Honckenyë peploïdes: Regional Gene Diversity and Global Karyotype Investigations

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Honckeny a peploides: Regional Gene Diversity and Global Karyotype Investigations

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Abstract

Integration of classical ecological measurements with molecular, cytogenetic and statistical analysis techniques is vital to a greater understanding of ecological and evolutionary relationships in time and space. Such understanding is the key to re-assembling and rehabilitating diversity in the face of current environmental and climate changes. In this study, molecular and cytogenetic techniques were used to evaluate both levels of genetic diversity and differentiation as well as karyotype diversity in *Honckenya peploides*.

Populations from Surtsey were studied using Amplified Fragment Length Polymorphisms (AFLP) and diversity measures were compared to populations from other regions including the island Heimaey, the southern coast of Iceland, Greenland and Denmark. Main results include: (i) Surtsey has the highest proportion of polymorphic markers as well as a comparatively high genetic diversity and Denmark the lowest, possibly indicating that *H. peploides* populations on Surtsey originate from multiple colonization events from several source locations; (ii) the total genetic differentiation (\(F_{ST}\)) among Surtsey and Heimaey populations was significant and less than half of that found among mainland Iceland populations, indicating significant gene flow within the islands; (iii) significant genetic distance was found within Surtsey, among sites, this appears to correlate with the age of plant colonization; (iv) most genetic variation was found within localities, possibly due to the outcrossing and subdioecious nature of the species; and (v) there is a positive and significant association between genetic differentiation and geographic distance at the broad scale indicating isolation by distance has an effect on the Surtsey and Icelandic populations.

Through collaboration with investigators worldwide, seeds of *H. peploides* were grown for karyotyping using the enzymatic root tip squash method. Results from Seltjarnarnes samples show a tetraploid genome containing 68 metacentric and sub-metacentric chromosomes, two of which contain satellites. The chromosome number found in the current study validates some previous work conducted on mainland Iceland but still leaves some questions regarding karyotype diversity both in Iceland as well as worldwide. Our results coupled with a literature review point to a diverse genetic constitution for the species, both within localities as well as worldwide. This warrants a deeper investigation into the interplay of environmental variables and phenotypic diversity with the polyploid nature of the species as well as its mating system.
Yfirlit

Til að auka skilning á sambandi umhverfis og þróunar er nauðsynlegt að samþætta hefbundnar umhverfismælingar við nútíma sameinda- og frumuerfðafreiði og tölfræðilega greiningatekní. Slik þekking er mikilvæg í viðhaldi og endurnýjun á breytileika á tínum örra umhverfis- og loftslagsbreytinga. Í þessari rannsókn voru þæði sameindamerking og frumuerfðagreining gerðar á völdum stofnum fjöruarfa, Honckenya peploides, til að ákvæða umfang erfðabreytileika í stofnum og aðgreiningar á milli stofna, auk þess sem breytileiki í litningagerð tegundarinnar var metinn.

AFLP aðferð var beitt á stofna frá Surtsey og breytileiki þeirra mældur og borinn saman við stofna frá Heimaey, Stokkseyri, Garði, Grænlandi og Danmörku. Eftirfarandi niðurstöður fengust: (i) stofnar frá Surtsey voru með mesta margleitni og háan alhliða breytileika, en dönsku stofnarnir hins vegar með minnstan breytileika; þetta bendir til að fjöruarfi hafi borist til Surtseyjar frá nokkrum svæðum; (ii) heildar erfðafreiðin (FST) milli stofna á Surtsey og Heimaey var tæplega helmingi lægri en hjá stofnum á meginlandi Íslands sem gefur til kynna viðtækt genafleiði innan eyjanna; (iii) marktækur erfðafreiðilegur munur greindist innan Svæðum Surtseyar og virðist hann hafa fylgni við hve langt er liðið frá landnámi tegundinna á hverju svæði á eyjum; (iv) mestur erfðabreytileiki fannst innan svæða, hugsanlega þess að tugundin er víxlfrjóvguð og tvíbýl og (v) jákvæð og marktæk fylgni var á milli stofnaðgreiningar og landfræðilegra fjarlægðar á stórum skala, sem bendir til að stofnar á Íslandi og á eyjunum hafi lengi verið til að lið til þeirra mældur og borinn saman við stofna frá Surtsey og frumuerfðafræði og tölfræðilega greiningatækni. Slik þekking er mikilvæg í viðhaldi og endurnýjun á tínum örra umhverfis- og loftslagsbreytinga. Í þessari rannsókn voru þæði sameindamerking og frumuerfðagreining gerðar á völdum stofnum fjöruarfa, Honckenya peploides, til að ákvæða umfang erfðabreytileika í stofnum og aðgreiningar á milli stofna, auk þess sem breytileiki í litningagerð tegundarinnar var metinn.

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1 General Introduction

1.1 Scope of Current Study

The following study is designed to test levels of genetic diversity and genetic differentiation in populations of the dioecious, tetraploid beach plant *Honckenya peploides* on the recently colonized, completely barren volcanic island of Surtsey and to compare these patterns to those found in older populations from previously colonized locations. A preliminary investigation into karyotype diversity was also started.

The focal location of the study is the island of Surtsey, a well protected UNESCO World Heritage Site devoid of all human influence. The island provides the perfect natural laboratory for a study of this kind as it is relatively young (50 years), plant colonization and succession have been extensively recorded there since its inception and the study organism, *H. peploides* was one of the first plant colonizers to arrive (Friðriksson, 1964). Furthermore, *H. peploides* is the most abundant plant found on the island and is a key facilitator of plant and animal colonization, providing substrate binding for plants as well as nesting material for birds (Sigurðsson and Magnússon, 2009). Studies such as this which catalog and characterize existing systems and identify the events that are critical in their evolution will play a pivotal role in their conservation and future restoration. This will be of ever increasing importance as anthropogenic habitat destruction and fragmentation along with global climate change is currently causing a biodiversity crisis of a scale of magnitude not seen since the Cretaceous, what some are calling the "Sixth Mass Extinction" (Barnosky et al., 2011).

Insight into the genetics of populations is of great significance as genetic variation is the key to adaptation and exploitation of variable environmental niches, playing a vital role in the survival of populations under changing environmental conditions (Lovelock and Margulis, 1974; Frankel and Soulé, 1981; Berry, 1992). Reduced population size, habitat area, chance events and inbreeding can have negative effects on genetic variation and often lead to decreased fitness by increasing susceptibility to diseases, pests (Whiteman et al., 2006), infertility (Heber and Briskie, 2010), inbreeding depression (Oakley and Winn, 2012) and population extinction (Franzen and Nilsson, 2009). As the detrimental impacts of reduced genetic variation become more apparent it is of increasing importance to develop a comprehensive understanding of the factors which mold and preserve it as well as of how such variation is structured in time and space. If the distribution and subdivision of variation as we see it today reflects the history of a population (Wright, 1969) then analysis of the spatio-temporal structure of that variation should provide us with a deeper understanding of the processes which have formed it.

In the following literature I will first discuss genetic variation and factors which influence the genetics of populations as well as some methods used to assess certain parameters relating to population genetic diversity. The relevance of cytological methods for answering questions relating to ecotypic variation and population differentiation will then be discussed briefly. Then the utility of islands for the study of the evolution of diversity
will be reviewed, followed by an introduction to the study system itself, the island of
Surtsey, Iceland. Some important aspects the study organisms, *Honckenya peploides*, life
history and morphology will also be discussed briefly. Lastly, the aims of the study will be
laid out followed by the respectable chapters.

### 1.2 Genetic Variation

The driving force behind genetic variation is mutation and recombination. The frequency
and intensity of which is influenced by events such as colonization, gene flow, genetic
drift, selection and breeding systems (Wright, 1969; Charlesworth, 2009). Establishment
of a new population starts with a founder event, the number of founders influences the
amount of variation in the new gene pool, with population reduction having a negative
effect on genetic diversity. Increased population differentiation and-or reduced fitness as a
result of random changes in allele frequency (genetic drift) sometimes follows after new
areas are colonized, again, with the effects being more pronounced in smaller populations
(Vandewoestijne and Van Dyck, 2010; Acquaah, 2012).

#### 1.2.1 Genetic Variation on Islands

Island populations and endemic species overall are thought to have lower genetic
diversity than their mainland cousins, with genetic diversity decreasing with island size
and increasing degree of isolation (Frankham, 1997, 1998). The effects of limited
population size can be a constraint on levels of genetic variation. Typically only a
small portion of individuals (genetic variation) from the gene pool are sampled in the
initial founding event (Mayr, 2006). The result of this is a loss of rare alleles along
with changes in allele frequencies in the new population (Wright, 1948). These effects
can be mitigated if the distance for the migration of new genotypes into the population
(gene flow) is not too great, often leading to population cohesion (Dixon et al., 2011).
Reduced gene flow due to distance or ecological isolation can lead to population
differentiation, reproductive isolation and eventually speciation. Further isolation
along with reduction in population size can lead to inbreeding, reduction of within
population genetic variation (reduced heterozygosity) and decreased fitness. Also, if
ecological conditions vary between populations and gene flow is hampered then
selection can favor successful genotypes at each location, possibly leading to
population differentiation (Slatkin, 1987).

#### 1.2.2 Island Biogeography and Gene Diversity

The Island Biogeography theory, first proposed by MacArthur and Wilson (1967)
offers a qualitative model for the relationship of species diversity with island isolation
and area. Simply put, as island distance from larger landmasses increases, species
number decreases, due to the effect of distance on immigration. Furthermore, species
number decreases as island size decreases, due to the heightened probability of
extinction. Jaenike (1973) proposes that isolation along with limited area should affect
genetic variation of populations on islands in a similar way. This is supported by many
studies showing a large number of taxa with significantly lower genetic diversity than
the same mainland species, with the effect increasing with decrease in island size
(Frankham, 1997). However, factors such as breeding systems, dispersal ability and
ploidy level all have a dynamic interplay, resulting in differences in genetic behavior
depending on the organism in question.
1.2.3 Dioecy and Genetic Variation

Dioecy, a condition in which sexes are on separate individuals, has been shown to positively affect gene flow and greatly reduce inbreeding (Charlesworth, 2006; Obbard et al., 2006). Nordal and Philipp (M. Philipp, pers. comm.) found that auto deposition in hermaphrodites of *H. peploides* (bags were placed over individuals) in Greenland did not produce any seeds and active selfing of hermaphrodites resulted in very low seed set with very low seed weight, while active out-crossing in hermaphrodites increased seed set. The occurrence of reduced fitness in seeds due to selfing of hermaphrodites has been found for other subdioecious species as well and seems to be a feature that has evolved to facilitate gene flow, prevent inbreeding and increase heterozygosity (Tsukui and Sugawara, 1992; Delph and Carroll, 2001; Weller and Sakai, 2004; Keller and Schwaegerle, 2006).

1.2.4 Genetic Variation in Polyploids

Genetic variation is often maintained through fixed heterozygosity in polyploid species. Fixed heterozygosity has been reported in a number of polyploid arctic plants and seems to be the norm rather than the exception in arctic polyploids. Brochmann et al. (2004) found that all Svalbard polyploids were genetic autoployploids with fixed heterozygosity at isozyme loci. Furthermore they found that heterozygosity in 65 arctic taxa increases dramatically from diploid to high-level polyploids. This high level of heterozygosity has been proposed by other authors to be the motive force behind polyploidy expansion into new habitats (Levin, 2002). Brochmann et al. (2004) speculate that fixed-heterozygosity as a result of polyploidy formation, buffering against inbreeding and drift, is the cause of the great evolutionary success of polyploids in the arctic region. Out-crossing diploid populations tend to become genetically homogeneous when confronted with bottlenecks and inbreeding, as is often the case when colonization of isolated areas takes place (Aguilar et al., 2008; Yuan et al., 2012). However, typical arctic plant populations (usually autoployploids), despite being faced with inbreeding and bottlenecks, have the possibility of maintaining genetic variation due to fixed heterozygosity maintaining allelic diversity within each individual (Otto and Whitten, 2000). The deposit of ancestral genetic material into duplicated, fixed heterozygous genomes guarantees that genetic diversity is maintained despite intense inbreeding and bottlenecks associated with long distance seed dispersal events or re-colonization of recently barren areas (Tate et al., 2005).

1.3 Quantifying Genetic Variation

Molecular marker techniques are often used to directly examine genetic variation as the interplay of genetic and environmental factors on phenotypic variation can be challenging to untangle (reviewed in Agarwal et al., 2008). The varying number and frequency of alleles per locus is used to determine the genetic variation in a population, typically separated into hierarchical levels such as the individual, the population or the region. Variation within these parameters is quantified using the number of polymorphic loci, the number of alleles at a locus and heterozygosity levels from which within-population heterozygosity (*H*_w*) and total heterozygosity (*H*_T*) can be determined. Between populations, variation is typically quantified as the variation in
allele frequency differences between them, a measure denoted as population differentiation or fixation index, $F_{ST}$ (Wright, 1965).

### 1.3.1 Amplified Fragment Length Polymorphism (AFLP)

A tool that has provided great insight into the genetics of populations is AFLP-PCR (Vos et al., 1995; Maughan et al., 1996; Muluvi et al., 1999; Kim et al., 2002; Bonin et al., 2007; Reeves and Richards, 2009; Sánchez-Vilas et al., 2010). AFLPs are selectively neutral, highly polymorphic molecular markers which have been used to evaluate patterns of genetic variation and genetic structure in a number of Arctic, Subarctic and Antarctic plant populations as well as isolated island plant populations (Sharma et al., 2000; Holderegger et al., 2003; Schonswetter et al., 2003; Alsos et al., 2007; Migliore et al., 2011). The method quickly generates large numbers of markers, prior knowledge of the genomic sequence in question is not necessary, very little starting template is required and it can be used for most organisms (van Haeringen et al., 2002; Groot et al., 2003; Brooker et al., 2008). Several studies have compared the effectiveness of AFLP to RFLP (Restriction Fragment Length Polymorphism), SSR (simple sequence repeats or microsatellites), and RAPD (Random Amplified Polymorphic DNA) at producing polymorphic loci and all have found AFLP to be the most effective and reliable method, in every case producing a much higher amount of polymorphic bands per primer used (Maughan et al., 1996; Nakajima et al., 1998; Barker et al., 1999; Xu et al., 1999; Meudt and Clarke, 2007; Reeves and Richards, 2009).

Through PCR amplification, AFLP methods allow the detection of polymorphisms of genomic restriction fragments (Vos et al., 1995). AFLP markers have been particularly useful in investigations of population structure and differentiation (Tohme et al., 1996; Heun, 1997; Triantaphyllidis et al., 1997; Arens et al., 1998). The power of AFLP analysis derives from its ability to quickly generate large numbers of marker fragments from minute pieces of any organism with no prior knowledge of the genomic sequence. Other advantages of the AFLP method are that the PCR is very fast and high numbers of different genetic loci can be simultaneously analyzed during each experiment (Powell et al., 1996). This high multiplex ratio guarantees that the whole genome is being sampled rather than just one piece of it. Another huge advantage of AFLP is its reproducibility, Jones et al. (1997) used a number of labs throughout Europe to show that through rigorous control of all variables it was possible to reproduce the same banding patterns in all the laboratories.

Using PCR the AFLP technique amplifies pieces of genomic restriction fragments (Figure 1). The first step of the AFLP process involves the cutting of DNA into small fragments using restriction enzymes and the ligation of double-stranded adapters of a specific sequence to both ends of all restriction fragments to create primer binding sites for the pre-selective and selective DNA amplification in the steps that follow. A pre-selective amplification is then performed in which specifically designed primers containing only one nucleotide at the 3’ end are ligated to the restriction fragments. Exact matching of the 3’ end of a primer is necessary for amplification because the polymerase can only copy DNA molecules which have a primer attached. Therefore only restriction fragments in which the 3’ primer extensions match the sequences flanking the restriction sites will be amplified. This phase of the AFLP process is called pre-selective due to the fact that only DNA
Figure 1: Outline of the methodology of Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995). (1) Total genomic DNA is digested with two restriction enzymes (one frequent and one rare cutter). (2) During the digestion reaction adapters with primer sites are ligated to the ends of the fragments. (3) PCR using primers with one selective nucleotide extending into the genome fragment is performed. This reduces the number of fragments and improves reproducibility of final fingerprints. (4) A second series of PCR follows using two or three selective nucleotides on each primer, thus (5) generating a highly polymorphic DNA fingerprint. ©SHA

molecules that have base pair sequences that are complementary to the primer plus the one selective nucleotide will be amplified. To verify amplification, the products of the pre-selective amplification are checked by running them on a 1.5% agarose gel. A selective
amplification is then performed. This amplification uses two primers with three selective nucleotides attached. The attachment of three nucleotides to the 3’ end of the primers makes this step in the AFLP process even more selective because now only DNA molecules which have sequences complementary to the primer plus the three nucleotides will be amplified. One of the selective amplification primers (EcoRI) includes a fluorescent label on the 5’ nucleotide. Selective amplification with an EcoRI and an MseI primer amplifies EcoRI-MseI ended fragments. The EcoRI-EcoRI fragments do not amplify well, the MseI-MseI fragments are not visualized because they do not contain fluorescent dye labels, however, the EcoRI-containing strands are detected because they contain the dye labels.

1.4 Karyotype Diversity

The overall form of the nuclear genome is quite variable among plants. A rich diversity in genetic constitution and morphology is known to be found between and thought to also be present within species (Watanabe et al., 1999; Bennett, 2008; Young et al., 2012). Differences in chromosome number, form and size can have profound phenotypic consequences as the nuclear DNA content itself is known to influence every stage of development, from the cellular to the somatic and reproductive phases. These differences often lead to differential survival of favorable ecotypes, resulting in variable spatio-temporal distributions both globally and locally (Ray, 2010; Bennett and Leitch, 2011; Leitch and Leitch, 2012).

The enzymatic root tip squash method for plant chromosome preparation has been used in order to obtain chromosomes suitable for various cytogenetic studies such as kayotyping, chromosome banding and fluorescence in situ hybridization (FISH) since as early as the nineteen fifties (Jacobsen, 1954; Malling, 1957; Schwarzacher and Leitch, 1994; Anamthawat-Jónsson, 2001; Valárik et al., 2004). Karyotypes combined with molecular phylogenetic analysis can provide information about taxonomic relationships, ecotypic variation, genetic peculiarities and the evolutionary origins of species (Watanabe et al., 1999; Leitch and Leitch, 2008; Chokchaichammanakit et al. 2007; Anamthawat-Jónsson et al., 2009; Lysak and Koch, 2011; Wolny et al. 2013). Furthermore, chromosome size differences, arm-length ratios and FISH can be used to distinguish identifying features and loci between ecotypes (Truta et al., 2010; Ebadi-Almas et al., 2012; Young et al., 2012; Catalán et al. 2012). This may provide a good basis for distinguishing between subpopulation, providing insight into mechanisms relating to population differentiation.

1.5 Natural Laboratories on Isolated Oceanic Islands

The utility of isolated oceanic islands for the study of evolutionary processes is not without justification. They are both centers of biodiversity and evolution as well as the setting for the majority or over 80% of known extinctions since the year 1500 (Rodriguez et al., 2012). This makes islands valuable tools in understanding the fundamental mechanisms involved in speciation and can provide insight into the causes and consequences of reduced diversity. Given that they make up such a small amount of the earth’s land area (5%), island populations, compared to their continental
counters, display prolific and extraordinary radiations (Cody and Overton, 1996; Givnish and Sytsma, 1997; Gemmill, 2002; Kapralov et al., 2009; Schmitz and Rubinoff, 2011). Attested to by the fact that islands contain a very high proportion of all known species, with 20% of plant species as well as 15% of known mammal, bird and amphibian species being found on islands (Brooks et al., 2002; Brooks, 2006). Therefore, conditions presented by the island environment (isolation, niches, and etc.) must favor species diversification. This is particularly clear on older, more isolated islands such as the Hawaiian, Galapagos and Canary islands (McCullen, 1987; Hortal et al., 2007; Cowie and Holland, 2008; Baldwin and Wagner, 2010). It can be argued then that islands themselves present us with a basic, replicable system whose finite size, isolated nature, niche diversity and sheer number offer an ideal environment in which to study the processes of evolution.

1.5.1 Surtsey - A Natural Laboratory

In the turbulent month of November 1963 the Nordic fire god Surtur plotted with the Hawaiian goddess Pele to produce an empty frame upon which evolution could weave its web, Surtsey was born (Figure 2). The island, located at 63.4°N, 20.3°W, was created during almost four years of continuous volcanic activity. Surtsey is in the Vestmannaeyjar Archipelago (Figure 3), an archipelago that was created just south (20-30 km) of the mainland of Iceland by a series of submarine volcanic eruptions following the last ice age. The initial size of Surtsey after the eruption was measured at 2.65 km², however today, due to the constant battering by wind and sea the island has been reduced to 1.47 km² by 1998 (Jakobsson et al., 2000). The highest point on the island is 155 m above sea level (Austurbunki). The climate is humid with cool summers, relatively warm winters, strong winds and is often overcast. The average winter temperature is 1.5 - 2°C with some 80 days below freezing. The summer average is around 10°C, but temperatures below -15°C or higher than 20°C are uncommon. The average annual rainfall is 1,600 mm, falling mostly between October and March, a third of it as a mixture of rain and snow. Winds prevail from the east off the mainland and are of hurricane strength at least 15 days a year. Waves are driven from the southwest and can be very high: 16.68 m was recorded at a measuring buoy, and the lava cliffs are heavily eroded. Like all the islands in the archipelago, Surtsey is of volcanic origin with palagonite tuff or basaltic lava (Steinthorsson, 1965).

In 1965, Surtsey was declared a protected area and secured for the sake of science. Since then it has been highly revered as a natural living laboratory for scientific discovery and was named a Unesco World Heritage Site in 2008.

The first biological observations were performed on Surtsey in the spring of 1964 (Friðriksson, 1964). Molds, bacteria and fungi were among the first organisms found (Brock, 1966). The first vascular plant, the sea rocket (Cakile arctica), was observed in 1965 and plants have been investigated annually since. A year later lymegrass (Leymus arenarius) began to grow on the island and by 1967 the sea sandwort (Honckenya peploides) joined the flora (Friðriksson and Johnsen, 1968). Six years after its arrival, H. peploides began to produce seed locally, making the population increase no longer dependent upon accidental seed transport. By 2008 H. peploides had managed to distribute itself all over the island and is currently the most frequent of all vascular plants found in study plots on Surtsey (Magnússon et al., 1996, 2009).
Figure 2. Surtsey: a) West side of Surtsey, b) close up of the western crater, c) the south coast of Surtsey, d) the east coast of Surtsey. Photo ©BM
Similar to other barren seashore habitats in the circumpolar north, *H. peploides* has formed a cohabiting community with lyme grass, *L. arenarius*, and lungwort or sea bluebells, *Mertensia maritima* (Friðriksson, 1987) (Figure 4). In 1978 *H. peploides* and *L. arenarius* began overlapping and spreading over sandy areas in the east part of the island. These two species and *M. maritima* have developed into a primitive, coastal community. In this relationship, *H. peploides* and *L. arenarius* serve as pioneer plants, holding the ground stratum, and later arriving species grow above it, taking advantage of the moist sand media held in place by the former species. By 2008 the number of vascular plant species colonizing the island had reached 69, of which 63 or 91% were found alive that year and 32 or 46% had formed viable populations.

As stated above, the initial colonization of the island was characterized by the arrival of shore plants such as *H. peploides*, *C. arctica* and *L. arenarius*. These are said to have facilitated the arrival of seabirds which used the plant material for nest building, especially *H. peploides*, which is said to have provided the nesting material for the first pair of the great black-backed gulls (*Larus marinus*) to breed on the island. Most of the first plant species had a high survival rate and formed viable populations. This initial period of colonization (1965 – 1975) was followed by a lag in new species colonization (1975 – 1984) in which relatively few new species entered the island and survival dropped. During this time, the shore plant community, mainly *H. peploides*
and *L. arenarius*, became more established and spread out. This was coupled by an increase in nesting gulls which seek out the developing patches of shore plants as nesting sites and enhance their growth through nutrient deposition (Magnússon and Magnússon, 2000; Sigurdsson and Magnusson, 2010).

The formation of a bird community on Surtsey has been thoroughly documented (Petersen, 2009). After a sharp increase in the gull population around 1985 and the formation of a dense breeding colony on the southern part of the island, a new wave of vascular plant invasion was facilitated (through seed dispersal and nutrient deposition by the birds) and survival rates increased. The period from 1985 – 1994 is characterized by the invasion of various sea gulls, mainly the herring gull (*Larus argentatus*), the lesser black-backed gull (*L. fuscus*) and the great black-backed gull (*L. marinus*). This invasion of gulls facilitated a big increase in new plant colonizers, the seeds of which are mostly dispersed by the gulls themselves. During this time, mycorrhizal fungi are first detected and are said to have facilitated the colonization and spread of rudimentary species such as annual meadow grass (*Poa annua*), chickweed (*Stellaia media*) and pearlwort (*Sagina procumbens*). At the end of this period, vegetative cover on the southern part of the island had reached 3 ha.

The period from 1995 – 2008 has been characterized by the development of a secondary plant community and the formation of a forb rich grassland in which the meadow-grass (*Poa pratensis*) and the arctic fescue (*Festuca richardsonii*) are the dominant species. This period has shown a large increase in soil organic matter and plant biomass within the gull colony, as well as an increase in the overall area of the vegetative material making up the bird colony from 3 ha to 10 ha. Also during this time, invertebrate species number and abundance have increased, facilitating the arrival of passerine, land bird species such as the snow bunting (*Plectrophenax*).
from the white wagtail (*Motacilla alba*) and the meadow pipit (*Anthus pratensis*), which all feed their young on insects. Along with the arrival of the land birds, came the arrival of ravens (*Corvus corax*), which feed their young mainly on the eggs and young of fulmars (*Fulmarus glacialis*), gulls and kittiwakes (*Rissa tridactyla*).

Dispersal methods of new seedlings to the island vary. Based on observations of the colonization sequence and locations of first encounters, the majority, around 75% of new colonists arrived by birds, 16% by wind and 9% by sea currents. It is also relevant to note that all species found on Surtsey so far are also found on the mainland of Iceland and about 80% may have derived from the other Vestmanna Islands (Magnússon et al., 2009). The vascular plant species brought by sea are all coastal plants adapted to sea dispersal such as *C. arctica*, *L. arenarius* and *H. peploides* and these species were among the initial colonizers of the island. These initial colonizers are all adapted to infertile habitats along sandy shores and barren inland areas with little nutrient content. It is also interesting to note that these species are all clonal perennials with high stress tolerance and large seeds.

Being a newly formed island Surtsey is a perfect natural laboratory for the study of this type. The island provides the rare opportunity to study the flora and fauna of an island as colonization takes place. This could bring novel understanding of the effects that colonization of barren island ecosystems has on the genetic behavior of populations, shedding further light on the mechanisms involved in population differentiation, reproductive isolation, speciation and extinction.

### 1.6 *Honckenya peploides*

The Sea Sandwort, *Honckenya peploides* (Figure 5) is the only species within the genus *Honckenya* (L.) Ehrh., belonging to the subfamily *Alinoideae* in the carnation family *Caryophyllaceae*. The plant reproduces both sexually and asexually and displays a large amount of genetic variation within spatially segregated unisexual populations in northwestern Spain (Sánchez-Vilas et al., 2010), Greenland and Svalbard (M. Philipp, pers. comm.). Typical of plants found in northerly and recently de-glaciated or bare areas, it is a tetraploid (Brochmann et al., 2004), with a relatively high chromosome number (2n = 4x = 66, 68, 70) (Malling, 1957) and large genome (8.57 to 10.66 pg/cell) when compared to other members of *Caryophyllaceae* (Kapralov et al., 2009).

#### 1.6.1 Habitat and Distribution

*Honckenya* is an early colonizer of barren seashores, facilitating both plant colonization and ecosystem development by binding soil particles together with its roots and providing nesting material for sea birds (Houle, 1997; Gagne and Houle, 2002; Sigurðsson and Magnússon, 2009). Adapted to habitats such as fore-dunes, drift lines, seashores and lake shores (Bry sting et al., 2007), it was one of the pioneer plant species colonizing Surtsey only a few years after the island appeared (Fríðriksson and Johnsen, 1968). The plant sometimes grows in large mats or clumps, forming small dunes through the spreading of leafy rhizomes whose shape and orientation facilitate sand accumulation (Gagne and Houle, 2002).
Four subspecies of *Honckenya* have been identified (Figure 6) (Jonsell, 2001): subsp. *peploides*, distributed from northern Norway to northern Portugal; subsp. *diffusa*, which has circumpolar distribution mainly in Arctic and northern Boreal zones; subsp. *major*, found in the North West Pacific area; and subsp. *robusta*, found in Northeastern North America. According to Hultén (1971), subsp. *diffusa* is a variety of subsp. *peploides* and this is the subspecies I will be focusing on in the present research. Subsp. *peploides* occurs mainly in the northern parts of Norway, Svalbard, Greenland, Iceland and subarctic Canada (Houle, 1997; Jonsell, 2001). In Iceland *H. peploides* can be found on most all shorelines from sea level up to 50m above sea level with Surtsey being the only place where the plant can be found growing up to 100 m above sea level (Figure 6).

**1.6.2 Flower Morphology**

*Honckenya peploides* flowers are axillary and solitary with one to six flowered terminal chymes. There are two sexual morphs, the hermaphrodites having large petals and stamens and the females whose flowers have very small stamens and petals
Such a condition is termed dioecy and is rare, being found only in roughly 6% of known angiosperm species (14,620 of 240,000) (Renner and Ricklefs, 1995). Some individuals (pistillate) have non-functional anthers, long styles and short petals while others (staminate) have long stamens that produce pollen grains, short styles and long petals (Tsukui and Sugawara, 1992). Individuals with pistillate flowers are females and those with staminate flowers are hermaphrodites. Females never produce pollen and are constant in their expression. Hermaphrodites (pollen producing morphs) rarely produce seeds and when they do, the number of seeds is very low compared to female flowers (Malling, 1957). This is characteristic of a subdioecious breeding system (reviewed by Delph and Wolf, 2005). Flower pollination occurs by insects (Lelej et al., 2012) and pollen is sometimes carried from flower to flower with the assistance of sand grains (Baillie, 2012). The fruit (Figure 7) produced is spherical or ovoid in shape; with three teeth/valves, which pop open to release seeds at maturity, after which the fruit is shed (Brysting et al., 2007). Later, seeds are spread by sea currents and wind (Jonsell, 2001).

Figure 7: Honckenya peploides; a) hermaphrodite flower showing large petals, b) female flower showing smaller petals and c) fruit capsule. ©SHÁ.

### 1.6.3 Breeding System

Hermaphrodite seeds develop into female and hermaphrodite plants in the approximate ratio 1:3. Seeds of female flowers produce about as many hermaphrodites as females (Malling, 1957; Sánchez-Vilas et al., 2010), implying that the sex determination system is heterogamous for the hermaphrodite; female = XX, hermaphrodite = XY or YY.

The evolutionarily relevant criterion for classifying breeding systems is fitness-based functional gender, i.e. the proportion of haploid genomes transmitted through pollen or through ovules (Lloyd, 1980). Examples of these fitness based systems in plants include gynodioecy (female and hermaphrodite), subdioecy (female, male and hermaphrodite), androdioecy (male and hermaphrodite) and dioecy (male and female functional parts). On the evolutionary pathway from co-sexuality to dioecy lie the intermediate breeding systems of gynodioecy and androdioecy (Charlesworth and Laporte, 1998), with subdioecy occupying an intermediate position between andro/gynodioecy and complete dioecy (Delph and Wolf, 2005; Wagner et al., 2005). This pathway (Figure 8) starts out with the invasion of a unisexual mutant, either female sterile (male) or male sterile (female). This in turn causes the hermaphrodites to allocate more of their sexual function towards the gender that is lacking, eventually leading, in some cases, to hermaphrodites becoming unisexual, hence making the population dioecious (Charlesworth and Laporte, 1998).
1.7 Thesis Overview and Objectives

There are two chapters in the thesis, each of them utilizing different tools to investigate the genetic behavior of \textit{Honckenya peploides}. The focal point of this thesis, chapter two, examines questions regarding the genetic effects that the colonization of the island of Surtsey has had on the genetics of \textit{H. peploides} populations. This chapter, entitled Spatial Genetic Structure of The Sea Sandwort, looks at genetic diversity patterns on island and mainland populations using AFLP markers. The main focus in this chapter is to determine if within population genetic diversity has changed following colonization to the island of Surtsey. Patterns of genetic differentiation are also compared to mainland areas and conservation implications are discussed briefly. Chapter three, Karyotype Variation in \textit{Honckenya peploides} Worldwide, deals with finding some evolutionary trends in karyotype diversity. This chapter sets the stage for further investigations into relationships between karyotypic variation within the species and environmental variables.
2 Spatial Genetic Structure of the Sea Sandwort

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2.1 Introduction

On the 14th of November 1963, just 30 km off the southern coast of Iceland, the young island of Surtsey arose violently to the sea surface, instantly producing a natural laboratory for scientific exploration. Surtsey surfaced out of the frigid North Atlantic Ocean during almost four years (November 1963 - June 1967) of continuous spewing of volcanic material from the mid-Atlantic ridge at lat. 63° 18’ 22” N, long. 20° 36’ 5” W, just 3 nautical miles west-south-west of Geirfuglasker, the southernmost island in the Vestmannaeyjar Archipelago (Þórarinsson, 1967). Plant seeds were already being documented washing up on shore during the first survey of the island on May 214st 1964. The first seedlings of the searocket, Cakile arctica, were recorded there on June 3rd 1965, later to be buried under layers of ash. It seems almost certain that the first plants to arrive to the island grew from seed carried either by floating debris or wind to the island, as the seedlings of the first colonizers, C. arctica, Leymus arenarius and Honckenya peploides, were found growing in a row at the high tide line (Friðriksson and Johnsen, 1968).

The meticulously documented history of colonization and relatively recent geologic origin of Surtsey offer a unique perspective for the scientist. The island provides a natural system whose community development can be monitored and studied devoid of all human influence. The focal species and the first plant to establish a viable seed producing population on the island, Honckenya peploides, was first recorded there in 1967 (26 individuals, Friðriksson and Johnsen, 1968), set seed first in 1968 and has grown on and dominated the vegetation on the island to this day (Magnússon et al., 1996, 2009). By 1968 there were 103 individuals, by 1971 their number was reduced to 52, five of which flowered, with one producing seed (most likely a female). In 1972, 71 plants were recorded, then a major colonization event took place before the summer of 1973 in which 548 H. peploides individuals were recorded (along with a dramatic
increase in both *L. arenarius* and *Cochlearia officinalis* individuals). This is thought to be due to an exceptionally good seed production seasons preceding this period.

The study of evolutionary processes has long been focused on isolated oceanic islands as their finite size, isolated nature and niche diversity provide a basic, replicable system in which to quantify and study the evolution of populations (Darwin, 1859; Wallace, 1880; MacArthur and Wilson, 1967; Whittaker et al., 2010). Although they make up only a very small fraction of the earths land surface area (5%), islands are home to roughly 15% of all known mammal, bird and amphibian species and 20% of all known vascular plant species (Brooks, 2006). Newly formed oceanic islands as well as areas recently affected by cataclysmic events such as volcanic eruptions present scientists with the unprecedented opportunity to collect data on and analyze evolutionary processes at spatial and temporal scales not possible with ecosystems in higher successional stages (Friðriksson, 1970; Fridriksson and Magnússon, 1992; Bush and Whittaker, 1991; Tsuyuzaki, 2009). Due to the relative isolation and novel ecological niches that these islands provide, colonization by small populations often leads to rapid evolutionary change, adaptive radiation and speciation (Wagner and Funk, 1995; Cody and Overton, 1996; Givnish and Sytsma, 1997; Gemmill, 2002; Ziegler, 2002; Wagner et al., 2005; Kapralov et al., 2009; Schmitz and Rubinoff, 2011). Such radiations are thought to be a result of mutational spread under different selection pressures as well as allele fixation and differential survival of genotypes best suited for the particular environment (Huxley et al., 1958; Barton and Mallet, 1996).

The use of molecular techniques along with greenhouse experiments has been instrumental in illuminating patterns of biogeography, genetic diversity and dispersal as well as in providing answers regarding genome size changes, polyploidy, breeding systems and speciation on oceanic islands (Bush and Whittaker, 1991; Givnish and Sytsma, 1997; Sakai et al., 1997; Nielsen, 2004; Cowie and Holland, 2008; Green et al., 2011; Himmelreich et al., 2012).

The distribution and segregation of current genetic variation is thought to be a reflection of the history of populations through time (Wright, 1969). Inbreeding and increased homozygosity are often the results of reduced variation within a population, frequently leading to infertility, increased sensitivity to disease and in the most extreme cases inbreeding depression and extinction (Charlesworth and Charlesworth, 1987; Ellstrand and Elam, 1993; Frankham, 1998; Futuyma, 1998; Eldridge et al., 1999; Ellis et al., 2006; Dostálek et al., 2009). Islands that are geologically older and extremely isolated from mainland sources of variation often times display an extraordinary propensity for species radiations and evolution. This evidence in the immense diversity is found within the Hawaiian (0.4 - 4.7 million years or Myr) flora and fauna (Cowie and Holland, 2008; Baldwin and Wagner, 2010), the Galapagos (0.7 - 4.2 Myr) vascular flora (McCullen, 1987) and the forest biota of the Canary Islands (0.8 - 20 Myr) (Hortal et al., 2007). The dynamic interplay of spatio-temporal factors, isolation-distance, breeding systems and dispersal ability results in differences in genetic behavior depending on the organism in question. Could it then be feasible to hypothesize that we will see any evidence of evolutionary processes on young islands such as Surtsey, where time and space have not existed until relatively recently (50 yrs.)?

A vast amount of theory and investigative methods have been established which illuminate how genetic variation in particular has been shaped by evolutionary processes over time (Huxley et al., 1958; Wright, 1969; Nei et al., 1975; Maynard
In light of the rapid anthropogenic habitat destruction and fragmentation that is currently causing an unparalleled loss of biodiversity around the planet, an analysis of the distribution of variation and an understanding of the processes which shape and conserve it at small spatial and short temporal scales is of great significance (Brooks et al., 2002; Travis, 2003; Brooks, 2006; Cardinale et al., 2006, 2009; Brittain and Craft, 2011; Rodriguez et al., 2012). In the following study, amplified fragment length polymorphism (AFLP) data along with classical and Bayesian statistical analysis methods were used to deduce the genetic structure of *Honckenya peploides* in sampling locations on Surtsey, Heimaey (Vestmannaeyjar), mainland Iceland, Greenland and Denmark. Due to its young geologic age, the fact that colonization and succession have been thoroughly studied there since its inception and the fact that *H. peploides* was one of the first and most prolific colonizers of the island, Surtsey provides the perfect natural laboratory for a study of this type and *Honckenya peploides* an excellent model species. This study is the first of its kind conducted on the island of Surtsey and provides the unprecedented opportunity to study the processes of evolutionary change at the molecular level as a population develops on a young (50 yrs.), small (< 2 km²) isolated (30 km from mainland) oceanic island located in the sub-arctic region (63°N, 20°W).

The objectives of this study were as follows: (i) to assess the genetic diversity and population genetic structure of *Honckenya peploides* on the island of Surtsey, (ii) to compare the genetic structure of *H. peploides* at locations on Surtsey to the genetic structure on older, more established locations on Heimaey, mainland Iceland, Denmark and Greenland and (iii) to examine the level and effects of isolation by distance on the sample locations.

### 2.2 Materials and Methods

#### 2.2.1 Study Species

The autotetraploid (2n = 4x = 66, 68, 70), gynodioecious, perennial, maritime, eudicot, *H. peploides* can reproduce both sexually with pistillate (female) and staminate (hermaphrodite) flowers as well as asexually using its rhizomes. Often daughter clones remain attached to parent clones via rhizomes whose connections sometimes run up to 2 m from the parent plant (Sánchez-Vilas et al., 2010). Hermaphrodite seeds develop into female and hermaphrodite plants in an approximate 1:3 ratio. Seeds of female flowers produce about as many hermaphrodites as females (Malling, 1957), implying that the sex determination system is heterogamous for the hermaphrodite. An experiment in which bags were placed over individual *H. peploides* plants in Greenland (M. Philipp, pers comm.) showed that hermaphrodites did not produce any seeds and active selfing of hermaphrodites resulted in very low seed set with very low seed weight, while out-crossing in hermaphrodites increased seed set. This indicates that although rare, self-fertilization is indeed possible for *H. peploides* and that, similar to most arctic species, the plant has a mixed mating system (Brochmann et al., 2004). However similar to most male heterogamous subdioecious species, the hermaphrodite seed production and fitness is quite low, possibly indicating some level of inbreeding depression (Tsukui and Sugawara, 1992; Delph and Carroll, 2001; Weller and Sakai, 2004; Keller and Schwaegerle, 2006). This seems to be a feature that has evolved to prevent inbreeding and reduced heterozygosity.
H. peploides habitat is mainly limited to dunes, drifts lines, lake-shores and seashores. It has a circumpolar distribution extending from the arctic to the temperate zone in Western Europe, North America and North Eastern Russia down to Japan (Jonsell, 2001). The plant is an early colonizer, contributing to the anchorage of the soil and is the first plant to set seed on Surtsey only a few years after the island first appeared (Friðriksson and Johnsen, 1968). H. peploides has been shown to exhibit a large amount of within population genetic variation in both northwestern Spain (Sánchez-Vilas et al., 2010), Greenland and Svalbard (M. Philipp, pers. comm.). However, little to nothing is known about the genetic makeup of populations located in Surtsey, Vestmannaeyjar or mainland Iceland.

2.2.2 Marker Choice

The selectively neutral, highly polymorphic technique, Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995), combines restriction digestion and PCR to produce Mendelian inherited, multi-locus, dominant markers which are highly reproducible and can be used for analysis of genetic structure (Jones et al., 1997). AFLP has been used in a number of studies of Arctic, Subarctic and Antarctic plant populations as well as isolated island plant populations to evaluate patterns of genetic variation and genetic structure (Sharma et al., 2000; Holderegger et al., 2003; Schonswetter et al., 2003; Alsos et al., 2007; Migliore et al., 2011; ). AFLPs have also shown that recently formed populations can have a propensity towards decreased genetic diversity after introduction to un-colonized areas (Muluvi et al., 1999; Amsellem et al., 2000). Also, with AFLPs, a positive relationship has been demonstrated between genetic diversity and populations size (Morden and Loeffler, 1999; Gaudeul and Till-Bottraud, 2008). Additionally AFLPs can reveal genetic structure in numerous plant species including Honckenya peploides the species under study (Sánchez-Vilas et al., 2010).

In the last decade, AFLP has become one of the most widely used molecular markers to study the genetic structure of natural populations (Meudt and Clarke, 2007; Foll et al. 2010). The method quickly generates large numbers of markers, prior knowledge of the genomic sequence in question is not necessary, very little starting template is required and it can be used for most organisms. The performance of AFLPs compared to other dominant, genome-wide markers or compared to traditional co-dominant markers like microsatellites or allozymes have been investigated on numerous occasions (Powell et al., 1996; Jones et al., 1997; Barker et al., 1999; Mariette et al., 2002; Nybom, 2004). All have found AFLP to be the most effective and reliable method, in every case producing a much higher amount of polymorphic bands per primer used. Due to the extensive polymorphisms generated, the AFLP method is very powerful in revealing intraspecific variation, hence applicable also for genotyping, fingerprinting and barcoding purposes (Meudt and Clarke, 2007; Huang et al., 2013; Cornille et al. 2014).

2.2.3 Plant Material and Collection Locations

During July of 2010 and May of 2011, a total of 397 samples were collected, of these 347 samples from 12 locations in the north Atlantic region were analyzed (Table 1, see also Figure 9). This includes; five locations on Surtsey: SC (63°18.246N / 20°36.751W), located inside the western crater Surtungur at 70 m above sea level,
growing on volcanic tuft: **SD** (63°18.500N / 20°35.987W), located on the northern sand spit at 16 m above sea level, growing on volcanic sand: **SE** (63°18.197N / 20°35.632W), located on the eastern slope at 104 m above sea level, growing on volcanic tuft and gravel: **SF** (63°17.942N / 20°36.348W), located in the gull colony on the southern shore: and **SK** (63°18.416N / 20°36.000W), located on the steep east-facing slope of the old tephra crater **Surtur** leading to the northern sandbar at 36 m above sea level, growing on volcanic tuft and gravel; two locations on Heimaey: **AH** (63°24.506/20° / 16.792W), located on the south side of the island, growing on volcanic sand and gravel at 1-3 m above sea level: and **BH** (63°26.911N / 20°16.250W ), located on the northern side of the island, growing on volcanic sand and gravel at 12 m above sea level; two locations on mainland Iceland: Stokkseyri (**ST** - 63°50.376N / 21°04.636W), located at the end of a fresh water marsh in between two large glacial rivers at 1-3 m above sea level on the southern coast, growing on glacial deposits and fine sand: and Gardur (**G** - 64°04.963N / 22°41.548W), located at the western-most tip of the Reykjanes peninsula at 1-3 m above sea level, growing on a rocky coast; two locations in Denmark: Skansehage (**DKS** - 55°58N / 11°46E), located on the north eastern tip of the island of Zealand at 1-3 m above sea level, growing above the high tide line on a limestone sand beach: and Knarbo (**DKK** - 55°50N / 11°22E), also located on the island of Zealand in very similar habitat at 1-3 m above sea level; and one location in Greenland: Qeqertarsuaq (**GR** - 69°15N/53°30W), located on the south coast of Disko Island on the west coast of Greenland at 1-3 m above sea level, growing on a rocky seashore in glacial till.

**Table 1 Sampling locations, GPS coordinates, dates, ID’s and number of samples.**

<table>
<thead>
<tr>
<th>Locality</th>
<th>GPS Coordinates</th>
<th>Date</th>
<th>Sample ID</th>
<th>N</th>
</tr>
</thead>
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<tr>
<td>Surtsey</td>
<td>63°18.24N/20°36.75W</td>
<td>12-15.07.10</td>
<td>SC 1-30</td>
<td>30</td>
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<td>63°18.50N/20°35.98W</td>
<td>12-15.07.10</td>
<td>SD 1-30</td>
<td>30</td>
</tr>
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<td>Surtsey</td>
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<td>12-15.07.10</td>
<td>SE 1-30</td>
<td>30</td>
</tr>
<tr>
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<td>63°17.94N/20°36.34W</td>
<td>12-15.07.10</td>
<td>SF 1-30</td>
<td>30</td>
</tr>
<tr>
<td>Surtsey</td>
<td>63°18.416N/20°36W</td>
<td>12-15.07.10</td>
<td>SK 1-30</td>
<td>30</td>
</tr>
<tr>
<td>Heimaey</td>
<td>63°24.50/20°16.79W</td>
<td>9.7.10</td>
<td>AH(1-12)(16-30)</td>
<td>27</td>
</tr>
<tr>
<td>Heimaey</td>
<td>63°26.91N/20°16.25W</td>
<td>9.7.10</td>
<td>BH 1-30</td>
<td>30</td>
</tr>
<tr>
<td>Gardur</td>
<td>64°04.96N/22°41.54W</td>
<td>17.7.10</td>
<td>G(1-12)(16-27)</td>
<td>24</td>
</tr>
<tr>
<td>Stokkseyri</td>
<td>63°50.37N/21°04.63W</td>
<td>17.7.10</td>
<td>ST(1-6)(10-30)</td>
<td>27</td>
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<tr>
<td>Qeqertarsuaq</td>
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<td>25.7.10</td>
<td>GR 1-29</td>
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<td>15.5.11</td>
<td>DKK 1-30</td>
<td>30</td>
</tr>
<tr>
<td>Skansehage</td>
<td>55°58N/11°46E</td>
<td>15.5.11</td>
<td>DKS 1-30</td>
<td>30</td>
</tr>
</tbody>
</table>

Samples were collected by placing a marker in a random location surrounded by clumps of *H. peploides* and sampling clumps within a 20 meter radius of the marker. At each locality 30 spatially segregated clumps were sampled, avoiding sampling of clones as best as possible. Fresh leaf samples were collected and stored in plastic bags with silica beads until further laboratory analysis could be conducted.
2.2.4 DNA extraction

Genomic DNA was extracted from dehydrated leaf tissue using the CTAB method as described by Aldrich and Cullis (1993), slightly modified for smaller samples. CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM TrisHCl pH 8.0, 0.2% 2-mercaptoethanol) was pre-warmed to 65°C. Approximately 15 - 20 mg of dried leaf tissue from each individual was placed in 1.5 mL eppendorf tubes and manually pulverized to a fine powder with sterile plastic pestles in liquid nitrogen. Warm extraction buffer (0.5 mL) was then added to the tube and mixed well until all material was suspended in the liquid, this was then incubated at 65°C in a water bath for 1 to 2 hours (longer incubation results in cleaner pellets) and mixed occasionally by inversion. An equal volume chloroform-isooamyl alcohol (24:1) was then added and mixed well before centrifuging at 1400 RPM for 10 minutes. The supernatant was recovered and placed in a 1.5 mL Eppendorf tube and the DNA precipitated using 1.5 mL (2.5 volume) of cold 96% ethanol for 25 minutes at room temperature. The tube was then centrifuged again at 3000 RPM for 10 minutes and the pellet washed twice with 0.5 mL wash buffer (76% ethanol and 10 mM ammonium acetate). The tube was then centrifuged again briefly at 3000 RPM, the supernatant removed and pellet air dried. Once dry, 200 µl of 1x TE buffer (10 mM TrisHCl, pH 8.0 and 1 mM EDTA, pH 8.0) and 1 µl of RNase (10 mg/ml) was added, the solution mixed well and incubated at 37°C for 30 minutes. After incubation, 78 µl of 5 M NaCl was added, the tube inverted several times and solution mixed well. DNA was then precipitated again by adding 0.9 mL of ice cold 96% ethanol, inverting and mixing. The tube was then centrifuged again at 3000 RPM for 10 minutes and the supernatant removed. The pellet was then washed with 0.5 mL of wash buffer (76% ethanol and 10 mM ammonium acetate) quickly spun, the supernatant removed and the pellet air dried. 100 µl of 1x TE buffer was then added and samples stored in a -27°C freezer until AFLP work could be done. This method works well for extraction of *H. peploides* DNA and when performed correctly can result in a large quantity of high quality DNA. The quality and concentration of DNA in all samples was measured using Thermo Scientifics NanoDrop 1000 Spectrophotometer at OD 260nm/280nm. Of the 347 samples collected, 300 were used for the AFLP analysis.

2.2.5 AFLP Fingerprinting

The AFLP technique was performed with fluorescent dye labeling and detection technology. Fragment detection was performed on the ABI Prism 3730 Genetic Analyzer (Applied Biosystems), an automated capillary electrophoresis device. The AFLP analysis followed the standard procedure as described by Vos et al. (1995) on quality *Honckenya peploides* DNA (250 ng in 5.5 µl) that was isolated from dry plant leaf tissue using the CTAB method. Prior screening for primers suitable for use with *H. peploides* was previously performed on individuals of both sexes across multiple localities (Sánchez-Vilas et al., 2010). Restriction and ligation reactions were carried out in accordance with the AFLP Plant Mapping Protocol (Applied Biosystems) for average sized genomes.
Table 2: Primers used in study (Applied Biosystems).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>5’-fam-GAC TGC GTA CCA ATT CAC T-3’</td>
</tr>
<tr>
<td>EcoRI</td>
<td>5’-joe- GAC TGC GTA CCA ATT CAG G-3’</td>
</tr>
<tr>
<td>MseI</td>
<td>5’-GAT GAG TCC TGA GTA ACT A-3’</td>
</tr>
<tr>
<td>MseI</td>
<td>5’-GAT GAG TCC TGA GTA ACT C-3’</td>
</tr>
</tbody>
</table>

Two primer pairs were chosen (MseI-CTA/EcoRI-ACT & MseI-CTC/EcoRI-AGG) and selective amplification was carried out on all samples (Table 2). Single PCRs were performed for each primer combination, and the products from two primer pairs (with different dyes) were multiplexed for electrophoresis. In all the reactions, only the EcoRI primers were 5’ labeled with a fluorescent dye (Applied Biosystems).

A total of 250 ng of DNA was added to a digestion-ligation mix in a final volume of 11 µL containing digestion ligation buffer (50 mM NaCl, 50 ng/µL BSA), 1 µL of both MseI and EcoRI adapters (Applied Biosystems), 1U T4 DNA ligase, 5 U EcoRI (Applied Biosystems), 1 U MseI (Applied Biosystems) and digested at 37°C for 2 hours. Following digestion-ligation, pre-amplification PCR was performed with EcoRI-A/MseI-C primer pairs (Applied Biosystems) having one selective nucleotide with 19 cycles of: 2 min at 72°C, 1 sec at 94°C, 30 sec at 56°C, 2 min at 72°C; followed by a final extension for 30 min at 60°C. Each 10 µL of the reaction contained 2 µL of the restriction/ligation dilution (1:20), 7.5 µL of AFLP core mix from the Applied Biosystems Amplification Core Mix Module (buffer, nucleotides, and AmpliTaq® DNA Polymerase) and 25 mM of each primer pair containing one selective nucleotide.

The PCR product was then diluted (1:20) and used for the selective amplification with two primer combinations containing three selective nucleotides this time (MseI-CTA/EcoRI-ACT and MseI-CTC/EcoRI-AGG). The selective amplification PCR reaction entailed: 1 cycle of 2 min at 94°C, 30 sec at 65°C, 2 min at 72°C; 8 cycles of 1 sec at 94°C, 30 sec at 64°C and 2 min at 72°C; 23 cycles of 1 sec at 94°C, 30 sec at 56°C, 2 min at 72°C; followed by a final extension for 30 min at 60°C. Each 10 µL of the reaction contained 1.5 µL of the pre selective amplification dilution (1:20), 7.5 µL of the AFLP Applied Biosystems® core mix, 0.5 µL EcoRI primer at 1µM (fluorecently labeled) and 0.5 µL MseI primer at 5µM. For analysis using capillary electrophoresis, each 11 µL reaction contained 1 µL of each of the two selective amplification products, 9.5 µL HiDiFormamid and 0.5 µL Genescan-500 Rox-labeled internal size standard (Applied Biosystems).

After preparation, samples were denatured for 2 min at 94°C and chilled on ice. Capillary electrophoresis was carried out on the 3730 series DNA Analyzer using POP 7 capillary polymer and ABI Prism 3730 electrophoresis buffer (Applied Biosystems). The capillaries were 47 cm in length with 50 µm ID. Samples were injected electrokinetically for 5-20 sec at 15 kV and were run at 15 kV for 24 min at 60°C. Fragments of each primer combination were then scored as described in the next section. Initial fragment analysis was performed at the center for Macroecology, Evolution and Climate at the University of Copenhagen, Denmark. Further data analysis was performed at home university, in the Laboratory of Plant Genetics at Askja, Reykjavík.
2.3 Data Analysis

2.3.1 Raw Data
In the AFLP analysis, only amplified fragments with sizes between 50 and 500 base pairs range that could be scored unambiguously and showed clear presence or absence patterns in all *H. peploides* samples were included. Fragments outside this range cannot be accurately sized, hence excluded. The raw AFLP data were scored using the programs GeneMapper 3.7 (Applied Biosystems) and the presence or absence of each fragment was scored for each individual manually in GeneMarker® (Softgenetics). As is usual for AFLPs, we assumed that each AFLP band corresponded to a separate locus with two alleles (Maguire et al., 2002). Therefore, the presence of a band indicates that the individual sample may have originated from a heterozygote or a dominant homozygote, not a recessive homozygote of the corresponding allele. Analysis of the AFLP data was based on the phenotypic frequency at a particular locus (i.e. the proportion of individuals having a band). We applied the band based approach due to the polyploidy nature of our study species and set the allele frequency equal to band frequency.

2.3.2 Phylogenetic Relationships and Isolation by Distance
The relationship among populations and individuals was first explored by using Nei’s (1972) genetic distances among populations estimated in AFLP-SURV 1.0 (Vekemans et al., 2002) (available at http://www.ulb.ac.be/sciences/lagev/) with 10,000 bootstrapped distance matrices. These matrices were then graphically displayed as un-rooted neighbour-joining (NJ) trees with the NEIGHBOR and CONSENSUS programs in PHYLIP 3.65 (Felsenstein, 1989). Nei’s pairwise genetic distance matrices as well as pairwise $F_{ST}$ matrices calculated with AFLP-SURV 1.0 were also plotted against geographic distances in order to test for the effects of isolation by distance. All locations in the full data set as well as select regions and locations were compared. Significance was tested with a Mantel correlation test in R with the ade4 package (Chessel et al., 2004). Multidimensional scaling (Kruskal, 1964) was also used to visualize patterns of relatedness within the $F_{ST}$ matrices.

2.3.3 Bayesian Clustering
A Bayesian clustering approach, implemented in STRUCTURE 2.2 (Pritchard et al., 2000) was used to determine the number of genetic clusters ($K$) in the data set without any prior information on the sampling locations, using the admixture model. The number of genetically distinct clusters ($K$) was set to vary from 1 to 12 (total number of populations). Twenty independent simulations were run for each value of $K$ with a burn-in length of $10^{4}$ and a run length of $10^{5}$ Monte-Carlo Markov Chain generations. The optimal value of $K$ was estimated by using the R script STRUCTURE-Sum-2009 (part of AFLPdat; Ehrich, 2006) to summarize the output files, calculate similarity coefficients between replicate runs and to plot the means of the estimated log posterior probability of the data over the replicate runs for each $K$ value or $L(K)$ in order to determine the highest level of hierarchical structure (Evanno et al., 2005). After detection of genetic divergence among populations, the analysis was repeated for each group to determine if any substructure existed below the highest hierarchical level. Graphical output was generated using CLUMP version 1.1.1 (Jakobsson and Rosenberg, 2007) and DISTRUCT (Rosenberg, 2003) software.
2.3.4 Genetic Data Analysis

Genetic differentiation among populations was calculated as Wright’s $F_{ST}$ (Lynch and Milligan, 1994) in AFLP-SURV 1.0 (Vekemans et al., 2002) using 10,000 permutations to test for significance. The percentage of polymorphic loci (PLP), gene diversity ($H_T$), expected heterozygosity ($H_E$) and average gene diversity ($H_W$) were also estimated in AFLP-SURV 1.0. To test for significant difference in PLP and $H_T$ between locations, a Wilcoxon rank sum test was implemented in R. The relationship between PLP and $H_T$ as well as between population size and PLP or $H_T$ was also tested with a Spearman’s rank correlation test in R. Analysis of molecular variance (AMOVA) was performed to assign components of genetic variation to sets of populations defined by location in Arlequin 3.5 (Excoffier and Lischer, 2010).

2.4 Results

2.4.1 Patterns of Polymorphism and Heterozygosity

Using 300 individuals (Table 1) and two primer combinations (Table 2), 173 AFLP loci were scored across all sampling locations. Of these, 65 were polymorphic (37.6%) and used for our analysis (Table 3). In general, highly reproducible AFLP patterns were obtained. An average error rate of 2.1% was estimated across the 10 pilot samples for all primer pairs. This value fell below the maximum error rate percentage accepted for good AFLP reproducibility (5%) (Piñeiro et al., 2007). The proportion of polymorphic loci per population (PLP) varied from 31.8% to 55.5%. The total heterozygosity ($H_T$) was 0.2118. The expected heterozygosity within populations varied from 0.1397 in the Denmark locations to 0.2130 in the SK Surtsey location which had the highest heterozygosity. Vestmannaeyjar south (AH) and Stokkeyri (ST) showed similar values, 0.1964 and 0.1972 respectively. The average genetic diversity within the populations ($H_W$) was 0.1746.

A significant difference was found between the Denmark and Surtsey populations for both the PLP (DKK = 31.8%, DKS = 31.8%, SC = 48%, SD = 48%, SE = 46%, SF = 44.5%, SK = 55.5%) and the total heterozygosity ($H_T$) (Denmark = 0.1362, Surtsey = 0.1974, with $p = 0.0243$ and $p = 0.0264$ respectively). Although PLP and $H_T$ differed slightly between all the populations, none of the other populations were found to differ significantly. PLP and $H_T$ values were also moderately correlated with each other (Spearman’s rank correlation, $R = 0.64$, $p < 0.05$) but were not correlated with population size ($R = 0.12$ and $R = -0.12$, respectively, $p > 0.05$) over the whole geographic range.

2.4.2 Genetic Differentiation and Diversity

The total genetic differentiation among all 12 Honckenyapa peploides sampling locations was moderate ($F_{ST} = 0.1769$, Table 3) and highly significant (permutation test, $P < 0.001$) in all collection locations except Denmark (Table 4). The $F_{ST}$ value for Denmark was found to be non-significant ($p = 0.367$). The total $F_{ST}$ among the Surtsey populations (0.0714) as well as the Vestmannaeyjar populations (0.0550) were less than half of that found among the mainland populations (0.1747). As seen in Table 4, the largest $F_{ST}$ values were found when comparing any population with the Denmark populations (DKK and DKS), especially Greenland (GR: 0.431 and 0.440), Garður (G: 0.356 and 0.367) and location SC on Surtsey (0.348 and 0.357).
Table 3  Genetic diversity in sampling locations of Honkenya peploides.

<table>
<thead>
<tr>
<th>ID</th>
<th>Polymorphic Bands</th>
<th>PLP</th>
<th>H_E</th>
<th>F_ST</th>
<th>H_T</th>
<th>H_W</th>
</tr>
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<tbody>
<tr>
<td>SC</td>
<td>83</td>
<td>48</td>
<td>0.1740</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SD</td>
<td>83</td>
<td>48</td>
<td>0.1808</td>
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<tr>
<td>SE</td>
<td>81</td>
<td>46.8</td>
<td>0.1789</td>
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</tr>
<tr>
<td>SF</td>
<td>77</td>
<td>44.5</td>
<td>0.1699</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK</td>
<td>96</td>
<td>55.5</td>
<td>0.2130</td>
<td>0.0714</td>
<td>0.1974</td>
<td>0.1833</td>
</tr>
<tr>
<td>AH</td>
<td>86</td>
<td>49.7</td>
<td>0.1964</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH</td>
<td>82</td>
<td>47.4</td>
<td>0.1696</td>
<td>0.0550</td>
<td>0.1938</td>
<td>0.1830</td>
</tr>
<tr>
<td>G</td>
<td>78</td>
<td>45.1</td>
<td>0.1765</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ST</td>
<td>89</td>
<td>51.4</td>
<td>0.1972</td>
<td>0.1747</td>
<td>0.2265</td>
<td>0.1869</td>
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<tr>
<td>GR</td>
<td>76</td>
<td>43.9</td>
<td>0.1663</td>
<td></td>
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</tr>
<tr>
<td>DKK</td>
<td>55</td>
<td>31.8</td>
<td>0.1397</td>
<td></td>
<td></td>
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<tr>
<td>DKS</td>
<td>55</td>
<td>31.8</td>
<td>0.1328</td>
<td>-0.0003</td>
<td>0.1362</td>
<td>0.1362</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>37.6</td>
<td>0.1769</td>
<td>0.2118</td>
<td>0.1746</td>
<td></td>
</tr>
</tbody>
</table>

Total means at the species level. PLP - percentage of polymorphic loci, H_E - Nei’s (1973) gene diversity (expected heterozygosity), F_ST  - Wright’s F_ST, H_T  - total gene diversity (total heterozygosity), H_W  - average gene diversity within populations (Nei’s gene diversity).

Table 4: Nei’s genetic distances are displayed on the upper diagonal and FST values on lower diagonal. *non-significant values

<table>
<thead>
<tr>
<th></th>
<th>AH</th>
<th>BH</th>
<th>DKK</th>
<th>DKS</th>
<th>G</th>
<th>GR</th>
<th>SC</th>
<th>SD</th>
<th>SE</th>
<th>SF</th>
<th>ST</th>
<th>SK</th>
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<tbody>
<tr>
<td>AH</td>
<td>0.013</td>
<td>0.095</td>
<td>0.102</td>
<td>0.005</td>
<td>0.020</td>
<td>0.009</td>
<td>0.006</td>
<td>0.026</td>
<td>0.018</td>
<td>0.025</td>
<td>0.037</td>
<td></td>
</tr>
<tr>
<td>BH</td>
<td>0.056</td>
<td>0.128</td>
<td>0.125</td>
<td>0.006</td>
<td>0.025</td>
<td>0.011</td>
<td>0.018</td>
<td>0.050</td>
<td>0.047</td>
<td>0.061</td>
<td>0.067</td>
<td></td>
</tr>
<tr>
<td>DKK</td>
<td>0.312</td>
<td>0.396</td>
<td>0.000*</td>
<td>0.109</td>
<td>0.147</td>
<td>0.104</td>
<td>0.081</td>
<td>0.059</td>
<td>0.070</td>
<td>0.053</td>
<td>0.050</td>
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<tr>
<td>DKS</td>
<td>0.331</td>
<td>0.398</td>
<td>0.000*</td>
<td>0.112</td>
<td>0.148</td>
<td>0.105</td>
<td>0.085</td>
<td>0.070</td>
<td>0.083</td>
<td>0.062</td>
<td>0.062</td>
<td></td>
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<tr>
<td>G</td>
<td>0.021</td>
<td>0.029</td>
<td>0.356</td>
<td>0.367</td>
<td>0.019</td>
<td>0.012</td>
<td>0.018</td>
<td>0.046</td>
<td>0.040</td>
<td>0.050</td>
<td>0.058</td>
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</tr>
<tr>
<td>GR</td>
<td>0.080</td>
<td>0.109</td>
<td>0.431</td>
<td>0.440</td>
<td>0.083</td>
<td>0.020</td>
<td>0.030</td>
<td>0.054</td>
<td>0.045</td>
<td>0.060</td>
<td>0.064</td>
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</tr>
<tr>
<td>SC</td>
<td>0.037</td>
<td>0.050</td>
<td>0.348</td>
<td>0.356</td>
<td>0.052</td>
<td>0.089</td>
<td>0.004</td>
<td>0.027</td>
<td>0.024</td>
<td>0.036</td>
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<tr>
<td>SD</td>
<td>0.026</td>
<td>0.078</td>
<td>0.291</td>
<td>0.305</td>
<td>0.077</td>
<td>0.124</td>
<td>0.020</td>
<td>0.015</td>
<td>0.012</td>
<td>0.023</td>
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<tr>
<td>SE</td>
<td>0.100</td>
<td>0.188</td>
<td>0.232</td>
<td>0.269</td>
<td>0.174</td>
<td>0.202</td>
<td>0.109</td>
<td>0.065</td>
<td>0.004</td>
<td>0.004</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td>0.073</td>
<td>0.185</td>
<td>0.271</td>
<td>0.309</td>
<td>0.158</td>
<td>0.180</td>
<td>0.102</td>
<td>0.051</td>
<td>0.017</td>
<td>0.005</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>ST</td>
<td>0.092</td>
<td>0.209</td>
<td>0.206</td>
<td>0.236</td>
<td>0.175</td>
<td>0.207</td>
<td>0.134</td>
<td>0.088</td>
<td>0.018</td>
<td>0.022</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>SK</td>
<td>0.123</td>
<td>0.216</td>
<td>0.189</td>
<td>0.227</td>
<td>0.190</td>
<td>0.212</td>
<td>0.138</td>
<td>0.103</td>
<td>0.031</td>
<td>0.056</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

2.4.3  Bayesian Analysis of Genetic Structure

In the Bayesian cluster analysis obtained from STRUCTURE, the model with the highest ΔK satisfactorily explained the data obtained from the AFLP analysis (Figure 9).
Figure 9: Bayesian inference clustering results for AFLP using the admixture ancestral model in STRUCTURE software. The two genetic groups detected (clusters I and II) are represented as pie charts (dark vs. light blue) on the google earth maps of all sampling locations.

In the estimated model based on the AFLP data set when $K = 2$, most individuals in the Denmark (DKK, DKS), Stokkseyri (ST), Surtsey E, Surtsey K and Surtsey F locations were assigned to cluster I; most of the individuals in the Greenland (GR), Gardur (G), Surtsey C, Surtsey D, as well as Vestmannaeyjar north and south (BH & AH) locations were assigned to cluster II. A similar pattern can also be seen in Figures 10 and 11. No sub-structures were detected within any cluster as a result of further analysis.
Furthermore, the sample locations on the island of Surtsey showed a clear genetic split between those on the north-west coast and those on the south-east coast, also supported by the NJ Tree (Figure 10) and the multidimensional scale plot (MDS) (Figure 11).

### 2.4.4 Neighbor Joining Tree

The neighbor joining (NJ) tree based on genetic distances produced from the full dataset (Figure 10A) shows a split into two clusters (cluster I dark blue; cluster II light blue). DKK, DKS, SK, ST, SE and SF group together in one cluster with bootstrap values above 88% while GR, G, BH, SC, AH and SD group together in another cluster with bootstrap values above 53%. This clustering shows a pattern similar to both the MDS plot (Figure 11) and the Bayesian analysis (Figure 9), with the south-eastern side of Surtsey (SK, SE and SF) clustering tightly together with Stokkseyri (ST) and closer to the Denmark populations (DKK & DKS) than the populations sampled on the north-western side of the island (SD & SC) which cluster closer to the Reykjaness peninsula population (G), the Vestmannaeyjar populations (AH & BH) and the Greenland population (GR). Looking closer at only the Surtsey population (Figure 10B), two clusters, supported by bootstrap values over 98% can be seen in which the populations on the north-west side of the island (SC & SD) cluster together (Nei’s D = 0.0044) and clearly separate (Nei’s D = 0.0115) from the populations located on the south-east side of the island (SF, SE and SK).

![Figure 10: Un-rooted neighbor joining trees based on Nei’s genetic distances for; A) The full data set and B) Surtsey only. Bootstrap values are shown at the nodes (10,000 iterations).](image)

### 2.4.5 Multidimensional Scaling

The multidimensional scaling plot based on $F_{ST}$ (Figure 11) shows SD, AH, SC and GR clustering closely. A similar clustering can also be seen in the neighbor joining (NJ) tree (Figure 10A) with SC, GR, BH and G forming a cluster that splits from the other sample locations. Similarly, SK, ST, SE and SF cluster together in both the MDS plot and the NJ tree. Furthermore, the genetic distance (Table 4) between SD and SC (0.004) is less than half the genetic distance between SD and SF (0.012) and the genetic differentiation ($F_{ST}$) between SD and SC (0.020) is also less than half the $F_{ST}$ between SD and SF (0.051), indicating a clear genetic split between the locations sampled on Surtsey (Figures 10A & 10B).

![Figure 11: Multidimensional scale (MDS) plot based on $F_{ST}$ values](image)
2.4.6 Mantel Test - Isolation By Distance

A mantel test on the full data set (not shown) indicated a positive and significant association between Nei’s (1972) genetic distance and geographic distance ($r = 0.6331$, $p = 0.001$) as well as genetic differentiation ($F_{ST}$) and geographic distance ($r = 0.8083$, $p = 0.001$) (Figure 12a). This indicates isolation by distance with geographic distance explaining roughly 80% of the variation in genetic differentiation. Non-significant associations between genetic distance (Nei’s) and geographic distance as well as between genetic differentiation ($F_{ST}$) and geographic distance were detected when the populations within each site or region were compared (Figures 12b & 12c; Figures 13a & 13c). This is an indication of significant gene flow and the limited effects of isolation by distance at the regional and local scale.

However within the island of Surtsey, there appears to be a negative yet non-significant correlation between geographic and genetic distances (Figures 13b & 13d). Although not significant this is of some interest as negative spatial autocorrelations are indicative of a pattern of dissimilar values (genotypes) (Manni et al., 2004) appearing in close spatial association, a pattern also seen in the STRUCTURE analysis as well as the NJ Tree (Figures 9 and 10). Also, the mantel test between all locations (Denmark excluded, Figure 12b) and Greenland revealed a smaller and less significant correlation than the mantel test between all locations (Greenland excluded, Figure 12c) and the Denmark locations. This points to a pattern of greater differentiation between the Denmark and Icelandic populations than between the Greenland and Icelandic populations.
Figure 12: Plot of pairwise estimates of $F_{ST}$ versus geographic distance between; a) all samples of Honckenya peploides, b) mainland Iceland, Surtsey, Heimaey and Greenland, c) Mainland Iceland, Surtsey, Heimaey and Denmark
2.4.7 AMOVA

The AMOVA (Table 5) showed that variation within populations (sample locations) accounted for most (77.01%) of the genetic variance. Variation among groups (areas or regions) accounted for 17.40% of the total genetic variance and variation among populations (sample locations) within groups (areas) accounted for only 5.59%. Genetic differentiation among groups was significant (p < 0.01) and highly significant among populations within groups as well as within populations (p < 0.001).
Table 5: Analysis of Molecular Variance.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>Variance</th>
<th>Variation (%)</th>
<th>Fixation Index</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 geographic groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among groups</td>
<td>4</td>
<td>3.23899</td>
<td>17.40</td>
<td>F&lt;sub&gt;CT&lt;/sub&gt; = 0.17402</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>7</td>
<td>1.04037</td>
<td>5.59</td>
<td>F&lt;sub&gt;SC&lt;/sub&gt; = 0.06767</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within populations</td>
<td>288</td>
<td>14.33320</td>
<td>77.01</td>
<td>F&lt;sub&gt;ST&lt;/sub&gt; = 0.22992</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>299</td>
<td>18.61256</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Populations were arranged into five groups according to location; G1) DKK & DKS, G2) G & ST, G3) All Surtsey (SC, SD, SE, SF, SK), G4) AH & BH, and G5) GR.

2.5 Discussion

Due to the isolated nature and restricted size of island habitats, population establishment is predicted to result in some reduction of genetic diversity and/or a population bottleneck (Barton and Charlesworth, 1984). Levels of genetic variation in such systems are thought to be dictated mostly by the loss of genetic variation at foundation, further loss due to finite population size since colonization and addition of genetic variation due to immigration and mutation (Jaenike, 1973). Also, the number of initial founders is predicted to have strong effects on levels of genetic variation, especially if the initial number is low and the populations stay small for several generations (Nei et al., 1975). As a result, island populations usually have lower genetic diversity than their mainland counterparts. This relationship however, depends on the intensity of isolation, island size and dispersal capabilities of the organism in question (Frankham, 1997). Although in general there are significant losses of both allelic richness and heterozygosity in introduced populations, and large gains in diversity are rare, multiple introductions have been shown to rescue the losses in diversity in a wide range of plant, animal and fungal taxa (reviewed in Dlugosch and Parker, 2008). Genetic diversity or allelic variation also appears to increase over long time scales, suggesting a role for gene flow in augmenting diversity over the long-term (e.g. Désamoré et al., 2012).

2.5.1 Patterns of Diversity on Surtsey Versus Other Sites

Contrary to original expectations, genetic diversity (H<sub>E</sub>) was found to be significantly higher in the Surtsey and Iceland populations than in the Denmark populations. Furthermore, when compared to Iceland, genetic diversity remains quite similar and is even increased in some Surtsey populations. The greater population size, age and wider geographic range of *H. peploides* in both Iceland and Denmark is expected to lead to higher genetic variation in those areas (Nei et al., 1975). However, the findings of this study contradict this and shed some doubt on the long held view that genetic diversity is generally lower in oceanic island populations (Frankham, 1997; Franks, 2010). Furthermore the present results, in parallel with a growing number of studies, find either similar (Su et al., 2010; García-Verdugo et al., 2013) or greater (Fernández-Mazurecos and Vargas, 2011; Désamoré et al., 2012) genetic diversity in oceanic island species when comparing them to their mainland counterparts. We therefore postulate that the resulting genetic structure on Surtsey and overall patterns of genetic diversity...
during colonization of oceanic islands for that matter, are a more complex than previously thought (Jaenike, 1973) and are likely driven by a dynamic interaction between multiple spatial, temporal and life history variables.

Facilitated by the species long distance dispersal capabilities, the colonization of *H. peploides* on Surtsey starts with a few individuals and increases rather quickly to cover most of the empty niches on the island (Sigurdsson and Magnnusson, 2010). The dispersal distance from mainland Iceland or any other islands in the archipelago (<35km) is most likely not a huge barrier to dispersal in this group (Jonsell, 2001). Furthermore, seeds of *H. peploides* are relatively small and are not prone to desiccation by salt water as the plant is a beach dune halophyte and actually requires some form of cold stratification and surface abrasion in order to break dormancy (Baskin and Baskin, 2001; see chapter 3). These dispersal characteristics likely promote enough gene flow between populations to negate the effects of isolation by distance at the regional scale. The breeding system, possibly in conjunction with dormancy requirements, then drives differentiation at the local scale. On Surtsey as well as on Heimaey the populations remain significantly differentiated despite their close proximity.

The significant genetic distance and differentiation found among the Surtsey populations indicates that the influx of genetic material is likely attributable to multiple introductions from various source locations. Multiple introductions increase both the effective population size and the population growth rate, and have been shown to lead to an increase in gene diversity in newly colonized populations (Dlugosch and Parker, 2008). Further increases in gene diversity may have also been promoted due to differentiation across the geographical distribution of populations in the source regions (Ellstrand and Schierenbeck, 2000). Moreover, multiple introductions followed by gene flow between formerly differentiated populations has been shown to result in levels of diversity that exceed those in the source populations (Novak and Mack, 2005; Dlugosch and Parker, 2008).

Therefore it seems probable that long distance dispersal from multiple locations is a key driver of the current genetic structure on Surtsey and that *H. peploides* populations in the Iceland region are highly genetically similar (panmictic). The negative genetic consequences associated with colonization (e.g. the bottleneck effect or genetic drift) are likely reduced through the constant influx of new genetic material (Nei et al., 1975; Barrett and Husband, 1990). The maintenance and/or expansion of populations with high gene diversity on the island is then most likely fostered by: a) the vast empty niche space available there (Friðriksson and Johnsen, 1968); b) the sea barrier inhibiting establishment of other (probably competing) dispersal groups; c) the polyploid nature of the study species which is accompanied by fixed heterozygosity that maintains gene diversity (Soltis and Soltis, 2000; Brochmann et al., 2004); and d) the breeding system of the plant itself preventing successful self-fertilization (Tsukui and Sugawara, 1992).

### 2.5.2 Genetic Differentiation Among Populations and Regions

At the level of intra-island genetic diversity, genetic differentiation (*F*<sub>ST</sub>) on both the island of Surtsey as well as on Heimaey is only moderate but highly significant. This indicates some population genetic structure with strong gene flow likely preventing
divergence within the islands (see Wright 1978). However, genetic differentiation on mainland Iceland is great and indicates that there is strong genetic structuring on the island and that limited gene flow between the two populations could promote divergence (Wright 1978). This strong differentiation is likely due to environmental heterogeneity and differential selection pressures (Audigeos et al., 2013; Pannell and Fields, 2014) as sampling locations in mainland Iceland are much older and separated by roughly 85 km of highly variable, mountainous topography. On the other hand, sampling locations within Surtsey and Heimaey are relatively recent and quite proximal (~1 & 5 km apart respectively), with limited topography separating them.

At the global scale, the greater FST between sampling locations leads us to believe that the dispersal of H. peploides over its entire range is similar to the propagule model in which the founding event is severe with few colonists coming from a single source (see Slatkin, 1977). This is thought to result in increased differentiation between locations. On the other hand, the FST between regional locations is far less. This paints a picture which is more similar to the migrant model in which colonization is the result of multiple immigration events from several populations (Slatkin, 1977), resulting in greater population cohesion. This spatial variation in intensity of genetic differentiation is once again most likely explained by the plants dispersal ability and breeding system.

Mantel tests of the full data set revealed a positive and highly significant correlation between genetic distance/differentiation and physical distance. Furthermore, a Mantel test that excluded only Denmark populations revealed a smaller and less significant correlation than the mantel test which excluded only the Greenland population. This indicates that gene flow between Denmark and all other sample locations is quite limited and that there is significant isolation by distance at the global scale, similar to the propagule model postulated by Slatkin (1977). Moreover, this also implies that isolation by distance is affecting gene flow between the Greenland and Iceland locations to a lesser degree than it is affecting gene flow between all the Iceland and Denmark locations. Furthermore, similar to Slatkins' (1977) migrant model, the non-significant mantel tests of select regions and locations indicate that there is significant gene flow between all locations except Denmark. These results parallel other studies which suggest that the presence of IBD (isolation by distance) patterns depends greatly on the spatial scale in question (Gaudeul et al., 2000; Stehlik et al., 2001; Ægisdóttir et al., 2009; Eidesen et al., 2013).

One possible explanation for this is that, similar to other plants found in Iceland (Thórsson et al., 2010), Western Europe is the source region from which the original genetic material was obtained. The most probable means of dispersal likely being drift via the North Atlantic current during the early Holocene (Buckland et al., 1986). Through time and space, the Iceland/Greenland region has become genetically isolated and is possibly still in the early stages of establishment compared to the Denmark populations. Recent molecular studies also support this view, showing that circumpolar species such as Juniperus (Adams et al., 2003), Saxifraga (Abbott, 2000) and Vaccinium (Alsos et al., 2005) are of Eurasian (western Siberia) origin. These studies show that circumpolar species tend to split into Eurasian and North American lineages. The Eurasian lineages often have distributions spanning from Eurasia and northern Scandinavia to Iceland and Greenland. However an analysis of the North American biota revealed that the majority of endemic species are a result of extensive post-glacial migration (Brochmann et al., 2003; Alsos et al., 2007).
Using only data from Surtsey, Mantel tests revealed a negative and non-significant correlation between genetic distance/differentiation and physical distance. This negative spatial correlation, although not significant, may suggest that at such a small scale, dissimilar genotypes are occurring in close spatial association to one another, like that found in other studies (Matesanz et al., 2011; Diniz-Filho and Bini, 2012; Diniz-Filho et al., 2013). This is also supported by the genetic structure seen in the un-rooted NJ tree, the moderate genetic differentiation ($F_{ST}$) found between locations on Surtsey and the bayesian analysis. This is a further indication that the breeding system and outcrossing nature of the plant might be keeping inbreeding depression in check via reduced fitness of self-fertilized embryos.

In accordance with this, an analysis of molecular variance indicates that despite the clonal capabilities of *H. peploides*, most of the genetic variance is within populations (sampling locations). These results parallel similar studies on *H. peploides* conducted in Spain and Portugal (Sánchez-Vilas et al., 2010). This high degree of within population genetic variation is likely explained once again by the outcrossing nature of the plant itself. Unpublished greenhouse experiments show that very few, small seeds are produced via active self-fertilization by hand, no seeds are produced by auto-deposition and active outcrossing produces a much higher seed set than open pollination (see section 2.2.1). We therefore postulate that the variable success of long distance dispersal of the species maintains great genetic differentiation at the global scale while the higher success of dispersal at the regional scale maintains population cohesion and negates IBD. The outcrossing nature of the plant then acts as a buffer to maintain moderate genetic differentiation at the local scale.

### 2.5.3 Phylogeography of *H. peploides* in the Iceland Region

There is a clear northwest-southeast split in the Surtsey populations. This clear split found with the Bayesian analysis on the island of Surtsey and in the data set as a whole is well supported by both the un-rooted NJ tree as well as the MDS analysis. Once again, this, along with the moderate differentiation found, indicates multiple colonization events to Surtsey from several sources as well as a clear genetic split between the Iceland/Greenland and Denmark locations. This is evident from the fact that populations from Garður, Greenland, Heimaey and the western coast of Surtsey (SC & SD) are clearly differentiated from the Denmark populations. While Stokkseyri (ST) and the locations on the eastern side of Surtsey (SK, SE & SF) show an intermediate between the Denmark and Greenland locations, with greater membership in the same genotype cluster as Denmark.

It is therefore most probable, as stated above, that the colonization of Iceland by *H. peploides* took place from the east and that through time and space the Iceland/Greenland populations have become isolated and diverged substantially from the Denmark populations. The initial migration to the region could have taken place in a stepping stone like fashion via drift on ice and wood during the early Holocene (Johansen and Hytteborn, 2008). As suspected by Magnússon et al. (1996) and revealed here by our genetic structure analysis, the colonization of Surtsey likely took place from several source locations including Heimaey, the Reykjanes peninsula and the southern coast of Iceland. Judging from the bayesian analysis, NJ tree, MDS plot and $F_{ST}$, the populations closer to the Reykjanes peninsula and from Heimaey are the
The most likely source of genetic material for the populations on the western side of the island (SC & SD). The southern shore of mainland Iceland is the most probable source of colonists for the eastern side of the island (SK, SE & SF).

The full NJ tree shows two distinct clusters which indicate a similar pattern as was found with the Bayesian analysis. However, the tree gives us clearer resolution on the relationship that the Denmark populations have with the rest of the samples, indicating a very clear separation from the Iceland/Greenland region. This relationship can most likely be attributed to the time since colonization of Iceland by \textit{H. peploides} from Europe following the end of the last glacial maximum (LGM) roughly 10,000 years ago (Hallsdóttir, 1995). As the plant colonized islands along the way from mainland Europe to the Iceland region it likely encountered variable habitats which might have favored survival of diverse genotypes not favored by mainland conditions. The dispersal of these genotypes in a stepping stone fashion towards the Iceland region could then have slowly increased divergence from the Denmark populations (Le Corre and Kremer, 1998). This could also have contributed to the increased gene diversity as the availability of nearby sources of variation following colonization has been shown to mitigate founder effects in newly colonized populations (Helsen et al., 2013)

The Surtsey NJ tree also shows two distinct clusters which indicate that there is significant genetic structure on the small island. This is likely explained by the relatively young age of the island itself and by multiple colonization events combining genotypes of differentiated populations from the source regions (Ellstrand and Schierenbeck, 2000; Novak and Mack, 2005). The original reports of plant colonization to the island indicate that \textit{H. peploides} initially colonized the northeastern shore (Friðriksson, 1970). This report indicates that during the summer of 1968 at location SK (F15 in report) there were two plants (plants # 28 & 34) which had five and two branches respectively. Likewise at site SD (B13 in report) there were two plants (plants # 75 & 76); however these plants had only one stem each meaning that they were younger. This indicates several colonization events, possibly at differing times as none of the plants had produced seed yet. This could be a feasible explanation for the significant genetic distance and differentiation found between sites on the island.

Also, two sampling locations on the northwest side of the island of Surtsey (SC & SD), which are separated by a distance of up to 800 m and a 100 m high volcanic crater, have a genetic distance that is less than half the genetic distance between SD and the closest sampling location, SK, located less than 150 m away. These sampling locations were colonized at different times. The SD location was colonized first in 1968 and the SC (I8 in report) location was not colonized until after 1978 (Friðriksson, 1982). The genetic similarity of SC and SD could possibly indicate that seed movement and spread of \textit{H. peploides} within the island of Surtsey is being facilitated by seabirds or migratory passerines such as the snow bunting (\textit{Plectrophenax nivalis}). This is a feasible scenario as previously Friðriksson (1975) reported finding seeds (not \textit{H. peploides}) in the digestive tracts of \textit{P. nivalis} caught on the island, suggesting that these birds are capable of distributing seeds long distances. These seeds were later germinated and produced viable offspring (S. Friðriksson, pers. comm., 2013). Moreover, tagged \textit{P. nivalis} from as far away as Norfolk England have been sighted near Stokkeyri in southern Iceland, Akureyri in the north and Seyðisfjörður in the east
(Middleton, 2012) and it is quite probable that these same birds use Surtsey as a stopping point on their long journey from western Europe.

### 2.5.4 Present Research and Future Prospects

It is through the dynamic interplay of spatio-temporal factors, breeding systems, seed dispersal and polyploidy that heterozygosity is maintained in colonizing arctic plant populations and the recently colonized *Honckenya peploides* population on Surtsey is no exception. Despite being faced with inbreeding and bottlenecks these populations still have the ability to maintain relatively high heterozygosity. It is likely that numerous, long distance seed dispersal events from multiple differentiated sources have lead to high genetic diversity during colonization. Heterozygosity was then maintained by the outcrossing nature of the plants dioecious breeding system and its tetraploid genomic constitution. In polyploid plants, this guarantees genetic variation through fixed heterozygosity in duplicated genomes (Brochmann et al., 2004; Soltis and Soltis, 2000).

When such populations are confronted with various novel, heterogeneous environments, the presence of duplicated genomes within each individual allows for selection to act on a greater number of available traits (Soltis and Soltis, 2000). This increases survival of diverse genotypes in variable niches. Such a statement has some implications for conservation efforts as it implies that conservation strategies in the Arctic should be focused on large numbers of small, spatially separated populations and the migratory-dispersal routes separating them rather than a small number of large populations. This will maintain pockets of diversity that can then be utilized in a similar manner as seed banks (León-Lobos et al., 2012). Such an effort can assure influxes of diverse genetic material for populations struggling to adapt to anthropogenic environmental changes, helping to secure ecological diversity for coming generations (Vitt et al., 2010).

The inferences drawn from the preceding analysis should be further validated with a more detailed investigation combining the methods used above with detailed cytogenetic, physiological and morphometric analysis. Such studies should sample populations from all shoreline areas of Iceland and compare them with populations from Greenland, Denmark, Norway, Northern Siberia, Spain, Portugal as well as the Shetland, Orkney and Faroe islands. If only sampling is considered, such an investigation is quite realistic and rather cost effective. Collaborations with other scientists can reduce travel expenses and consolidate time so that additional funding can be utilized to improve analytical methods. A phylogeography study of this scale will give a more comprehensive understanding of the dispersal history of *H. peploides* in the north Atlantic region, provide valuable insight into the long standing glacial “refugia” debate (reviewed by Habel et al., 2010) as well as perhaps most importantly provide a greater and novel understanding of the processes which drive the evolution of gene diversity.
3 Karyotype Variation of Honckenya peploides Worldwide

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3.1 Introduction

The global morphology of the nuclear genome displays great variation among plant species (Johnson et al., 2003; Stuessy et al., 2004; Perný et al., 2005; Suda et al., 2007; Sonnleitner et al., 2010; Travniecek et al., 2010; Spaniel et al., 2011). As effective plant cytological methods are being developed (e.g. Anamthawat-Jónsson, 2004) they are increasingly being used to explore ploidy levels and chromosome morphology within plant groups. The presence of widespread cytotypic and karyotypic variation within plant species is causing some re-evaluation of previously held assumptions on the degree of intra-specific karyotype variation found in plants (Delaney and Baack, 2012; Fehlberg and Ferguson, 2012; Kono et al., 2012). For some time the prevailing paradigm has been that a species has only one chromosome number, ploidy level and karyotype structure. This was thought to be due to the fact that variable chromosome numbers usually bring about meiotic irregularities and/or sterility (Levin, 2002; Stebbins, 1971). However, recent studies have identified plant species which have more than one diploid somatic chromosome number, most frequently due to intraspecific polyploidy (Frello and Heslop-Harrison, 2000; Stuessy et al., 2004; Suda et al., 2007). Yet other species have shown complex patterns of chromosome numbers thought to have arisen through a dysploid series at the diploid level accompanied by hybridization and polyploidy. These include *Prospero autumnale* (Hyacinthaceae) which has 2n = 10, 12, 14, 25, 26, 27, 28, 42 (Ainsworth et al., 1983; Ebert et al., 1996) and *Scilla scilloides* (Hyacinthaceae) with 2n = 16, 18, 26, 27, 34, 35, 36, 43 (Haga and Noda, 1976; Choi et al., 2008), to name a few. These intraspecific karyotype variants have been termed cytotypes or cytoraces (Stuessy, 2009). Some even suggest that various reproductively isolated cytotypes within species may indeed represent cryptic species (Soltis et al., 2007) as various cytotypes might be found allopatrically, parapatrically, or in mixed populations.

Slight morphological differences among cytotypes within species, particularly in floral and fruit characteristics have been shown in several studies (Perný et al., 2005; Cires et al., 2009; Spaniel et al., 2011). Investigations into the Crowberry, *Empetrum*, from locations in the Czech Republic found that cytotype is strongly associated with flower sex, with diploids having unisexual flowers and the tetraploids having exclusively bisexual flowers (Suda et al., 2004). Also, in some instances cytotypic differences can lead to changes in pollinator species, creating limitations on gene flow between cytotypes. For instance, varying cytotypes of the rhizomatous herb, Alumroot, *Heuchera grossulariifolia*, growing sympatrically in Northern Idaho have been shown...
to attract differing pollinators. Diploid and autotetraploid *H. grossulariifolia* plants act essentially as separate ecological species and may experience partial reproductive isolation through differential visitation and pollination by their floral pollinators (Thompson and Merg, 2008). Such morphological and physiological variation among different cytotypes can lead to the use of diverse ecological niches (Johnson et al., 2003), often leading to the reduction of one or more of the cytotypes or to further genetic differentiation between them (Garcia et al., 2008). In other cases there may be no detectable differences in appearance among cytotypes, but higher ploidy simply provides the plants with the ability to cope with environmental stresses which is an adaptive advantage (Leitch and Leitch, 2008). Numerous series of cytotypes and polyploid complex via hybridization have been evident among alpine-arctic plants, e.g. in Brassicaceae (Grundt et al., 2005).

Four subspecies of *Honckenya peploides* have been described. These include: subsp. *peploides* which is found on the coasts of northern Norway down to northern Portugal; subsp. *diffusa* which has a circumpolar distribution mainly in Arctic and northern Boreal zones; subsp. *robusta*, found in Northeastern North America; subsp. *major/oblangifolia*, found in the North West Pacific area and on the coasts of northeastern Russia down to Japan (Houle, 1997; Jonsell, 2001; Probatova et al., 2004) (see Section 1.6.1). Studies from several locations over the whole of the species range report varying chromosome numbers (2n = 4x = 66, 68, 70) as well as possible differing ploidy levels including 2n = 34 (Probatova et al., 2004) (see Table 6).

**Table 6: All published chromosome numbers for Honckenya peploides.**

<table>
<thead>
<tr>
<th>Location</th>
<th>Source</th>
<th>2n =</th>
</tr>
</thead>
<tbody>
<tr>
<td>Svalbard</td>
<td>Flovik (1940)</td>
<td>66</td>
</tr>
<tr>
<td>Dragor, Denmark</td>
<td>Malling (1957)</td>
<td>68</td>
</tr>
<tr>
<td>Sakhalin Island, Russia</td>
<td>Sokolovskaya &amp; Strelkova (1960)</td>
<td>66, 68, 70</td>
</tr>
<tr>
<td>Wrangle Island, Russia</td>
<td>Agapova (1993)</td>
<td>66, 68, 70</td>
</tr>
<tr>
<td>North Eastern Russia</td>
<td>Zhukova (1966)</td>
<td>68</td>
</tr>
<tr>
<td>Eastern Russia</td>
<td>Zhukova (1966)</td>
<td>66</td>
</tr>
<tr>
<td>Moneron Island, Russia</td>
<td>Probatova (2004)</td>
<td>34*</td>
</tr>
<tr>
<td>North Eastern Asia</td>
<td>Zhukova (1966)</td>
<td>68</td>
</tr>
<tr>
<td>Ogotoruk Creek, N.W. Alaska</td>
<td>Johnson &amp; Packer (1968)</td>
<td>68</td>
</tr>
<tr>
<td>Meade River, N. Alaska</td>
<td>Packer &amp; McPherson (1974)</td>
<td>68</td>
</tr>
<tr>
<td>Western Alaska</td>
<td>Murray &amp; Kelso (1997)</td>
<td>68</td>
</tr>
<tr>
<td>Arctic Canada</td>
<td>Löve &amp; Löve (1982)</td>
<td>68</td>
</tr>
<tr>
<td>Iceland</td>
<td>Löve &amp; Löve (1950, 1956, 1970)</td>
<td>66, 68, 70</td>
</tr>
<tr>
<td>Sweden</td>
<td>Lövkvist and Hultgård (1999)</td>
<td>68</td>
</tr>
</tbody>
</table>

* indicates diploid.

The relationship between this cytotypic variation and variation in physiological, morphological or life history characteristics remains largely unexplored. For example, seed germination of the diploid (2n = 34) *H. peploides* subsp. *oblangifolia/major* occurred...
within three weeks at temperatures of no less than 28°C without prior cold stratification (Voronkova et al., 2011). However seeds of the tetraploid (2n = 68) *H. peploides* began to germinate only after cold stratification for up to 12 weeks at 2°C and germinated only at temperatures of 5 - 15°C (Baskin and Baskin, 2001; S. H. Árnason, unpublished work). Variation in morphology can also be seen between *H. peploides* in Greenland, Svalbard and Norway, with the Svalbard ecotypes being generally smaller in all traits measured, as well as having greater genetic diversity (M. Philipp, pers. comm.). Furthermore, on the island of Iceland, three different chromosome numbers have been reported by Löve and Löve (1950, 1956, 1970), yet to the authors knowledge, no investigation has been carried out which attempts to explain these differences in karyotype using physiological, morphological or life history variables.

In light of these apparent cytological uncertainties in the species as a whole, cytogenetic methods used to characterize genome structure such as karyotyping and fluorescence in situ hybridization (FISH) should prove to be valuable tools. Such investigations can shed light on the roles that karyotype variation together with environmental heterogeneity plays in the evolution of species (Lodh and Basu, 2013). Differences in chromosome number, form and size within species can have substantial phenotypic consequences (Balao et al., 2011) as the nuclear DNA content itself is known to influence every stage of development, from the cellular to the somatic (Richards, 1997). These differences often lead to differential survival of favorable ecotypes, resulting in variable spatio-temporal distributions both globally and locally (Ray, 2010; Bennett and Leitch, 2011). Therefore, karyotypes combined with molecular phylogenetic analysis can provide information about taxonomic relationships, ecotypic variation, genetic peculiarities and the evolutionary origins of species (Watanabe et al., 1999; Ruffini Castiglia et al., 2010; Lysak and Koch, 2011). Furthermore, chromosome size differences, arm-length ratios and FISH can be used to distinguish identifying features and loci between ecotypes (Truta et al., 2010; Ebadi-Almas et al., 2012; Young et al., 2012), furthering our understanding of the effects that karyotypic variation has on population genetic differentiation.

In order to obtain chromosome spreads suitable for karyotyping using the enzymatic root tip squash method, the chromosomes must be very well spread apart and the background clear of fragments of cytoplasm (Anamthawat-Jónsson, 2004). The cells from which the chromosomes are obtained must be arrested in metaphase and be appropriately condensed and free of damage. In order to obtain such chromosomes the parts of the plant containing actively dividing cells such as the root tips, leaf meristems, calli or protoplasts must be used. To obtain information regarding chromosome number and karyotype variation between ecotypes, individuals of *H. peploides* were chosen for chromosome isolation using the enzymatic root tip squash method and stained using DAPI (4 ’,6-diamidino-2-phenylindole), a fluorescent stain that binds to the A-T rich regions of DNA. The data obtained can provide a good basis for distinguishing between subpopulations and will provide insight into the mechanisms driving population differentiation.

The objectives of this study were as follows: (i) determine the chromosome number for populations of *H. peploides* located on the Seltjarnarnes Peninsula, in Reykjavík Iceland; (ii) obtain a collection of individual representatives for further studies intended to examine karyotype variation in the worldwide distribution of the species; (iii) determine the proper protocol for germination of *H. peploides* seed from the sample locations.
3.2 Materials and Methods

3.2.1 Plant Material

Samples for karyotyping were collected during September 2012 from Seltjarnarnes peninsula (64° 09' N / 22° 01' W) in Reykjavík, Iceland. Live plants were placed into plastic containers with the native substrate and kept at 15°C under a grow lamp with a 12/12 light/dark regime. In order to obtain plant material spanning the worldwide distribution of the species, a correspondence with multiple research institutions around the globe was initiated by the author SHÁ. Using this method, seeds of \textit{Honckenya peploides} were obtained from Keibu, Estonia (59° 14’ 47.29” N, 23° 39’ 50.17” E), Kolobrzeg, Poland (54° 17’ 00.57” N, 16° 09’ 45.79” E), Cornwall, England (50° 32’ 45.26”N, 5° 02’ 20.00W), Maryport, Scotland (54° 40’ 13.23” N, 4° 52’ 52.68” W), Madeleine Island, Canada (47° 23’ 44.94” N, 61° 50’ 58.10” W), Miquelon Island, Canada (47° 04’ 32” N, 56° 22’ 46.61” W) and Cold Bay, Alaska (55° 12’ 00.27” N, 162° 42’ 47.25” W) (Figure 14). These seeds were treated to cold stratification, germinated and grown under 12/12 light/dark regime. All plants were maintained and grown in the Plant Genetics research laboratory growth room located on the second floor of the Natural Sciences building at the University of Iceland, Reykjavík.

\textbf{Figure 15:} Collection locations worldwide. From east to west: Keibu, Estonia; Kolobrzeg, Poland; Cornwall, England; Maryport, Scotland; Madeleine Island, Canada; Miquelon Island, Canada; Cold Bay, Alaska. Map from Google Earth.
3.2.2 Seed Stratification, Sterilization and Germination

Seeds of *H. peploides* obtained from the various locations (Figure 14) were split into two groups, those to undergo stratification and those to be germinated right away. For each location, one half was stratified by placement into plastic bags with a moist, sandy medium and refrigerated at 2 - 4°C for 12 weeks. The other half of the seeds obtained from each location was sterilized right away using the procedure described below and placed into germination directly after. Seeds that had been stratified for up to 12 weeks as well as those that were germinated right away were sterilized by placement in 5% CL solution for 2 minutes, then rinsed with distilled water and placed in a Petri dish between two sterile filter papers. Petri dishes were then placed under a 12/12 l light regime at 15°C during light hours and then moved into a dark refrigerator and kept at 5°C during dark hours until germination. Germination success was estimated visually and noted as % germination per plate. Once germinated, five seedlings from each location were placed in plastic pots with a mixture of sand and soil, labeled and separated according to location of collection. Root tips were then collected from the Seltjarnarnes individuals in order to obtain chromosomes for karyotyping using the enzymatic root tip squash and DAPI fluorochrome staining method described below. Pending further funding for a more detailed analysis of global karyotype variation using FISH, individuals from the other locations are being stored at the Plant Genetics Laboratory growth room at the University of Iceland.

3.2.3 Enzymatic Root Tip Squash Method

Enzymatic root tip squash was performed according to Anamthawat-Jónsson (2010). Young root tips were harvested from the live plants at mid-day. Root tips were cut to approx. 1 - 3 cm. and placed in 15 mL tubes with ice water and kept at 4°C with ice for 24 - 27 h in order to synchronize mitosis and arrest as many cells as possible in metaphase. Root tips were then placed in another 15 mL tube containing a fixative solution of glacial acetic acid/96% ethanol (1:3) and allowed to sit at room temperature for about two hours before keeping them in a freezer at -28°C. After fixation, root tips were submerged in an enzyme buffer twice in 20 minutes at room temperature and then placed into an enzyme mixture at 37°C for 30 minutes. Roots were then submerged again in the enzyme buffer and the solution changed after 5 minutes. Root tips were then placed one at a time onto microscope slides that had been pre-cleaned with chromic acid (chromium trioxide in 80% sulphuric acid) for 3 hours, rinsed thoroughly in tap water and placed in 96% EtOH before use. The ends of the roots were removed, leaving only the tip (1 - 2 mm) containing the meristematic tissue on the slide. The slide was then placed under a stereo microscope at 20x magnification, a drop of 45% acetic acid added and the meristem teased apart, allowing the mitotically dividing cells to flow into the acetic acid. A cover slip was then placed on top and pressed flat. The slides were analyzed for quality in a phase contrast microscope. Good quality slides were then dipped in liquid nitrogen, the cover slip removed and slides stored in an air tight storage box at 4°C until further use. Prepared slides were stained with 20 μL of the fluorochrome DAPI (1 μL/ml) (4 ’,6-diamidino-2-phenylindole) for 1 minute and viewed under Nikon Eclipse 800 epifluorescent microscope using a UV filter block with 340-380 excitation and 430-450 emission wavelengths (blue). The images obtained with the 100x objective were captured with a CCD camera and used for chromosome number determination and karyotyping.
3.3 Results

3.3.1 Seed Stratification, Sterilization and Germination
The sterilization procedure proved effective at removing most bacterial, mold or fungal contaminants from the seed coat as only 7.1% (22 of 311) of Petri dishes containing germinating seeds were contaminated. Of the unstratified seeds, only the samples obtained from Cold Bay, Alaska, germinated under the 12/12, 5°C/15°C conditions, with most germination occurring within five to seven days. None of the seeds obtained from other locations germinated without prior cold stratification. Germination rates following cold stratification for 12 weeks were quite high, with roughly an 80% germination rate in all the non-contaminated plates at 12/12, 5°C/15°C.

3.3.2 Karyotype
After root tip samples had been prepared and stained with DAPI the slides were scanned at 200-400x magnification to fine metaphases and then at 1000x magnification to obtain the chromosome number. Microscope pictures indicate the presence of 68 chromosomes, such as the metaphase shown here (Figure 16a). In this cell, two chromosomes with satellites are indicated and this is clearly the pair of homologous chromosomes carrying active ribosomal loci NOR, Nucleolar Organizing Region (Figure 16b). Chromosome sizes ranged from 1.36 μm – 4.66 μm and most seem to be metacentric and sub-metacentric. A karyotype constructed shows chromosomes lined up by size and paired with possible homologs (Figure 16c).

Figure 14: Honckenya peploides chromosomes stained with DAPI taken at 1000x magnification (a), with drawn outlines (b) and karyotypically arranged. Arrows indicate locations of satellites
3.4 Discussion

3.4.1 Significance of Chromosome Numbers

A total of 68 chromosomes were counted for *H. peploides* from Seltjarnarnes peninsula, Iceland. This is the same number as Malling (1957) reported for plants from N. America, Germany and Denmark. Furthermore it is also one of the three values that Löve & Löve (1956) found for locations in Iceland. These results, lead us to believe that 2n (4x) = 68 is an intermediate cytotype in Iceland and that this is one of three tetraploid cytotypes found on the island (i.e. 2n = 66, 68, 70). Such thinking is not far fetched as a survey of a wide range of species reports that 12-13% of all angiosperms display some cytotypic variability (Wood et al., 2009). Furthermore, a literature review of cytotype comparisons from the sub-species *H. oblongafolia* (Probatova, 2004) from the western Pacific region revealed a possible genome duplication event. Populations on the southern shores of the island of Moneron (~46°N, 141°E) were found to be diploid (2n = 34), however populations from the nearby island of Sakhalin (~50°N, 142°E) were found to be tetraploid with two cytotypes (2n = 4x = 68, 70) (Sokolovskaya & Strelkova, 1960). Moreover, populations from the more northerly Wrangle island (~71°N, 179°E) were found to be tetraploid and contained three cytotypes (2n = 4x = 66, 68, 70) (Agapova, 1993; Goldblatt, 1985).

The co-existence of such variable cytotypes could be facilitated by ecological barriers to gene flow or by numerous biological reproductive barriers which prevent hybridization between proximal cytotypes (Husband and Sabara, 2003). Such barriers can be present at various steps of reproduction. For instance, differences in both insect pollinator composition (Seagraves and Thompson, 1999) and foraging patterns (Kennedy et al., 2006) was found to reduce pollen exchange between cytotypes of *Heuchera grossulariifoli* and *Chamerion angustifolium* respectively. Moreover, cytotype hybridization is also thought to be prevented by gametophytic selection via pollen/ovule non-conformity (Howard et al., 1998). Also, spatial division of cytotypes via ecological segregation is often observed (Bretagnolle and Thompson, 1995; Felber-Girard et al., 1996; Duchoslav et al., 2010; Sonnleitner et al., 2010) and is thought to have a large influence on ploidy coexistence.

The spatial relationship between ploidy states within this study species can be characterized as either sympatric, parapatric or allopatric. A sympatric and/or parapatric cytotype distribution is likely found on the island of Iceland, resulting in several cytotypes on the island. This is possibly the result of some yet unknown physiological or morphological reproductive barriers. Changes in morphological or physiological characteristics are frequently accompanied by changes in cytotype, often resulting in ecological differentiation between cytotypes (Levin, 2002). An allopatric cytotype distribution is likely found in the south-western pacific region, resulting from geographical barrier between ploidy groups that maintains the diploid and tetraploid cytotypes. Examples of all three forms of cytotype distribution have previously been described in the literature. An extreme case of allopatric distribution can be found with one pantropical species, *Nymphoides indica*. This aquatic plant of the Menyanthaceae family is diploid in the Old World but tetraploid in the New World (Ornduff, 1970). In comparison, a sympatric distribution was found throughout the overlapping range of the diploid and tetraploid cytotypes of the Blue Ridge Mountain endemic *Galax ureolata* (Burton and Husband, 1999). Lastly, an example of parapatry can be found in the Central European
distribution of *Vicia cracca* in which tetraploid cytotypes are found in the western limits of the species range and diploids in the east (Travnicek et al., 2010).

The abundance of polyploidy within plants has led some to speculate as to the effects of genome duplication on species diversification (Otto and Whitton, 2000; Leitch and Leitch, 2008). Some argue that polyploidy simply accumulates as a unidirectional mutation, having little influence on the degree or direction of evolutionary divergence (Stebbins, 1971; Meyers and Levin, 2006). Proponents of this view hypothesize that the evolution of polyploidy is a one-way process in which chromosome number can increase but not decrease. If reversals do take place, the haploids derived are quite rare, have reduced fitness and high sterility (Stebbins, 1980; Ramsey and Schemske, 2002). Others propose that evolutionary diversification may be enhanced by polyploidy and that it could lead to elevated levels of divergence and species richness (Otto and Whitton, 2000). Elevated diversification could take place if polyploidy increases the rate of adaptive divergence and the evolution of reproductive isolation or decreases species extinctions (Otto and Whitton, 2000). Furthermore, the capacity to differentiate extra gene copies (sub/neofunctionalization) or the effects of genomic restructuring on current genetic and phenotypic variation could enhance such divergence (Schranz and Osborn, 2004; Tate et al., 2005). Polyploidy is widespread in the flowering plants (Bennett, 2004) and the success of polyploids is known to be attributable to their highly plastic genome structure and the ability to restructure in response to external changes (Leitch and Leitch, 2008).

Variation in the cytotype of *Honckenya peploides* likely contributes in some way to the morphological variation found within the species. Slight morphological variability has led to the classification of the four recognized sub-species (Jonsell, 2001). However, since morphological divergence is often used as a criterion for sub-species delineation, some of the reported polyploid lineages may not be recognized at the sub-species level. This is especially true for autoploids such as this as they have a close resemblance to their diploid progenitors (Soltis et al., 2007). A more indepth cytogenetic study conducted throughout the species range is likely to confirm the existing cytotypes and may reveal more cytotypes than have been reported so far.

### 3.4.2 Future Prospects

Further studies must be conducted on the individuals grown from seed from all locations and new locations must be sampled throughout the species range. The use of chromosome banding should assist in the evaluation of the global variation in karyotype for the species (Guerra, 2008; Jang et al., 2013). Furthermore, correlating this variation with individual morphological features as well as environmental aspects from the plants with different origins will further our understanding of the mechanisms influencing changes in karyotype morphology, in turn shedding further light on the factors driving population differentiation and speciation similar to that found in other plant groups (Li et al., 2010; Balao et al., 2011; Schmickl and Koch, 2011). In the future we would like to use Fluorescent in situ hybridization (FISH), specifically targeting the highly conserved 45S and 5S ribosomal regions in order to study *H. peploides* karyotype evolution. In numerous plant groups the FISH mapping of these ribosomal genes has been shown to have a very high physical resolution for cytotaxonomic purposes (e.g. Taketa et al., 2005; Chokchaichammankit et al., 2008; Barros e Silva et al., 2013). Morphometric data on *Honckenya peploides* attained from
this pilot study and environmental data from the original habitats would then be correlated with cytotype variation in order to paint a clearer picture of what is driving diversity within this system. Combining these data with similar analysis of *Schiedea*, a sister genus of herbs, vines, and small shrubs endemic to the Hawaiian Islands, consisting of 34 known species which are all thought to have arisen from a single colonization event (Wagner et al., 2005), will also pose some interesting questions and hopefully answers in future studies.
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