only included the data from 2019. The morphological

variables from different years and different species would influence the univariate in the analyses. Hence, the comparison between species and subspecies would not be included in the univariate analysis of variance. The variation within and among populations of *C. arcticum* and *C. a.* subsp. *arcticum* between leaf quality morphological variables (leaf shape and leaf margin) was compared by a Chi-square ($\chi 2$) test for equal distribution across the five classes for the leaf shape and leaf margin data ($1:1:1:1:1\chi 2$).

Results

Pedological environment condition. The soil test results revealed considerable disparity between the recommended greenhouse soil standards and collection sites' samples (Table 5; *cf.* Fig. 4, Liu, 2020). The concentration of Nitrate-nitrogen (NO_3-N) from both the mainland Alaska and Attu island collection sites were significantly lower than the greenhouse standard, especially Attu island which had <0.05 ppm N. The electrical conductivity (EC) or relative dissolved soluble salt levels were in the range of 0-2 mmhos/cm (millimhos per centimeter/cm) are non-saline, which occurred for soil samples from Ninilchik, Old Valdez-1, -2, -3, and -4 collection sites, the mainland Alaska, and the soil samples from Attu island. The Kenai-1 population had 2.1-4 mmhos/cm, or very slightly saline, whereas 4.1-8 mmhos/cm (moderately saline) was found for the soil samples from Anchor Point and Kenai-2 populations on mainland Alaska.

Additional soluble salt concentration and sodium (Na) level tests provided additional data on salt tolerance. The saturated paste extract EC could only be run for four populations (Table 5) due to insufficient quantities of soil for testing. The standard reference values and relative salt tolerance of crops ranges from 0 to 2, 3-4, 5-7 with a maximum of 8-10 mmhos/cm. Old Valdez-4 population had the lowest of 2.5 mmhos/cm, followed by Kenai-1 at 4.8 mmhos/cm, to Kenai-2 of 7.9 mmhos/cm and Anchor Point with the highest level of 14.7 mmhos/cm (Table 5). According to the soil testing laboratory, the soil sample from Old Valdez-4 would be considered slightly saline. The Kenai-1 population would be considered moderately saline whereas Kenai-2 and Anchor Point would be saline.

Exchangeable NH_4OAc-Na or sodium concentrations in all populations of both *C. arcticum* and *C. a.* subsp. *arcticum* collected were many levels of magnitude greater than the recommended greenhouse soil standard of 0-10 mg/kg (Table 5). For example, Attu Island (*C. a.* subsp. *arcticum*) had the lowest level of 123.94 mg/kg, followed by increasingly higher concentrations of Na^+ in the *C. arcticum* populations, with the highest recorded in Anchor Point, AK at 2445.74 mg/kg (Table 5).

The water pH level of soil samples from the mainland Alaska sites ranged from 6.1 to 6.9, within the normal range for greenhouse crops whereas Attu Island pH=4.8 is considerably more acid (Table 5). The Bray-P test was used when the soil pH is <7.4 (otherwise, the Olsen-P test will be used). These soil samples collections, except for Anchor Point and Ninilchik, were within the standard range as greenhouse standard (5-15 mg/kg soil; Table 5). Anchor Point and Ninilchik collection sites had higher levels of phosphorus ($\geq$15 mg/kg; Table 5). The concentration of NH_4OAc-K (mg/kg soil) of soil samples from Anchor Point (280 mg/kg), Kenai-1 (278 mg/kg), and Kenai-2 (247 mg/kg; Table 5) collection sites were greater than the range of the Greenhouse standard 75-200 mg/kg soil, while the other samples fell within this range. Extractable Zinc, Copper, Iron, and Manganese concentrations, reported as DTPA-Zn, DTPA-Cu, DTPA-Fe, and DTPA-Mn (mg/kg soil) respectively, were frequently higher than the greenhouse standards (Table 5). Meanwhile, the exchangeable Potassium, Calcium, and Magnesium concentrations were reported as Exchangeable NH_4OAc-K, NH_4OAc-Ca, and NH_4OAc-Mg, respectively, also indicated high

variation among the samples with exchangeable Ca and Mg having the highest range of values.

% Survival in Cultivated Conditions.

Since the growing requirements for species and subspecies are completely unknown, we rooted the rhizomes and grew the clones in our standard greenhouse conditions (no added Na in the soilless medium) used for cultivated chrysanthemums (as described earlier) (Anderson, 2006; Anderson et al., 2008; Dole & Wilkins, 1999). The *C. arcticum* populations survived sub optimally with all populations experiencing losses, ranging from 7.7% (Ninilchik) to 45.9% plant death (Old Valdez-1; Table 6). In contrast, all of the *C. a.* subsp. *arcticum* populations had 0% plant death (Table 6).

Morphological data.

A total of 21 morphological characteristics for 225 *C. arcticum* individuals from nine populations and 326 *C. a.* subsp. *arcticum* individuals from 21 populations were evaluated and found to be significantly different for the majority of characteristics measured (Table 7). Since the Attu-21 population contained n<3 individuals (n=1) it was automatically eliminated by the SPSS program for ANOVAs. Hence, a total of 29 populations were analyzed. Except for flower fresh, dry and ΔFlower weight variables, all species, populations and species * population interactions were very highly significant ($p \leq 0.001$).

Mean plant height ranged from 21.3 cm (Attu-1; which overlapped with all other *C. a.* subsp. *arcticum* populations) to 47.1 cm (Ninilchik; Table 7) and there was very highly significant variation among *C. arcticum* and *C. a.* subsp. *arcticum* species (F= 11420.89, $p \leq 0.001$). The *C. a.* subsp. *arcticum* populations differed significantly from *C. arcticum* for mean plant height, except for all of the Old Valdez populations (Table 7). The Ninilchik population was significantly different from all other populations of *C. arcticum* as well as *C. a.* subsp. *arcticum* whereas the three Kenai populations overlapped with both Ninilchik and Anchor Point. Plant height cannot be considered a diagnostic trait for these species and subspecies.

Mean inflorescence lengths, ranging from 1.6 cm (Attu-2) to 21.4 cm (Old Valdez-2; Table 7) consistently showed highly significant variation among both species and subspecies (F= 2314.247, $p \leq 0.001$). The shortest mean inflorescence lengths were contained in all of the *C. a.* subsp. *arcticum* populations which did not overlap with any of the *C. arcticum* (Table 7). Thus, inflorescence length differs significantly between species and subspecies, and is a diagnostic trait. The Ninilchik population differed significantly from only the Old Valdez-1, -2, and -3 populations but overlapped with the other *C. arcticum* populations. Whereas, there was no significant difference among *C. a.* subsp. *arcticum* populations for inflorescence length (Table 7).

The number of leaves on each primary inflorescence stem ranged from 9.8 (Old Valdez-2) in *C. arcticum* to 33.7 (Attu-7; Table 7) in *C. a.* subsp. *arcticum*. The nine *C. arcticum* populations differed from Attu-1 to Attu-7

populations significantly ($p \leq 0.001$). Interestingly, despite most of the *C. arcticum* subsp. *arcticum* populations having shorter plant height than *C. arcticum*, Attu-1 through Attu-6 populations had significantly greater numbers of leaves on the primary stems (Table 7). The internode length ranged from 0.8 cm (Attu-7) to 4.0 cm (Old Valdez-1; Table 7) with a highly significant difference between *C. arcticum* and *C. a.* subsp. *arcticum* species (F=3420.38, $p \leq 0.001$). The Attu-1 to -7 and Attu-12 and Attu-14 populations differed significantly from all *C. arcticum* populations on the internode length morphological trait. Thus, they are distinctly different for this trait. As a result, both leaf number and internode lengths are not diagnostic traits between species and subspecies.

Leaf lengths ranged from 5.7 cm (Old Valdez-3) to 18.3 cm (Attu-17; Table 7), while a significant difference was found between *C. arcticum* and *C. a.* subsp. *arcticum* species (F=5199.363, $p \leq 0.001$). The Attu-15 to -17 populations differed significantly from the *C. arcticum* populations (Table 7). Among the *C. arcticum* populations, Old Valdez-3 and Ninilchik differed from the rest significantly (Table 7). Mean leaf widths ranged from 2.1 (Old Valdez-3) to 7.1 cm (Attu-15; Table 7) with a significant difference between *C. arcticum* and *C. a.* subsp. *arcticum* species (F=6042.344, $p \leq 0.001$). *C. arcticum* populations differed significantly from all *C. a.* subsp. *arcticum* populations except for Attu-1 (Table 7). Among the *C. a.* subsp. *arcticum* populations, mean Attu-15 leaf width was significantly different from all other Attu populations, except for Attu-7, -16, -17 and -20. Mean petiole lengths, ranging from 3.3 cm (Old Valdez-3) to 12.4 cm (Attu-17; Table 7), varied significantly between the species and subspecies (F=3977.258, $p \leq 0.001$).

The Attu-15 and -17 populations differed significantly from all *C. arcticum* populations except for Ninilchik. Mean lamina lengths ranged from 2.4 cm (Old Valdez-3) to 6.0 cm (Attu-17; Table 7), showing highly significant variation between the species and subspecies (F=4485.451, $p \leq 0.001$). Attu-15 and -17 populations differed significantly from all *C. arcticum* populations except for the Ninilchik population. Overall, interestingly, the Attu-15 and -17 differed from the majority of *C. arcticum* populations except Ninilchik for leaf morphological traits. Due to the outlying populations within species, e.g. Ninilchik, Attu-17, and Old Valdez-3 which caused overlap among leaf morphological traits, none of these can be identified as diagnostic.

Leaf color (RHS) on the adaxial surface of *C. arcticum* population individuals was RHS 137 Green (Table 7) whereas that of *C. a.* subsp. *arcticum* populations were primarily the same color, although the Attu-6 and Attu-16 populations were RHS 138 Green whilst Attu-18 and Attu-21 populations were RHS 139 Green (Table 7). Thus, most variation in adaxial leaf surface coloration occurred in *C. a.* subsp. *arcticum;* this trait is not diagnostic.

The majority leaf shapes for the nine *C. arcticum* populations were as flabellate, although a few individuals had hastate, oblong, pandurate and round (Table 7). The Ninilchik population did not have Hastate leaf shape although it was the second most commonly occurring leaf shape in *C. arcticum* (Table 7). The Anchor Point population was the only *C. arcticum* that had oblong leaf-shaped individuals (Table 7). The *C. a.* subsp. *arcticum* Attu-2 population had 100% flabellate leaf shape (Table 7). Unlike *C. arcticum populations, C. a.* subsp.

arcticum did not have any oblong leaf-shaped individuals (Table 7). Hastate and pandurate leaf shapes were the most common among *C. a.* subsp. *arcticum* populations (Table 7). The 1:1:1:1:1 χ^2 for leaf shape (flabellate : hastate : oblong : pandurate : round) did not differ significantly within populations (Table 7). Whereas, the pooled populations 1:1:1:1:1 χ^2 for leaf shape was highly significantly different (χ^2=272.639, $p \leq 0.001$; Table 7) and did not fit an equal distribution. Thus, a specific leaf shape is not diagnostic of the species and subspecies.

A tripartite leaf margin was the most common type in all *C. arcticum* populations (Table 7). Except for the Attu-2 population, tripartite was also the most common leaf margin in all the other *C. a.* subsp. *arcticum* populations. The 1:1:1:1:1 χ^2 for leaf margin types (cleft : crenate : entire : lobed : tripartite) did not differ significantly from expected, while a highly significant difference occurred among pooled populations (χ^2=283.693, $p \leq 0.001$). Since most populations, regardless of species, had one or two to five of the leaf margin types (cleft, crenate, entire, lobed, tripartite), this trait is not diagnostic.

Chrysanthemum arcticum and *C. a.* subsp. *arcticum* species were significantly different (F=15797.324, $p \leq 0.001$) for inflorescence diameter. This trait ranged from a mean of 4.3 cm (Old Valdez-1) to 5.8 cm (Attu-16; Table 7) and overlapped significantly within populations and among species. The Old Valdez-1 population was significantly different from the Attu-10, -12, -16 and -20 populations (Table 7). Disc floret diameter ranged from 1.2 cm (Attu-17) to 1.7 cm (Old Valdez-4, Attu-3; Table 7), with highly significant variation between

species and subspecies (F=20431.789, $p \leq 0.001$). The *C. a.* subsp. *arcticum* Attu-17 population was significantly different from all *C. arcticum* populations except for Kenai-1 and Kenai-2 (Table 7). Mean petal lengths were highly significantly different between *C. arcticum* and *C. a.* subsp. *arcticum* (F=7606.809, $p \leq 0.001$), ranging from 1.4 cm (Old Valdez-1, Attu-1 and -2) to 3.2 cm (Attu-11; Table 7) among populations. The Attu-11 mean petal length was significantly longer from all other populations. None of these three floral traits (inflorescence diameter, disc floret diameter, and petal length) can be classified as diagnostic between the species and subspecies.

C. arcticum populations lack ray floret color and disc floret color data since it was not collected during or after plant collecting in 2018 and only seven plants flowered or reached VBD in 2019 (Table 6), although all ray petals observed in the field were white. Ray floret colorations of all *C. arcticum* subsp. *arcticum* populations were uniformly expressed as RHS 155 white (Table 7) while the disc floret colors ranged from RHS 14 yellow to RHS 15 and RHS 17 (Table 7).

None (0%) of the *C. arcticum* individuals flowered during the 2018 experimental year. However, a limited number of plants within some populations of *C. arcticum* subsequently flowered in late 2019, long after *C. a.* subsp. *arcticum* had completed flowering. Thus, only limited flowering data is available for this species since flowering stems collected in the wild needed to be used for all floral traits. Data missing from *C. arcticum* include the number of days to visible bud date (VBD) and to flowering (anthesis) for the majority of the populations of *C. arcticum,* since 96.9% (218/225 individuals) did not initiate

flower buds and 97.8% (220/225) did not reach anthesis during 2018 or 2019 in the greenhouse test environment (Table 6). For the seven plants that did reach VBD and/or flower at the end of 2019, it took >1 year or 457 - 486 d (65.3 - 69.4 wks) among the seven individuals to reach VBD and 462 - 492 d (66 - 70.3 wks) to reach anthesis or flowering (Table 7), the longest period for either trait reported in chrysanthemum (Anderson, 2006; Dole & Wilkins, 1999). The seven individuals were from Anchor Point, Kenai-1, Kenai-3, and Old-Valdez-3 populations (Table 7). Thus, flowering data was collected in different years for both species and subspecies, due to the lengthy delays in *C. arcticum* flowering (Table 7). In contrast with the lengthy amount of time (>1 year) it took the seven *C. arcticum* individuals to flower, the mean number of days for *C. arcticum* subsp. *arcticum* individuals to reach VBD ranged from 48.33 d or 6.9 wks (Attu-5 population) from the start of the experiment to 59.17 d or 8.45 wks (Attu-3) under the same long day photoperiods. The *C. arcticum* subsp. *arcticum* mean number of days to flowering, also termed “response group” (Dole and Wilkins, 1999), ranged from 60 d or 8.57 wks (Attu-5) to 72.25 d or 10.3 wks (Attu-16; Table 7). The mean duration of flower bud development from VBD to anthesis (flowering) in this species was extremely fast, taking as few as 11.67 d or 1.8 wks (Attu-5) to 21.55 d or 3.1 wks (Attu-12) with a pooled mean across all populations of 15.73 d or 2.2 wks.

Due to lack of flowering in 2019, insufficient quantities of flowers occurred in several *C. arcticum* populations. Since fresh/dry weights could not be determined with the inflorescences collected in the wild in 2018 (due to seed

ripening to obtain open-pollinated progeny), most of the flower weight data are missing (Table 7). Mean fresh weight of the flowers ranged from 0.59 g (Old Valdez-3) to 1.28 g (Attu-21; Table 7). In contrast, water loss created mean dry weights ranging from 0.06 g (Old Valdez-3) to 0.17 g (Attu-21; Table 7) / inflorescence. The ΔFlower weight (Fresh weight minus dry weight) values ranged from 0.53 g (Old Valdez-3) to 1.11 g (Attu-21; Table 7). None of these traits would be diagnostic for the species and subspecies.

Pairwise correlations among 16 quantitative variables were primarily positively and significantly correlated (Table 8). Plant height was significantly and positively correlated with inflorescence length (r=0.610), the number of leaves (r=0.311), disc diameter (r=0.207), number of days to VBD (r=0.366) and flowering (r=0.315), fresh (r=0.157) and dry weights (r=0.196; Table 8). Plant height also had a highly significant negative correlation with internode length (r=-0.509), leaf length (r=0.-329), and leaf width (r=-0.466; Table 8). Inflorescence length was positively and significantly correlated with the number of days to VBD (r=0.644) and flowering (r=0.678) but negatively and significantly correlated with internode length (r=-0.718), number of leaves (r=-0.604), leaf length (r=-0.576), leaf width (r=-0.651), petiole length (r=-0.605), lamina length (r=-0.380), and inflorescence diameter (r=-0.199). Inflorescence length was positively correlated with flower dry weight (r=0.034), while negatively correlated with flower fresh weight (r=-0.040) and ΔFlower weight (r=-0.051; Table 8). Overall, plant height and inflorescence length were all negatively correlated with all four quantitative leaf morphological traits (Table 8). The number of leaves was significantly and

positively correlated with internode length (r=0.210), leaf length (r=0.286), leaf width (r=0.326), petiole length (r=0.321), lamina length (r=0.142) but negatively correlated with number of days to VBD (r=-0.180) and flowering (r=-0.177; Table 8). Internode lengths were significantly and positively correlated with leaf length (r=0.610), leaf width (r=0.684), petiole length (r=0.624), lamina length (r=0.444), and flower petal length (r=0.313) but negatively correlated with number of days to VBD (r=-0.365) and flowering (r=-0.295; Table 8). Interestingly, the internode length was highly significantly and positively correlated with inflorescence diameter (r=0.204) whereas it was negatively correlated with disc floret length (r=-0.326).

Leaf length was highly correlated with leaf width (r=0.839), petiole length (r=0.972), lamina length (r=0.837), but not as correlated with flower diameter (r=0.247), flower petal length (r=0.267), flower fresh weight (r=0.052), and ΔFlower weight (r=0.063; Table 8). Leaf length was negatively correlated with disc diameter (r=-0.221), number of days to VBD (r=-0.205) and flowering (r=-0.153). Leaf width was positively correlated with petiole length (r=0.795), lamina length (r=0.749), inflorescence diameter (r=0.212), and flower petal length (r=0.237), while negatively correlated with disc diameter (r=-0.221), number of days to VBD (r=-0.273), flowering (r=-0.231). Leaf width was positively correlated with flower fresh weight (r=0.051) and ΔFlower weight (r=0.061), but negatively correlated with dry weight (r=-0.026). Petiole length was positively correlated with lamina length (r=0.684), inflorescence diameter (r=0.240), while highly significant and negatively correlated with disc diameter (r=-0.206), number of days to VBD

(r=-0.209) and flowering (r=-0.163). Petiole length was positively correlated with flower fresh weight (r=0.045), and ΔFlower weight (r=0.057), but negatively correlated with dry weight (r=0.030). Lamina length was highly significant and positively correlated with inflorescence diameter (r=0.209), flower petal length (r=0.229); was highly significant and negatively correlated with disc diameter (r=-0.207). Lamina length was positively correlated with flower fresh weight (r=0.050), and ΔFlower weight (r=0.057), while negatively correlated with dry weight (r=-0.001).

All four quantitative leaf morphological traits (leaf length, leaf width, petiole length and lamina length) were very significant and highly positively correlated with each other (Table 8) while all were negatively correlated to disc floret diameter, the number of days to VBD and flowering (except for lamina length), respectively (Table 8). Additionally, these four quantitative leaf morphological traits were all positively correlated with fresh weights and ΔFlower weight, while negatively correlated with dry weights, respectively.

The inflorescence diameter was positively and significantly correlated with all other floral traits (disc floret diameter, r=0.273; petal length, r=0.838; fresh weight, r=0.229; dry weights r=0.159; and ΔFlower weight, r=0.238)) while negatively but not significantly correlated with the number of days to flowering (r=-0.003; Table 8). The disc floret diameter was significantly and positively correlated with flower fresh (r=0.208), dry weights (r=0.231) and ΔFlower weight (r=0.202); whereas, negatively correlated with flower petal length (r=-0.022). Apparently, flower petal length was significantly positively correlated with

inflorescence diameter (r=0.838) and negatively correlated with disc floret diameter (r=-0.022). The flower petal length was positively correlated with ΔFlower weight (r=0.150) and fresh weights (r=0.137), while negatively correlated with the number of days to VBD (r=-0.010,) and flowering (r=-0.028; Table 8). The number of days to VBD was positively correlated with the number of days to flowering (r=0.989), and positively correlated with fresh (r=0.086) and dry weights (r=0.075) and ΔFlower weight (r=0.086). The number of days to flowering was positively correlated with flower fresh weight (r=0.104), dry weight (r=0.082) and ΔFlower weight (r=0.105). Fresh and dry weights and ΔFlower weight traits were significantly and positively correlated with each other, as would be expected.

Principal Components Analyses (PCA). For Group one (both species and subspecies analyzed together), the first two principal components (PC1 and PC2) accounted for 50.1% of the variation (Fig. 18a). PC1 accounted for 31.3% of the total variation and was positively associated with number of leaves, leaf length, petiole length, lamina length and leaf width, ray floret diameter, flower petal length, flower fresh weight, dry weight and ΔFlower weight. PC1 was negatively associated with disc floret diameter, plant height, inflorescence length, internode length and the number of days to VBD and flowering (Fig. 18b). PC2 accounted for 18.8% of the total variation and was positively associated with most morphological variables, but negatively associated with leaf and petiole length and leaf width (Fig. 18b). The variable biplot revealed that fresh and dry weights and ΔFlower weight variables were closely clustered; leaf number, leaf

length, petiole length, leaf width and lamina length were clustered together. All individuals were categorized as two groups into *C. arcticum* and *C. a.* subsp. *arcticum* species (Fig. 18c). In the scatter plot, two clusters showed overlapping distribution, yet separated distinctly along the PC1 for 30.1% of the total variance. Compared with *C. arcticum*, the *C. a.* subsp. *arcticum* group was dispersed widely along the PC2 for 18.8% of the total variance and presented more outliers from the ellipses.

For Group two, PCA of 16 morphological variables of the nine populations of *C. arcticum*, 10 principal components were determined with 100% cumulative contribution (Fig. 19a). The first two principal components for Group two analysis, PC1 and PC2 accounted for 43.7% of total variation (Fig. 19b). PC1 accounted for 25.6% of total variance and was positively associated with leaf length, petiole length and lamina length, leaf width, plant height, flower diameter, flower petal length and all three flower weight characteristics. However, it was negatively associated with inflorescence length, flower disc diameter, internode length, and number of days to VBD and flowering. PC2 accounted for 18.1% of the total variance and was positively correlated with leaf width and length, petiole length and lamina length, internode length and inflorescence height, number of days to VBD and flowering. However, it was negatively correlated with inflorescence diameter, disc diameter, plant height, flower petal length, number of leaves, fresh and dry weights, and ΔFlower weight. Four quantitative morphological leaf variables were closely clustered together which were positively associated with PC1 negatively with PC2. Fresh, dry weights, and ΔFlower weights were

clustered closely, associated with both PC1 and PC2 positively. However, instead of the relationship between species and subspecies, *C. arcticum* revealed a highly mixed distribution among the nine populations based on multivariate analysis of the morphological characteristics. According to the scatter plot (Fig. 19c), the Ninilchik population had a wider range of variation within population than other populations for *C. arcticum* species. Four individuals from the Ninilchik population were outliers from the overlapping distribution along with three individuals from Old Valdez-1, Old Valdez-3, and Old Valdez-4.

In contrast, Group three PCA were relatively indistinguishable for the first two principal components (Fig. 20a), compared with Group one (Fig. 18a) and two (Fig. 19a) PCAs. The first two principal components accounted for 43.6% of total variation derived from 16 quantitative morphological traits in the 21 populations of *C. a.* subsp. *arcticum* (Fig. 20a). PC1 accounted for 22.9% of total variance and was positively associated with all variables except for the number of leaves. PC2 accounted for 20.7% of total variance and was highly positively associated with fresh and dry weights, ΔFlower weight, number of days to VBD, flowering, the number of leaves, inflorescence and disc floret diameters, and flower petal length (Fig. 20b). It was negatively associated with leaf, petiole, lamina length and leaf width, plant height and inflorescence length (Fig. 20b). The fresh and dry weights and ΔFlower weight variables were closely clustered together, which were positively associated with both PC1 and PC2. The leaf length, leaf width, petiole and lamina lengths also clustered closely, which were positively associated with PC1 but negatively with PC2. Similar to the Group two analysis, the *C. arcticum*

subsp. *arcticum* individual scatter plot showed a highly overlapping distribution among the 21 populations, based on the morphological characteristics. Individuals within populations tended to disperse along the PC1 instead of PC2.

Discussion

Soil test results overall showed lowered N levels across species and subspecies collection sites (Table 5), which may be consistent with heavy precipitation levels experienced in these sites, ranging from 7.26 cm to 16.84 cm in Attu island, while 6.76 cm to 22.89 cm at Old Valdez, 1.88 cm to 8.46 cm at Kenai, 2.54 cm to 7.57 cm at Anchor Point, and 2.39 cm to 8.66 cm at Ninilchik (Center, W. R. C., 2014). This doesn't mean that either species or subspecies are low N feeders; however, since no evidence of N toxicity occurred in either species or subspecies among all populations throughout this study despite being fed 125 ppm N as constant liquid feed (see Materials & Methods) used for commercial chrysanthemums (Dole & Wilkins, 1999; Van Eysinga, & Smilde, 1980). Thus, these *Chrysanthemum* species utilize available nitrate N (NO_3^-) during the growth phases.

Soil pH levels among both species and subspecies may be a physical diagnostic trait since all of the *C. arcticum* populations grow naturally in the standard soil pH range for the genus (pH=6.2 - 6.8; Table 5) (Dole & Wilkins, 1999; Van Eysinga, & Smilde, 1980) *C. a.* subsp. *arcticum* populations grew in acidic soils at a pH=4.8 (Table 5). The collection sites on Attu Island had high concentrations of sphagnum moss on the soil surface which favors lower pH levels, regardless of whether they were on cliff faces by the ocean or adjacent shoreline areas. Reasons for this site differences may be partially attributable to the high precipitation on Attu Island which allowed for predominance of sphagnum moss growth throughout the island wherein precipitation falls as either

rain or snow every day of the year, although significant rain- and snowfall also occur in the mainland Alaskan sites. Despite the acid-tolerant trait for *C. arcticum* subsp. *arcticum,* no evidence of nutrient deficiencies occurred in any of the individuals during the 2018-2019 experimental period (Van Eysinga, & Smilde, 1980) when the soil pH was maintained within the pH=6.2-6.8 range. Thus, the pH tolerance of *C. arcticum* subsp. *arcticum* has a wider range than that found in the Attu Island soil.

While most of the EC levels were within recommended ranges of low soluble salts (SS), both Anchor Point and Kenai-2 locations were in the saline range (Table 5). However, since these tests only measure SS, rather than Na levels in the soils, the NH_4OAc-Na amounts showed all soil sample sites to be excessively high in Na, in contrast with the greenhouse recommended standard (Table 5) (Roorda van Eysinga & Smilde, 1980). Even the lowest levels at Attu Island (123.94 mg/kg Na) were high but surpassed by the increasingly higher amounts in the maritime sites on the Alaskan mainland where as much as 2445.74 mg/kg was found at Anchor Point, AK (Table 5). These incredibly high levels of Na in the soils adjacent to the ocean indicate a high level of salt tolerance in both species and subspecies which is unusual for any other chrysanthemum species (Anderson, 2006; Dole & Wilkins, 1999). This trait would be of significant interest to chrysanthemum breeding programs throughout the world, providing options for growing chrysanthemums in locations with saline water, in saline soils such as along roadways in northern latitudes where salt is used for ice melt in the winter months, as well as saline conditions in the desert

southwest. Future studies will be devoted to analyzing the levels of salt tolerance in these species and subspecies as well as understanding the mechanism(s) involved.

While the levels of P and K were in the recommended range among the soil samples (Table 5), Anchor Point and Kenai-1 and -2 collection sites were high in K. Other nutrients, such as Zn, Cu, Fe, Mn, Ca, and Mg, were frequently higher than the norms (Table 5) (Van Eysinga, & Smilde, 1980). It was observed, particularly in the Attu Island sites, that significant WWII military waste may have seeped into the soils given the predominance of oil barrel dumps throughout the island where the 21 populations of *C. a.* subsp. *arcticum* were collected. Such tolerance to these micronutrients and heavy metals may indicate potential use of either species or subspecies in soil mitigation. Future studies will examine the levels of heavy metals in the leaves of Arctic daisy to determine whether they are sequestered therein.

High mortality rates of 7.7% to 45.9% among the *C. arcticum* populations is noteworthy, particularly since the pH range was kept within that found for the native soils (Table 6). These are in contrast with 0% mortality among all *C. a.* subsp. *arcticum* populations (Table 6). We observed that the dead individuals had root rot from unknown pathogens despite having routine applications of fungicide rotations applied. The specific reasons for this high mortality are unknown and deserve attention in future research.

Plant height of the Attu Island *C. a.* subsp. *arcticum* populations were all significantly shorter than most *C. arcticum* populations (Table 7), with the notable

exception of all Old Valdez populations. It is unclear why the Old Valdez populations were significantly shorter than their counterparts from Ninilchik, Anchor Point or Kenai locations; it could be an evolutionary adaptive change since all populations are reproductively isolated due to highly mountainous terrain, although inbreeding depression could also be a possibility. Further research would be necessary to determine the reproductive barriers operating in both species and subspecies. Presumably, the species is self-incompatible, since most other *Chrysanthemum* species possess this reproductive barrier (Anderson et al., 1988; Zagorski et al., 1983). However, if either or both of these species and subspecies were diploid it is possible they are self-compatible, which could limit gene exchange within isolated populations and lead to reduced plant height (Anderson et al., 1992).

Other morphological traits recorded did not prove to be distinguishing diagnostic characteristics between the species and subspecies, including leaf number of the primary stem, internode and leaf lengths, leaf width, petiole and laminar lengths, leaf color, leaf shape, leaf margins, inflorescence and disc floret diameters, ray and disc floret colors, and flower petal lengths (Table 7). Frequently, related leaf or floral traits were highly and significantly correlated with each other, as would be expected (Table 8). The number of leaves is similar in range to those reported for cultivated *C.* x*grandiflorum* and *C.* x*hybridum* (Anderson et al., 2008). Inflorescence length is a diagnostic trait, based on the significant differences among the species and subspecies with all individuals of

C. a. subsp. *arcticum* having significantly shorter inflorescences than all *C. arcticum* (Table 7).

In this study we integrated conventional multivariate analysis techniques for 16 quantitative and five qualitative morphological characteristics for 525 individuals (Group one) to determine phenotypic differences between *C. arcticum* and *C. a.* subsp. *arcticum* and among populations within species and subspecies. The phenotypic data we collected were consistent with the historic record for *C. arcticum* and *C. a.* subsp. *arcticum* and were applied to identify the variation between the species and subspecies (Bremer & Humphries, 1993; Hultén, 1968; Nishikawa & Kobayashi, 1989; Porsild, 1957). According to Nishikawa and Kobayashi's study (1989), *C. a.* subsp. *arcticum* leaves tend to have a finely shallow sinus compared with *C. arcticum* species' deep and regularly toothed leaf margins. As expected, our collection (Table 7) matched the historic taxonomic records. *Chrysanthemum a.* subsp. *arcticum* populations tend to have more crenate leaf margins, while *C. arcticum* had more tripartite and lobed leaf margins (Table 7). The significant variation pooled among populations revealed by the Chi-square test for leaf margins indicated the diagnostic difference between *C. arcticum* and *C. a.* subsp. *arcticum* species (Table 7).

Consistently, it was found in the Group one PCA that *C. arcticum* and *C. a.* subsp. *arcticum* were primarily separated as taxa (Fig. 18c). This distinguishable classification agrees with the genetic variation of SNP markers between *C. arcticum* and *C. a.* subsp. *arcticum* found previously (Liu, 2020). The common morphological traits that *C. arcticum* and *C. a.* subsp. *arcticum* shared

could be related to the overlapping distribution on the individual scatter plot for all individuals. However, this relatedness may be decreased due to the lack of gene flow between species, caused by the restrictive geographical separation (Liu, 2020).

Since both species normally flower during the summer months (long day photoperiods) throughout the distributional range of Alaska (United States), Kamchatka (Russia), Hokkaido (Japan), and British Columbia, Hudson Bay (Canada), the species are considered to be long day plants for flower bud initiation (occurring prior to VBD) and flowering. All *C. arcticum* plants were at peak flowering in late July 2018 during collection trip, which means they would have initiated and developed flower buds during long day photoperiods. Our data with greenhouse forcing confirm this particularly well with *C. a.* subsp. *arcticum* (Table 7), 100% of which flowered in both years under long day conditions (16 hr photoperiod). The significant lack of flowering within all populations of *C. arcticum* over a two-year period in the present study (2018-2019; Table 6) is curious. While a few individuals reached VBD (3.1%; Table 6) and flowered (2.2%) successfully, albeit after ~1.5 years had passed under inductive long day photoperiods, clearly another factor(s) of plant growth is required for *C. arcticum* to reach VBD and flower successfully, as occurs in the wild. We postulate that Na levels in the soil or salt spray along the oceanic coasts may be a potential primary factor in the flowering process for this species. Future research will be devoted to this question to understand the factor(s), particularly Na, and physiological mechanisms of this unusual phenomenon within *C. arcticum.* The

long day flowering in the *Chrysanthemum arcticum* complex germplasm is the opposite of what is found in cultivated *C.* x*grandiflorum* and *C.* x*hybridum* (Anderson et al., 2008; Dole & Wilkins, 1999) which are short day plants (8 hr photoperiod). However, *C. arcticum* and *C. arcticum* subsp. *arcticum,* as long day plants, would be similar to some other chrysanthemum species, such as pyrethrin, *C. cinerariifolium* (Brewer, 1968).

Flower bud initiation (occurring prior to VBD) and development (flowering or anthesis) occurred extremely rapidly in all populations of *C. arcticum* subsp. *arcticum* (Table 7), i.e. 48.33 to 59.17 d (or 6.9 to 8.45 wks) and 60 to 72.25 d (or 8.57 to 10.3 wks). At the time of collection, all terminal meristems of *C. arcticum* subsp. *arcticum* were examined under a dissecting microscope and determined to be in a vegetative state. The mean number of days to VBD = 15.73 d or 2.2 wks is the fastest ever reported within the genus (Dole & Wilkins, 1999), with some individuals initiating flower buds in the mist house while rooting. In contrast, the number of days to VBD and flowering in the seven individuals of *C. arcticum* took significantly longer periods of time, i.e. 457 to 486 d (or 65.3 to 69.4 wks) and 462 to 492 d (or 66 to 70.3 wks), respectively (Tables 1, 3).

By mid-July 2018 (~week 29), all of the *C. arcticum* plants in the wild were at peak flowering (Anderson N. O., unpublished data) and, based on field observations as well as greenhouse trials in the current experiment, neither species re-flowers in the same season. Thus, the observed flowering period was shorter than previously reported for either species or subspecies, e.g. flowering was noted in historic specimens to occur from May 21 (week 21) to September

25 (week 39) during the growing season across the geographical distributional range (Liu, 2020). This shortened flowering period was assumed to be related to environmental factors, possibly global warming temperatures, that may have caused widespread extinction of the species, since they were reported as "common" in the historic records (as far back as 1865; N. Anderson, unpublished data) as well as taxonomic reports (Polunin, 1948). This trend agrees with the previous warming simulation studies (Kaplan & New, 2006), which showed major northward shifts and significant reductions of the tundra biomes in the Arctic, becoming restricted to coastal and mountainous areas. From the perspective of conservation, future research will launch analogous analyses on our extensive collection of herbarium specimens on morphological traits for *C. arcticum*, *C. a.* subsp. *arcticum* and *C. a.* subsp. *polaré* and other related species, which will contribute to determine the extent and magnitude of a potential genetic bottleneck in the species occurring over time. Likewise, a morphological and genetic (SNP) study of *C. a.* subsp. *polaré* populations in Alaska and Canada will be possible as soon as extant populations are collected to confirm whether or not this subspecies is similar to or divergent from both *C. arcticum* and *C. a.* subsp. *arcticum*.

Previous studies on chrysanthemum species and cultivar variation based on morphological characteristics, tended to focus on specific morphological traits with ornamental market value, such as inflorescence morphology and chemical composition (Hodaei et al., 2017; Song et al., 2018) or descriptive traits for plant patents (Anderson et al., 2008). The extensive morphological data sets,

especially on qualitative and quantitative traits that best discriminate between species and populations of *C. arcticum, C. a.* subsp. *arcticum* and *C. a.* subsp. *polaré* is a valuable resource for future research. This morphological dataset will be enhanced with additional traits to facilitate the identification of phenotypes among species and populations and provide opportunities for marker assisted selection.

As noted earlier, the morphological variation among populations may not be distinguished significantly based on principal components analysis, although taxa were for specific traits. This may be associated with a close relatedness for individuals within and among populations. Meanwhile, the morphological variables we selected could fall into the common traits shared for species and subspecies or indistinguishable enough as diagnostic traits. However, the univariate analysis of variance provided a significant variation among populations consistent to our previous genetic variation studies. For example, for the *C. arcticum* extant populations, Old Valdez and Kenai populations tended to be significantly different from each other based on a majority of variables (Fig. 19): inflorescence length, number of leaves, internode length, inflorescence diameter, flower petal length. In contrast, Anchor Point and Ninilchik populations showed an intermediate tendency between Old Valdez and Kenai populations. The ANOVA among populations of *C. arcticum* showed a good consistency with our previous genetic structure analyses, suggesting that the variation between Old Valdez populations cluster and Kenai populations cluster contributed to the most significant variation among populations within the species. The variance among

Anchor Point and Ninilchik populations contributed to the total variance secondarily (*cf.* PCoA analyses, Liu, 2020).

The phenotypic variation noted in this study is in agreement with the genetic relatedness revealed in the UPGMA phylogenetic tree (based on using Jaccard genetic distance) described in Liu, 2020. A detailed genetic distance within each collection sites, such as for Old Valdez -1 and -2 populations in the same genetic ward whilst Old Valdez -3 and -4 populations were successively distant related (Fig. 14, Liu, 2020). The phenotypic variation obtained from this study in agreement with the genetic relatedness, Old Valdez populations, especially for Old Valdez -1 and -2 groupings, very significantly different from other populations for the majority of morphological traits (Table 7). Additionally, the variance among populations based on morphological characteristics reconfirmed that the geographical isolation might be the major reason leading to the genetic and phenotypic variation among populations within *C. arcticum*.

In the ANOVAs, *C. a.* subsp. *arcticum* populations differed significantly from *C. arcticum* populations for the majority of morphological traits, although the range in variation within and among populations and species created overlap of many morphological traits (Table 7). This might be expected, since similarly indistinct clustering with the SNP cluster analysis for *C. a.* subsp. *arcticum* occurred*,* which verified the possibility of more frequency gene flow among Attu island collection sites (Liu, 2020). The Attu populations geographical distributions were not as far apart as the *C. arcticum* populations were from each other. However, the UPGMA phylogenetic tree from SNPs revealed a detailed genetic

distance among populations and presented a relatively close genetic relatedness in groupings (Liu, 2020), which were consistent with some of the phenotypic relationships among populations (Table 7; Fig. 20). For example, the Attu-8 and Attu-9 populations differed from Attu-1 to -7 populations significantly for internode length, leaf length, leaf width, which is in agreement with the Attu-8 and Attu-9 SNP populations groupings in the phylogenetic tree (Liu, 2020). Interestingly, the Attu-15, -16 and -17 populations differed significantly from other populations for the majority of morphological traits whereas the genetic SNP analysis did not present significant variation between these groupings and other populations (Liu, 2020). Testing these populations in additional environments would provide useful data on genotype x environment interactions or the stability of trait expression.

Correlations showed that plant height was positively related to all the flower morphological variables except flower petal length (including inflorescence length, inflorescence diameter, disc floret diameter, flower weight), which confirmed a robust vegetative growing would benefit the reproductive growth (Table 8). As expected, leaf morphology showed a significantly positive correlation among leaf variables, such as leaf, petiole, lamina lengths and leaf width for both *C. arcticum* and *C. a.* subsp. *arcticum* (Table 8). Floral morphological traits were also interrelated with significantly positive correlations among variables: inflorescence diameter, disc diameter, flower petal length, and a series of flower weight characteristics (Table 8). Other pairs of variables were inevitably correlated, such as inflorescence diameter and fresh weight (Table 8). As noted before, more morphological traits will be considered in the future

research, such as pollen and seed morphological characteristics, presence of chemical compounds such as pyrethrin, ploidy and/or reproductive barrier(s) (Hodaei et al., 2017; Kim et al., 2014; Uehara et al., 2017). A thorough cytological study would be useful with this expansive germplasm collection within the *C. arcticum* species complex, since reported ploidy levels differ and differing levels may be diagnostic traits for the species and subspecies (Brouillet, 1987).

Besides the perspective of conservation, a better understanding of variation among species and populations will facilitate selection and use of advantageous traits. Since the production of chrysanthemum in the greenhouse often encounters high salinity, which is caused by the high irrigation frequency and high evapotranspiration (Liu et al., 2013), salt tolerance in chrysanthemum is becoming imperatively in the response to the growing demands of comprehensive chrysanthemum products worldwide, and the spreading application of automatic irrigation and environmental control systems (Anderson, 2006; Anderson et al., 2008; Tsirogiannis et al., 2010). At the same time, soil salinization is a growing problem in Minnesota and worldwide. Salt accumulation in soils is mainly derived from snow melting agents, which would harm the garden mums. *Chrysanthemum arcticum* and its subspecies only grow in maritime habitats throughout Alaska and Canada as well as in acidic soils on Attu Island (Table 5), making it perfectly suited for developing salt-tolerant landscape perennials from these species and subspecies.

With the anticipated addition of *C. arcticum* subsp. *polaré* populations to the current germplasm bank, we will have a comprehensive genetic and

morphological dataset. A series of studies will be aimed to combine the genetic (SNP marker) data and phenotypic datasets would be expected. Genome-wide association study (GWAS) would be an effective approach that can associate individuals with phenotypes effectively and simultaneously detect allelic variations and candidate genes from a pre-established set of germplasm (Chong et al., 2017; Li et al., 2016; Sun et al., 2017). Previous studies have detected on detecting SNPs associated with important horticultural traits via GWAS (Chong et al., 2017). Sequencing the genomes, coupled with marker assisted selection will be valuable tools in furthering research on the species in the *Chrysanthemum arcticum* species complex.

List of tables.

Table 5. Chapter 3 Soil test results (Spurway Greenhouse, Florist, & Nursery Crops test, Soil Testing Laboratory, University of Minnesota) (Erwin 1998) from select Alaskan populations of *C. arcticum* (Anchor Point-1, Kenai-1, Kenai-2, Ninilchik-1, Old Valdez-1, -2, -3, -4) and *C. a.* subsp. *arcticum* (Attu Island) with greenhouse standard for crops, including chrysanthemum.

Species	Sample ID	NO_3-N (mg/kg soil)	SO_4-S (mg/kg soil)	Bray P (mg/kg soil)	NH_4OAc-K (mg/kg soil)	LOI OM (%)	Water pH	1:1 Elec. Conductivity (mmhos/cm)	Sat. Paste Elec. Cond. (mmhos/cm)	Hot Water Boron (mg/kg soil)	DTPA-Fe (mg/kg soil)	DTPA-Mn (mg/kg soil)	DTPA-Zn (mg/kg soil)	DTPA-Cu (mg/kg soil)	Exchangeable NH_4OAc-K (mg/kg soil)	Exchangeable NH_4OAc-Ca (mg/kg soil)	Exchangeable NH_4OAc-Mg (mg/kg soil)	Exchangeable NH_4OAc-Na (mg/kg soil)
C. arcticum	Anchor Point	0.54	130	15.5	280	13.8	6.1	7.6	14.7	2.182	373.2	8/418	0.986	2.001	247.52	515.65	710.89	2445.74
C. arcticum	Kenai-1	0.78	14	3	278	6.7	6.7	2.6	4.8	2.14	197.86	18.420	1.806	5.619	204.27	362.59	462.59	873.28
C. arcticum	Kenai-2	1.06	37	5	247	15	6.4	5.5	7.9	3.545	261.20	17.562	4.197	4.848	237.79	557.90	646.67	1689.44
C. arcticum	Ninilchik	0.90	6	15	150	3.8	6.8	0.1		0.629	145.15	5.537	1.364	1.423	155.08	261.24	291.22	224.88
C. arcticum	Old Valdez-1	0.65	13	8	147	12.2	6.7	0.5		2.217	141.29	16.769	3.845	5.111	145.55	611.51	519.35	310.64
C. arcticum	Old Valdez-2	2.02	12	6	95	9.7	6.8	0.4		1.893	97.913	5.715	2.487	3.643	106.07	471.71	437.85	336.79
C. arcticum	Old Valdez-3	3.85	11	5	124	5.8	6.9	0.4		2.319	45.515	6.071	2.206	4.697	131.37	631.12	481.05	185.78
C. arcticum	Old Valdez-4	0.56	16	6	120	6.3	6.9	1.5	2.5	2.134	186.25	91.393	4.419	4.922	119.15	381.27	346.09	472.29
C. a. subsp. *arcticum*	Attu Island	<0.05 ppm	12	7	147	49.5	4.8	0.5		0.490	296.83	25.374	12.962	3.306	136.57	316.68	211.31	123.94
	Greenhouse Standard – Spurway	150-180	N/A	5-10	50-60	33-100	6.2-6.5	1.2-1.8		0.25-0.5	0.25-0.5	0.25-0.5	0.25-0.5	N/A	50-60	120-180	40-60	N/A
	Greenhouse Standard – Saturated Paste	100-199	N/A	10-15	150-250	33-100	6.2-6.5		2.0-4.0	0.1-0.5	0.3-3	0.3-3	0.3-3	0.01-0.1	150-250	200-300	50-125	N/A

Table 6. Chapter 3 *Chrysanthemum. arcticum* (nine populations) and *C. a,* subsp. *arcticum* data for clonal ramets (rhizomes rooted from the individuals collected in the wild) grown in the greenhouse during the 2018-2019 experimental period: plant death (number, %), number of plants flowering (%), range (mean) number of days to visible bud date (VBD) and range (mean) number of days to flowering.

Species	Population	Plant death number (%)	Number of plants flowering (%)
C. arcticum	Old Valdez-1	17/37 (45.9%)	0 (0%)
	Old Valdez-2	5/18 (27.8%)	0 (0%)
	Old Valdez-3	8/23 (34.8%)	1/23 (4.3%)
	Old Valdez-4	5/15 (33.3%)	0 (0%)
	Anchor Point	11/40 (27.5%)	3/40 (7.5%)
	Ninilchik	1/13 (7.7%)	0 (0%)
	Kenai-1	11/32 (34.4%)	2/32 (6.2%)
	Kenai-2	7/22 (31.8%)	0 (0%)
	Kenai-3	11/25 (44.0%)	1/25 (4.0%)
C. a. subsp. *arcticum*	Attu	0/326 (0%)	321/326 (98.5%)

Table 7. Chapter 3 Mean values of *C. arcticum* (nine populations) and *C. a.* subsp. *arcticum* (21 populations) for 21 plant morphological traits (2018 data: *C. arcticum* plant height, inflorescence length, number of leaves, internode length, inflorescence diameter, disc floret diameter, flower petal length, flower fresh weight, flower dry weight and Δflower weight; 2019 data: *C. arcticum* leaf length, leaf width, petiole length, lamina length and three qualitative traits (adaxial leaf color, leaf shape, leaf margin), number of days to visible bud date, number of days to flowering and *C. a*. subsp. *arcticum* all 21 morphological traits). Mean separations within traits (columns), are based on Tukey's 5% HSD. Chi-square tests of two qualitative phenotypic traits (leaf shape, leaf margin) tested with equal probability of occurrence (1:1:1:1:1 χ2). One asterisk (*) indicate a significant variation (p<0.05); two asterisks (**) indicate a highly significant variation (p<0.01).

Population	Plant height (cm)	Inflorescence length (cm)	No. of leaf (ea)	Internode length (cm)	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)	Lamina length (cm)	Adaxial leaf color (RHS)	Leaf shape							Leaf margin							Inflorescence diameter (cm)	Disc floret diameter (cm)	Petal length (cm)	Ray floret color (mature; RHS)	Disc floret color (mature; RHS)	No. days to VBD (days)	No. days to flowering (days)	Fresh weight (g)	Dry weight (g)	Water in fresh flower (ΔFLower weight = Fresh weight – dry weight; g)
										Flabellate	Hastate	Oblong	Pandurate	Round	χ2	p value	Cleft	Crenate	Entire	Lobed	Tripartite	χ2	p value										
C. arcticum																																	
Old Valdez-1	37.1 cde	20.9 cd	10.0 ab	4.0 h	7.5 abcd	2.5 ab	4.6 abc	3.0 abcd	137	17	3	0	0	6	111	0.402	0	2	5	12	7	148	0.393	4.3 a	1.6 bcd	1.4 a	-	-	-	-	0.71	0.98	0.61
Old Valdez-2	36.0 bcde	21.4 d	9.8 a	3.9 h	6.5 ab	2.7 ab	4.0 ab	2.6 ab	137	9	1	0	4	1	72	0.347	1	2	0	4	8	72	0.347	4.5 ab	1.6 bcd	1.4 ab	-	-	-	-	-	-	-
Old Valdez-3	29.0 abc	20.3 cd	10.5 abc	2.9 efgh	5.7 a	2.1 a	3.3 a	2.4 a	137	8	5	0	1	2	92	0.364	0	2	0	5	9	69	0.376	5.1 ab	1.8 d	1.7 abcd	-	-	464	472	0.59	0.06	0.53
Old Valdez-4	37.3 cdef	17.9 bcd	11.3 abcd	3.5 gh	7.5 abcd	2.6 ab	4.8 abcd	2.7 ab	137	8	1	0	1	3	60	0.333	0	3	1	5	4	60	0.333	4.6 ab	1.7 cd	1.5 abc	-	-	-	-	0.41	0.04	0.37
Anchor Point	42.6 ef	18.7 bcd	12.1 abcde	3.8 h	9.5 abcdefgh	2.9 abc	5.8 abcdef	3.7 abcdefg	137	15	5	1	3	4	200	0.388	3	9	2	7	8	200	0.388	5.3 ab	1.5 bcd	1.9 abcd	-	-	466.7	473.3	1.02	0.15	0.87
Ninilchik	47.1 f	12.2 b	18.1 abcdefgh	2.7 defgh	10.6 bcdefghi	3.0 abc	6.4 abcdefg	4.2 bcdefghi	137	9	0	0	2	0	26	0.353	0	0	1	8	2	39	0.336	5.2 ab	1.6 bcd	1.8 abcd	-	-	-	-	1.32	0.20	1.12
Kenai-1	41.1 ef	14.1 bc	13.8 abcdefg	3.1 fgh	8.7 abcdef	2.7 ab	5.5 abcde	3.3 abcde	137	8	12	0	2	1	128	0.385	5	0	0	6	12	96	0.395	4.9 ab	1.5 abcd	1.7 abcd	-	-	464	464	1.11	0.18	0.93
Kenai-2	44.2 ef	15.6 bcd	13.8 abcdefg	3.3 gh	7.2 abc	2.6 ab	4.4 abc	2.8 abc	137	12	4	0	1	1	88	0.361	0	7	1	6	4	88	0.361	5.4 ab	1.5 abcd	1.9 abcd	-	-	-	-	-	-	-
Kenai-3	40.1 def	16.3 bcd	13.3 abcdef	3.2 fgh	8.0 abcde	2.6 ab	4.8 abcd	3.1 abcde	137	12	8	0	1	0	75	0.381	3	5	1	4	8	125	0.359	5.6 ab	1.6 bcd	2.0 abcd	-	-	464	475	0.69	0.13	0.56
C. a. subsp. *arcticum*																																	
Attu-1	21.3 a	3.4 a	27.1 ijkl	0.9 a	9.1 abcdefg	3.7 abc	6.2 abcdefg	2.9 abcd	137	12	0	0	7	0	72	0.379	1	7	0	4	11	96	0.367	4.9 ab	1.6 bcd	1.7 a	155	17	52.8	67.3	0.90	0.13	0.77
Attu-2	30.1 abc	1.6 a	28.5 jkl	1.1 ab	11.3 cdefghi	4.3 cde	8.6 efgh	2.7 abc	137	2	0	0	0	0	-	-	2	0	0	0	0	-	-	4.9 ab	1.6 bcd	1.7 a	155	17	48.5	61.0	0.91	0.11	0.81
Attu-3	23.1 a	3.0 a	27.5 ijkl	1.0 ab	10.3 bcdefghi	4.4 cde	7.2 bcdefg	3.1 abcde	137	2	1	0	1	3	21	0.279	1	3	0	2	7	21	0.279	4.8 ab	1.7 cd	1.6 abcd	155	17	59.2	75.0	1.21	0.16	1.05
Attu-4	24.9 a	3.2 a	23.6 hijk	1.3 abc	12.7 fghij	4.4 cde	8.9 fgh	3.8 abcdefgh	137	15	1	0	13	7	108	0.401	0	12	0	9	15	72	0.411	5.2 ab	1.5 abcd	1.9 abcd	155	14	57.5	73.6	1.04	0.14	0.90
Attu-5	21.4 a	2.4 a	23.5 hijk	0.9 a	11.6 cdefghi	4.4 cde	8.0 defgh	3.6 abcdef	137	1	1	0	2	2	18	0.263	0	3	1	0	2	12	0.285	5.3 ab	1.5 abcd	1.9 abcd	155	14	48.3	60.0	0.84	0.11	0.74
Attu-6	26.4 ab	3.5 a	30.6 jk	0.9 a	11.2 cdefghi	5.4 ef	7.5 cdefgh	3.7 abcdefg	138	0	4	0	1	0	5	0.287	0	0	0	0	5	-	-	5.0 ab	1.5 bcd	1.7 abcd	155	14	48.6	60.4	1.18	0.14	1.03
Attu-7	25.6 a	3.1 a	33.7 k	0.8 a	13.1 fghij	5.7 efg	8.9 fgh	4.1 abcdefgh	137	5	0	0	2	0	16	0.313	0	3	0	2	2	24	0.293	5.3 ab	1.6 bcd	1.9 abcd	155	14	56.6	70.6	1.12	0.15	0.97
Attu-8	26.0 a	4.0 a	17.7 abcdefgh	1.6 abcd	13.2 fghij	4.9 de	8.8 efgh	4.4 cdefghi	137	23	3	0	13	3	126	0.408	6	13	0	8	15	126	0.408	5.6 ab	1.5 bcd	2.1 bcd	155	14	52.1	67.8	0.95	0.11	0.83
Attu-9	26.8 ab	4.1 a	17.6 abcdefgh	1.6 abcde	13.7 hij	4.8 de	9.5 ghi	4.2 bcdefgh	137	28	5	0	3	8	180	0.402	1	15	6	14	8	225	0.394	5.3 ab	1.5 abcd	2.0 abcd	155	17	54.8	72.5	1.00	0.11	0.90
Attu-10	30.0 abc	3.5 a	19.0 bcdefghi	1.6 abcde	13.0 fghij	5.1 def	9.0 fghi	4.0 abcdefgh	137	4	2	0	3	1	30	0.314	0	5	0	1	4	20	0.333	5.6 b	1.4 abc	2.1 cd	155	14	57.0	75.8	0.89	0.11	0.78
Attu-11	30.3 abcd	4.4 a	19.5 defghij	1.7 abcde	12.1 efghij	4.4 cde	8.2 cdefgh	4.0 abcdefgh	137	2	0	0	4	0	6	0.306	0	1	0	2	3	12	0.285	5.6 ab	1.4 abcd	3.2 e	155	7	55.5	72.2	0.91	0.11	0.80
Attu-12	29.0 abc	3.3 a	22.4 ghijk	1.4 abc	12.2 efghij	4.3 cde	8.3 efgh	3.9 abcdefgh	137	5	3	0	10	3	63	0.371	4	4	3	4	6	84	0.358	5.7 b	1.5 abcd	2.1 bcd	155	14	53.8	75.3	0.90	0.11	0.80
Attu-13	26.8 ab	3.8 a	19.7 defghij	1.4 abcd	12.0 defghij	4.8 de	8.1 cdefgh	3.9 abcdefgh	137	19	4	0	5	4	96	0.395	0	18	1	6	7	96	0.395	5.4 ab	1.4 abc	2.0 abcd	155	14	54.8	71.3	1.02	0.12	0.90
Attu-14	25.3 a	3.7 a	21.5 ghij	1.3 abc	13.0 fghij	5.1 def	8.8 efgh	4.2 bcdefgh	137	8	2	0	11	5	78	0.384	0	9	3	6	8	78	0.384	5.5 ab	1.4 abc	2.1 bcd	155	14	55.2	70.4	1.15	0.15	1.00
Attu-15	28.3 abc	2.8 a	14.7 abcdefgh	2.1 abcdef	16.2 jk	7.1 g	10.7 hi	5.5 hi	137	1	1	0	1	0	6	0.199	0	1	0	0	2	3	0.223	4.8 ab	1.4 abcd	1.7 abcd	155	-	49.0	62.7	1.02	0.12	0.90
Attu-16	29.9 abc	3.9 a	13.5 abcdefg	2.4 cdefg	14.5 ijk	5.6 efg	9.5 ghi	5.0 fghi	138	0	2	0	2	0	4	0.261	0	2	0	1	1	8	0.238	5.8 b	1.4 abc	2.2 d	155	15	57.8	72.3	0.94	0.12	0.82
Attu-17	28.3 abc	5.5 a	13.5 abcdefg	2.2 bcdefg	18.3 k	6.5 fg	12.4 i	6.0 i	137	0	1	0	1	0	2	0.157	0	0	0	0	2	-	-	5.6 ab	1.2 a	2.2 d	155	-	50.0	67.5	0.75	0.10	0.65
Attu-18	30.1 abc	4.3 a	18.8 abcdefghi	1.7 abcde	13.6 ghij	5.2 def	9.0 fgh	4.7 efghi	139	7	5	0	5	1	54	0.36	0	7	0	5	6	36	0.375	5.0 ab	1.3 ab	1.8 abcd	155	14	54.7	70.8	0.92	0.11	0.80
Attu-19	30.0 abc	3.9 a	20.4 efghij	1.5 abcd	13.7 hij	5.3 ef	9.2 fghi	4.5 defghi	137	8	2	0	2	2	32	0.342	5	5	0	1	3	42	0.342	5.2 ab	1.4 abcd	2.0 abcd	155	14	52.6	67.6	0.98	0.13	0.85
Attu-20	28.3 abc	3.6 a	19.2 cdefghi	1.5 abcd	14.0 hijk	5.6 efg	8.7 efgh	5.3 ghi	137	8	1	0	2	2	56	0.327	1	1	9	1	2	56	0.327	5.7 b	1.4 abcd	2.1 cd	155	14	51.2	69.2	1.12	0.14	0.98
Attu-21	29.8	6.7	-	1.5	13.6	6.15	8.2	5.4	139	0	1	0	0	0	-	-	0	0	0	0	1	-	-	4.5	1.2	1.7	155	17	51	69	1.28	0.17	1.11
Among populations	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	0.000**	-						272.639	0.000**						283.693	0.000**	0.000**	0.000**	0.000**	-	-	-	-	-	-	-

Table 8. Chapter 3 Correlations between 16 quantitative plant morphological traits for *C. arcticum* (225 individuals) and *C. arcticum* subsp. *arcticum* (326 individuals) (2018 data: *C. arcticum* plant height, inflorescence length, number of leaves, internode length, inflorescence diameter, disc floret diameter, flower petal length, flower fresh weight, flower dry weight and Δflower weight; 2019 data: *C. arcticum* leaf length, leaf width, petiole length, lamina length, number of days to visible bud date, number of days to flowering; and *C. a.* subsp. *arcticum* all 16 quantitative morphological traits). An asterisk (*) indicates a significant correlation coefficient ($P \leq 0.05$), two asterisks (**) indicate a highly significant correlation coefficient ($P \leq 0.01$) whereas a lack of any asterisk(s) denotes not significant.

	Plant height (cm)	Inflorescence length (cm)	No. of leaf (ea)	Internode length (cm)	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)	Lamina length (cm)	Inflorescence diameter (cm)	Disc floret diameter (cm)	Flower petal length (cm)	No. days to VBD	No. days to flowering	Fresh weight (g)	Dry weight (g)	ΔFlower weight (g)
Plant height (cm)	1															
Inflorescence length (cm)	0.610**	1														
No. of leaf (ea)	-0.311**	-0.604**	1													
Internode length (cm)	0.647**	0.800**	0.210**	1												
Leaf length (cm)	-0.329**	-0.576**	0.286**	0.610**	1											
Leaf width (cm)	-0.466**	-0.651**	0.326**	0.684**	0.839**	1										
Petiole length (cm)	-0.367**	-0.605**	0.321**	0.624**	0.972**	0.795**	1									
Lamina length (cm)	-0.169**	-0.380**	0.142**	0.444**	0.837**	0.749**	0.684**	1								
Inflorescence diameter (cm)	0.027	-0.199**	0.226**	0.204**	0.247**	0.212**	0.240**	0.209**	1							
Disc floret diameter (cm)	0.207**	0.333**	-0.013	-0.326**	-0.221**	-0.238**	-0.206**	-0.207**	0.273**	1						
Flower petal length (cm)	-0.019	-0.273**	0.198**	0.313**	0.267**	0.237**	0.258**	0.229**	0.838**	-0.022	1					
No. days to VBD	0.366**	0.644**	-0.180**	-0.365**	-0.205**	-0.273**	-0.209**	-0.135*	0.018	0.083	-0.010	1				
No. days to flowering	0.315**	0.678**	-0.177**	-0.295**	-0.153**	-0.231**	-0.163**	-0.087	-0.003	0.096	-0.028	0.989**	1			
Fresh weight (g)	0.157**	-0.040	0.096	0.048	0.052	0.051	0.045	0.050	0.229**	0.208**	0.137*	0.086	0.104	1		
Dry weight (g)	0.196**	0.034	0.130*	-0.105	-0.023	-0.026	-0.030	-0.001	0.159**	0.231**	0.047	0.075	0.082	0.904**	1	
ΔFlower weight (g)	0.148*	-0.051	0.089	0.071	0.063	0.061	0.057	0.057	0.238**	0.202**	0.150**	0.086	0.105	0.997**	0.873**	1

List of figures.

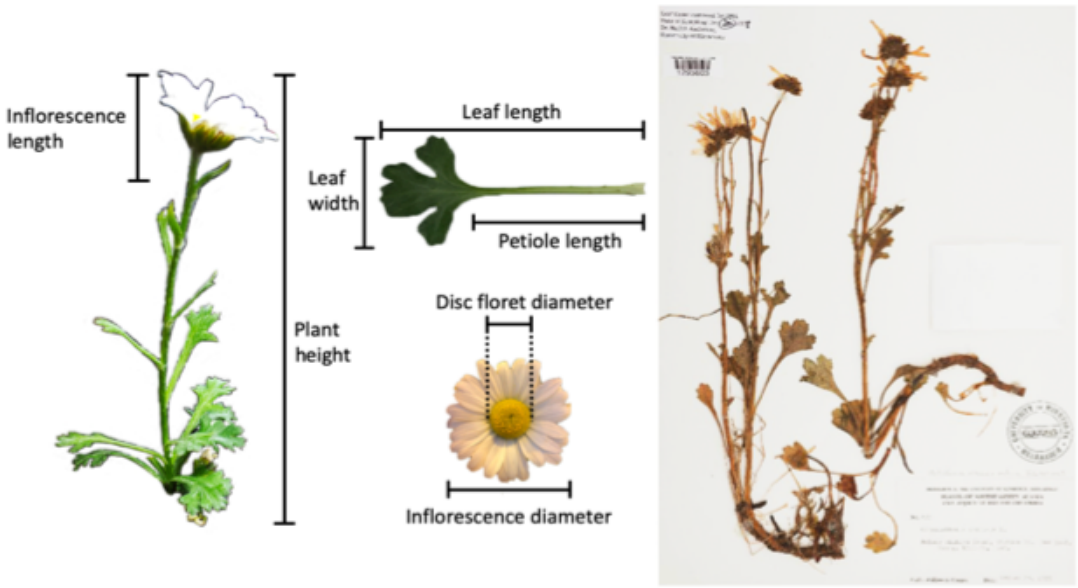

Figure 17. Chapter 3 Basic survey standard (left) of an extant specimen versus an historic or herbarium specimen (right) of *C. arcticum* species (Old Valdez-1).

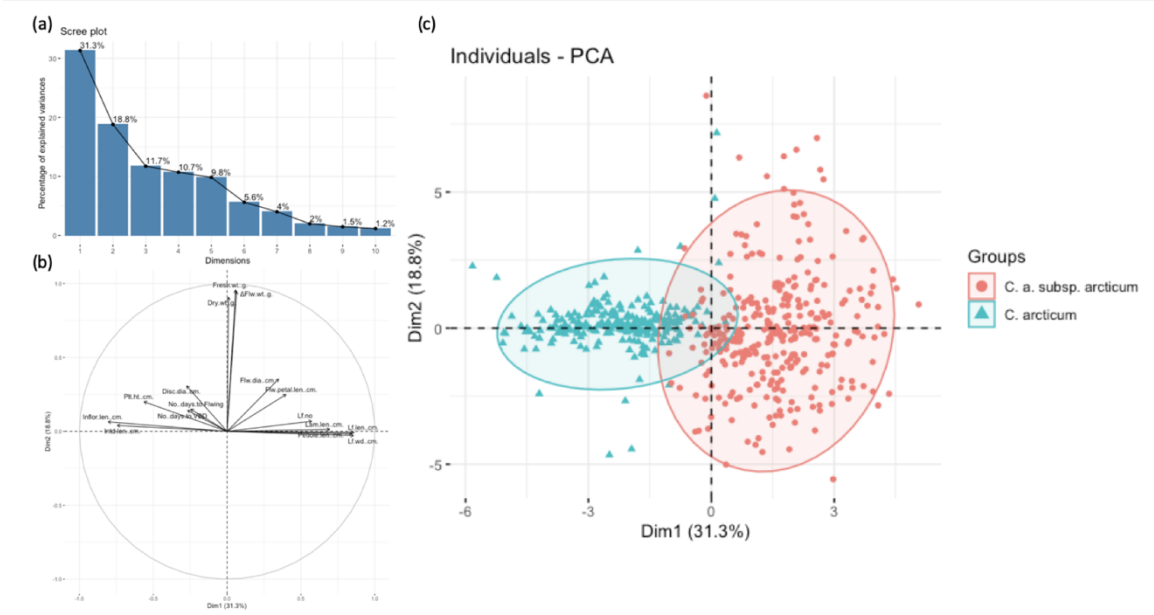

Figure 18. Chapter 3 Principal components analysis (PCA) for *C. arcticum* and *C. a.* subsp. *arcticum* for 16 quantitative morphological traits. (a) Scree plot of principal component analysis of *C. arcticum* populations between eigen value and principal components; (b) variables plot revealed by two principal components analysis; (c) Individual scatter plot grouping by species with two principal components analyses.

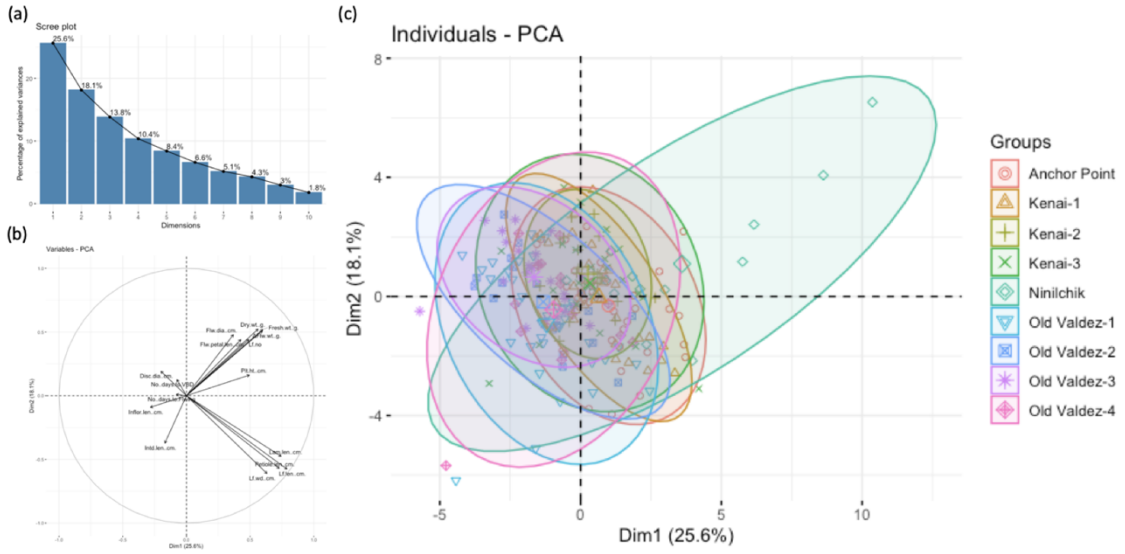

Figure 19. Chapter 3 Principal components analyses (PCA) for *C. arcticum* 16 quantitative morphological traits. (a) Variables plot revealed by two principal components analysis. (b) Scree plot of principal component analysis of *C. arcticum* populations between eigen value and principal components. (c) Individual scatter plot grouping by populations revealed by two principal components analysis.

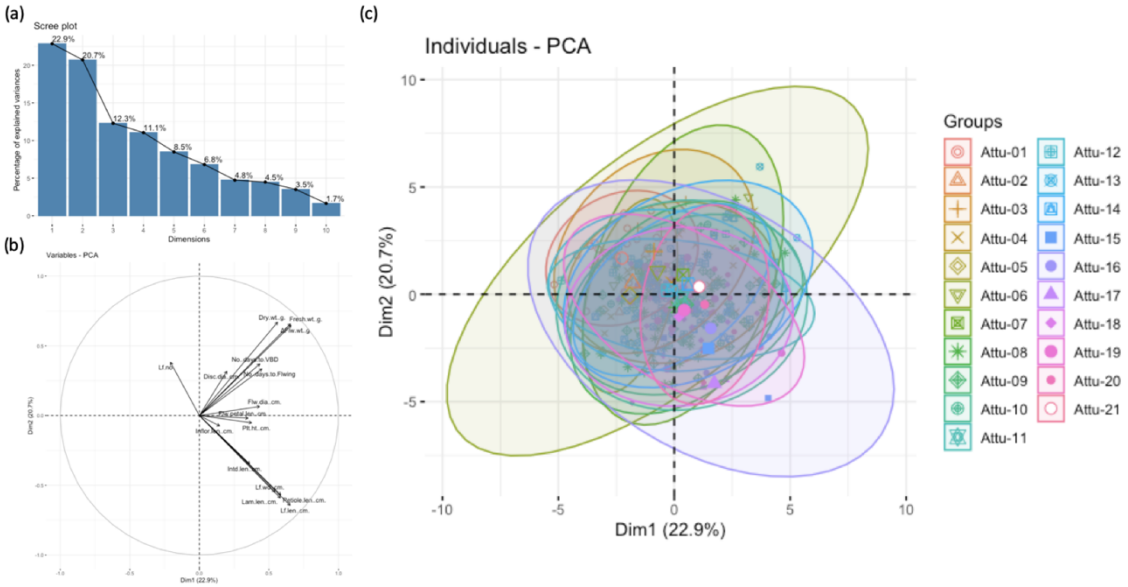

Figure 20. Chapter 3 Principal components analyses (PCA) for *Chrysanthemum arcticum* subsp. *arcticum* 16 quantitative morphological traits. (a) Variables plot revealed by two principal components analysis. (b) Scree plot of principal component analysis of *C. a.* subsp. *arcticum* populations between eigen value and principal components. (c) Individual scatter plot grouping by populations revealed by two principal components analysis.

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Appendix

1. Detailed protocol of DNA extraction

Preparation:

a. Get dry ice in polystyrene box, during the extraction keep samples in the polystyrene box and on the dry ice, to maintain cold environment.

b. Get a smaller container that 96 Well SynergyTM Homogenization Plate can fit in. Put an appropriate amount of dry ice into the container and keep the plate on these dry ices.

c. Label all the plates in the 96 Well SynergyTM Plant DNA Extraction Kit (including 96 Well SynergyTM Homogenization Plates, PVDF Filter Plates, Glass Fiber Filter Plates, Collection Plates, Elution Plates).

d. Prepare 3 beakers for cleaning scissors and tweezers. One with soapy water and two for rinsing. Wash and rinse after every sampling. Then dry the scissors and tweezers with paper towel, replace the paper towel for every time.

Sampling:

a. Get Chrysanthemum arcticum healthy young leaf tissues from -80C freezer. Keep the sampled and will be sampled leaf tissues in the polystyrene box with dry ice. Get one sample's envelope for each time.

b. with gloved hands, remove a small sample of tissue from envelope, cut into appropriate size and put them into the matched homogenization plate well. Cut in half or crosswise or trim to fit inside without forcing the sample into the tube.Sample size is about 25 mg.

Purification:

a. Remove the strip caps and transfer 400 ul of Plant Homogenization Buffer into each homogenization well.

b. Reseal the wells with the strip caps. Press the seals firmly with wheel.

c. Place the Well Support Mat underneath the homogenization plate to support wells during the grinding by GenoGrinder. (Appendix: Turn on the GenoGrinder and open the cover. Place two plates in the GenoGrinder evenly, put the foam mat on them, tighten the plates carefully. Set the GenoGrinder to 5 minutes and 1,500 rpm, using the left knob to adjust time and right knob to adjust rpm. Run the GenoGrinder for three times for a total of 15 minutes. Check the samples after each grinding, to make sure the wells not be foamy. Turn off the GenoGrinder.)

d. Centrifuge the plates for 10 minutes at 4,000 rpm.

e. Transfer 160 ul of supernatant from each well to the PVDF Filter Plate (the transferred supernatant should not be over 180 ul).

f. Place a Collection Plate under the PVDF Filter Plate and centrifuge for 10 minutes at 4000 rpm.

g. Add 5 ul RNase A Solution to each well in the Collection Plates. Incubate at room temperature for 15 minutes.

h. Add 0.7 volumes of isopropanol to the solutions, mix and incubate at -20C for 15 minutes.

i. Place a new Collection Plate under the Glass Fiber Filter Plate. Transfer the lysates to the Glass Fiber Filter Plate and centrifuge for 10 minutes at 4000 rpm. Discard the filtrate from the Collection Plate and place the Collection Plate under back to the Glass Fiber Plate.

j. Wash the bound DNA by adding 250 ul 70% cold ethanol to each well. Centrifuge for 10 minutes at 4000 rpm. Discard the filtrate from the Collection Plate and place the plate under back to the Glass Fiber Plate.

k. Repeat the wash.

l. Replace the Collection Plate with an Elution Plate. Elute the DNA by adding 50 ul of Molecular Biology Grade Water. Centrifuge for 10 minutes at 4000 rpm.

m. Cover the Elution Plate with the lid, and store at -20 C fridge.

2. Nanodrop SOP

Active the NanoDrop 2000 software on PC. Select the Nucleic Acid Application. Raise the sampling arm, and apply 2 ul molecular biology grade water as blank onto the lower pedestal using a micropipette. Lower the sampling arm. Click the Blank button on the PC software interface. When the blanking is complete, raise the sampling arm and wipe the solution from both the upper and lower pedestals using a clean lint-free laboratory wipe. Repeat the sampling procedure for multiple samples measurement. When measurement is complete, save the data.

3. Agarose gel

Herbarium specimens' DNA are typically highly degraded, which always leads to fragmented DNA extraction results. It may happen that the herbarium specimen's concentration is high, but they are mono-deoxynucleotides instead of DNA sequences, which may provide fewer effective materials for genotyping. Therefore, we filtered the herbarium specimens based on both molecular weight (over 1000 bp) and quantity (lightness on agarose gel) (insert fig).
High Range DNA Ladder: consist of 12 DNA fragments ranging from 500bp to 10kb.

4. DArtR in R studio file preparation

Combine the DArTR single row file with individual Metadata (choose specific metafile as needed). The Metadata file used in this analysis includes id, pop. These metrics are supplied by the way of a metafile, provided at the time of inputting the SNP data to the genlight object. (>Fresh_locations <- gl.read.dart(filename="freshdart.csv", ind.metafile = "pop1.csv")

A metafile is a comma-delimited file, which have a column headed id, contains the individual and a column headed pop, which contains the populations to which individuals are assigned.

Filtering of data was applied in the R studio with a series function as "gl.filter.callrate", to make sure high quality loci and a consistent quality of individuals. Two filterings were applied sequentially, filtering individuals by amount of missing data (gl.filter.callrate, method = "ind") and filtering by amount of missing data per locus (gl.filter.callrate, method = "loc").

To analyze the relatedness within/among the populations in one island separately, the genlight object was isolated as one island populations by dropping other “pop”.
Fresh_Attu_loc <- gl.drop.pop(Fresh_all_loc,pop.list = c("Old Valdez-1", "Old Valdez-2", "Old Valdez-3", "Old Valdez-4", "Kenai-1", "Kenai-2", "Kenai-3", "Ninilchik", "Anchor Point"))
Fresh_Alaska_loc <- gl.keep.pop(Fresh_all_loc,pop.list = c("Old Valdez-1", "Old Valdez-2", "Old Valdez-3", "Kenai-1", "Kenai-2", "Kenai-3", "Ninilchik", "Anchor Point"))

5. DAPC preparation

A. The genlight objects for managing SNP data are a relatively recent development, and the analysis options are limited for DAPC. Conversion to {adegenet} genind object was necessary, which can be achieved with the script gl <- gl2gi(gl, v=0).
B. pop names list. Extracted the “pop” from the genlight objects and stored as “.csv” file for strata.

DAPC was implemented by the function dapc <- dapc(“gind objects”, n.pca= 100, n.da= 4), which first transforms the data using PCA (Jombart & Collins, 2015), and then performs a Discriminant Analysis on the retained principal components. “n.pca” = 100 were default value for DAPC, the best statistical number for specific data could be calculated by script optim.a.score(). We refilled the best statistical number back into the “dapc” function, and obtain the scatter plot for the DAPC.)

6. PCoA preparation

The eigenvalues converts to percentages and some additional diagnostics by the script “gl.pcoa()” {Alaska_loc_pc <- gl.pcoa(Fresh_Alaska_loc, nfactors = 5)}. The results of the PCoA can be plotted using “gl.pcoa.plot()”. The script is essentially a wrapper for plot {ggplot2} with the added functionality of {directlabels} and {ploty}.