Developing Microsatellite Markers for *Cypripedium passerinum* (Sparrow's Egg Lady's Slipper)

Lina Lim

Mentor: David McFadyen

Department of Biological Sciences, MacEwan University

liml24@mymacewan.ca

Abstract

Natural and anthropogenic disturbances contribute to increased habitat loss and fragmentation and subsequently, species loss. Integrated conservation approaches combine both *in-situ* and *ex-situ* approaches whereby natural habitats of endangered species are conserved, and the genetic diversity of the threatened population is retained outside of their natural habitat. Therefore, an essential component of an effective conservation strategy is to assess genetic variation to ensure that the conservation approach employed is effective in preserving the diversity of the whole population. Microsatellites, highly polymorphic repetitive DNA sequences in the genome of all organisms, have proven to be a valuable tool in the assessment of genetic diversity. This project aimed to isolate microsatellite markers from *Cypripedium passerinum,* a native North American terrestrial orchid at risk of extinction. Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) was employed to generate a genomic DNA library enriched for AT, AC, and AAG microsatellites. Clones were selected from the libraries and bidirectionally sequenced to identify those which contain microsatellites. A total of 158 microsatellite loci were identified, of which 83% were perfect microsatellites. PCR primers were developed using the unique sequences flanking the identified microsatellites and were evaluated for their utility. Primers amplifying polymorphic loci can be used to assess the genetic diversity of *C. passerinum* populations both within the Wagner Natural Area, Alberta, Canada and elsewhere in its range of distribution. The project findings will contribute to the integrated conservation efforts to protect species found in Wagner Natural Area and contribute to our understanding of *C. passerinum.*

Keywords Conservation, Microsatellites, *Cypripedium,* FIASCO, Genetic Diversity

Introduction

In the age of the Anthropocene, there has been a steady and exponential decline in population biodiversity (Butchart et al., 2010). Both natural and anthropogenic disturbances such as deforestation, overexploitation, and climate change have contributed to this loss of biodiversity. These pressures have led to habitat loss, which is increasing establishment of invasive species and pollution events which have contributed to substantive species loss over the last decade (Knapp et al., 2021; Meerabai and Pullaiah, 2016). Furthermore, habitat loss or fragmentation are considered to be the leading cause in current species extinction events as it reduces population sizes and increases the susceptibility of species found within the habitat to evolutionary forces (Fahrig, 1997).

The rate of species extinction is rising at an exponential rate, which may have serious ecological impact (Sodhi et al., n.d.). The consequences of extinction may vary with the role of the extant species within the ecosystem (Sodhi et al., n.d.). The loss of keystone species may cause ecosystems to collapse from top-down effects that lead to cascades of secondary extinctions in a process called trophic cascade (Eklöf and Ebenman, 2006). Such cascades were observed when large coastal sharks were overexploited for their fins and meat in the North Atlantic Ocean (Sodhi et al., n.d.; Myers et al., 2007). This led to the subsequent increase in cownose rays and a concomitant decrease in bivalves leading to potential ecosystem degradation due to increasing stress (Myers et al., 2007). Furthermore, important species such as pollinators of flowering plants (angiosperms) can become extinct due to trophic cascades (Sodhi et al., n.d.). Therefore, extinction cascades can lead to an imbalance in the food web connection and disrupt ecosystem function and structure. Hence, it is essential that the consequences of species loss are minimized.

Conservation efforts have increased to mitigate biodiversity loss through *ex-situ* and *in-situ* approaches (Cecco et al., 2020). *In-situ* conservation measures involve the protection of ecosystems and habitats "in place" by establishing natural reserves and conservation corridors and subsequently conserving the species biodiversity found within these ecosystems (Cecco et al., 2020; Li and Pritchard, 2009). An example of an *in-situ* conservation approach within Alberta

is the natural area, Coates Conservation Land. The landscape of this area is mostly parkland forest, which is a type of habitat that is rapidly lost and undergoing fragmentation (Edmonton & Area Land Trust, 2021). *Ex-situ* conservation aims to preserve groups of species that are genetically diverse outside of their natural habitat by establishing zoos or botanical gardens (Li and Pritchard, 2009). The goal of *ex-situ* conservation is to preserve population genetic diversity when certain alleles are lost in nature, perhaps due to evolutionary mechanisms (Li and Pritchard, 2009). Other than maintaining a variety of plant species and their seeds in a botanical garden, there are also captive breeding programs in zoos that help retain genetic diversity (Lewis et al., 2019). Conservation programs attempt species recovery by reintroducing or assisting colonization of species whose genetic diversity was maintained in *ex-situ* conservation approaches (Cecco et al., 2020; Fath, 2018; Reiter et al., 2016). An example of an *ex-situ* conservation approach within Alberta is the Muttart Conservatory. Muttart Conservatory is a botanical garden found in Edmonton, where three different types of biomes with differing climates are used to sustain different growth condition requirements for more than 700 species of plants (City of Edmonton, 2021). Although these conservation approaches are effective, there has been instances where it was not sufficient to prevent species loss.

The efficacy of *ex-situ* and *in-situ* conservation approaches alone are not always capable of preserving species genetic variation as the rate of species extinction exceeds species regeneration (Cecco et al., 2020). Previous conservation approaches such as the one employed by International Rhino Foundation (IRF) have failed due to excessive poaching of northern white rhinos, leading to the near-extinction of this species. The IRF began by establishing the Garamba National Park in the Democratic Republic of Congo to retain the genetic diversity of these rhinos *in-situ.* Due to the large-scale poaching of rhinos for their horns for the illegal market, the *in-situ* conservation approach could not prevent species loss (International Rhino Foundation, 2020). Northern white rhinos are considered extinct in the wild, and only two remain in captivity (International Rhino Foundation, 2020). Moreover, studies have shown that existing protected areas will not be sufficient in overcoming the projected loss of marine and terrestrial biodiversity due to anthropogenic stresses unless additional strategies to prevent species loss are implemented (Mora and Sale, 2011). A study conducted by Rada et al. (2018)

aimed to assess whether protected areas could prevent biodiversity loss for butterflies by comparing biodiversity within and outside of protected areas. The authors found that there was a higher number of butterfly species within the protected area than the unprotected areas (Rada et al., 2019). Despite that, there was an overall decline in butterfly species between the years of 2005 and 2015, which showed that protected areas could not prevent species loss over time. These studies highlight the importance of implementing multiple strategies to halt the high species extinction rates. Thus, many conservation strategies use the integrated approach, which involves *ex-situ* and *in-situ* approaches to enhance the rate of species recovery and prevent species loss.

The integrated conservation approach has been used in many different conservation programs and has successfully recovered endangered species. In 1987, the Running Buffalo Clover, *Trifolium stoloniferum,* was declared endangered, prompting authorities to implement an integrated conservation approach (U.S. Fish & Wildlife Service, 2011). Authorities monitored, restored, and protected the clovers' habitat and stored the clover's seeds in the National Center for Genetic Resource Preservation to provide a seed source for future reintroduction and augmentation (U.S. Fish & Wildlife Service, 2011). The conservation effort for the Running Buffalo Clover was successful and led to the recommendation for the species to be downlisted from endangered (U.S. Fish & Wildlife Service, 2011). The successful recovery of the Running Buffalo Clover illustrates the efficacy of an integrated conservation approach.

To maximize the efficacy of conservation strategies, endangered species should be extensively studied. Ecological, geographic, and genetic variation data can elucidate the evolutionary and biogeographic history of endangered species, which can help develop the most efficient conservation program for that species (Li et al., 2005). Species with low genetic diversity are highly susceptible to extinction due to evolutionary forces (genetic drift, population bottlenecks, natural selection) and low adaptability (Moss et al., 2003; Pritchard et al., 2012). *Aconitum austrokoreense,* a medicinal herb endemic in Korea, was declared endangered due to its extremely low genetic diversity, increasing its susceptibility to extinction from population bottlenecks resulting from geographical isolation, illustrating the significance of assessing a population's genetic diversity (Lee et al., 2018). The assessment of genetic

diversity of a population can help predict its susceptibility to environmental pressures and prevent species loss.

Amongst the molecular tools used in conservation studies to assess genetic diversity of a population are DNA microsatellites. Microsatellites, also referred to as simple sequence repeats (SSRs), are a type of tandem repeat found in the DNA of all organisms (Butler, 2007; Li et al., 2004). The repeating unit is typically a 2-7 nucleotide unit iterated 5 to 50 times (Fan and Chu, 2007). The repetitive nature of microsatellites creates a mutation hot spot due to strand slippage during replication that results in an expansion or contraction in the number of repeating units (Fan and Chu, 2007). As a result, microsatellites with high variation are formed, providing a means to deduce genetic diversity of a population (Fan and Chu, 2007). Thus, polymorphic microsatellites have been extensively used in conservation studies to study population genetic diversity.

Microsatellites have been extensively used to study genetic diversity in various plants and animals (Almeida et al., 2013; Karl et al., 2014; Arnold et al., 2002). Karl et al. (2011) used microsatellites to determine the implications of geographical isolation seen in sharks and provided insight into sexual differences in philanthropy amongst sharks. Numerous studies in barley have used microsatellites to characterize barley genotypes, origin, and relatedness between cultivar and wild type forms which helped develop conservation strategies for barley (Struss and Plieske, 1998). Primers designed to flank microsatellites for one study were shown to be able to isolate microsatellites in closely and distantly related species (Almeida et al., 2013; Arnold et al., 2002). Almeida et al. (2013) were able to isolate microsatellites in *Cattleya* species, which is one of the most endangered species of orchids. They isolated microsatellites and designed primers to isolate more microsatellites in *C. labitata* and later on used the same primers to isolate microsatellites in a closely related species, *C. warneri.* The ability of the primers to amplify microsatellites and detect polymorphisms between two *Cattleya* species indicates the possibility of cross-species amplification using the same primers. Similarly, Arnold et al. (2002) was able to isolate microsatellites from the *Vitacae* species using microsatellites loci from a distantly related grape species. This would allow primers designed to isolate microsatellites in projects like this one to be used in species closely or distantly related to *C.*

passerinum. Microsatellites are therefore a valuable tool in conservation studies to study genetic variation and increase the effectiveness of conservation strategies.

Globally, plants are going extinct 3.5 times faster than vertebrate species, with an estimated 450 000 plant species currently considered extinct (Knapp et al., 2021). Plants are fundamental for most thriving terrestrial ecosystems as they are involved in forming soil, reducing pollution, storing and recycling nutrients, and stabilizing the climate (Meerabai and Pullaiah, 2016). Besides their ecological importance, plants also have nutritional, medicinal, cultural, and ornamental importance (Meerabai and Pullaiah, 2016). The extinction of such an important species will affect the interactions within an ecosystem and lead to global ecosystem collapse.

One of the largest family of flowering plants is the Orchidaceae. The family Orchidaceae contains about 28, 484 species and are incredibly diverse in flower shape, size, and colours (Khasim et al., 2020). The various uses of orchids such as pharmaceutically and ornamentally, have led to overexploitation. However, the biggest threats to orchid extinction are the loss of habitat due to deforestation, urbanization, and agriculture (Gale et al., 2018). As the largest family of angiosperms, orchids are particularly prone to extinction if pollinator populations were affected in trophic cascade events (Givnish et al., 2016). Global temperatures are increasing due to human and natural disturbances (Knapp et al., 2021). The increase in temperature causes changes in habitat conditions and increases habitat loss, and these combined factors are creating environments that are no longer suitable for orchids (Seaton et al., 2013). This was evident in some orchid species found on top of Monteverde Cloudforest in Costa Rica, where orchid populations have been reduced due to habitat loss resulting from climate change (Seaton et al., 2013). Although most orchids can be grown from seeds, terrestrial orchids require specific growth conditions such as appropriate symbiotic fungi, pollinators, temperature range, and relative humidity (Seaton et al., 2013). Additionally, researchers are unable to dry immature seeds and have trouble storing the seeds long term (Seaton et al., 2013). More than 51.5% of Orchidaceae species were listed as critically endangered, vulnerable, or near threatened (IUCN, 2020). Species such as *Liparis olivaceae* have been declared extinct in the wild due to overexploitation in the trade and consumption

business (Pant, 2013). *Dactylorhiza hatagireae* is a type of medicinal orchid found in the Himalayas that have also been listed as critically endangered due to its increase in western medicine usage (Pant, 2013). The rapid decline in orchid species due to overexploitation, habitat loss from anthropogenic causes, and highly specific growth requirements encourage wide spread research of orchids due to their cultural, ecological, and biological importance. Therefore, there is an increased incentive immediate need for conservation efforts directed at the family Orchidaceae.

The importance of orchids makes them an attractive target for integrated conservation approaches. There are many ecological relationships formed between orchids and their environment for survival and reproduction (Weston et al., 2005). A type of symbiotic relationship formed by orchids is the association with fungi in the roots (Swarts and Dixon, 2009). The most common type of orchid mycorrhizal fungi is from the genus *Rhizotonia;* the formation of a fungal associate is crucial for seed germination in most orchids (Swarts and Dixon, 2009). It has also been observed that mycorrhizal associates have an effect on surrounding habitats and nutrient distributions and may affect nearby organisms (Weston et al., 2005). Moreover, orchids are flowering plants that are dependent on insect pollination (Swarts and Dixon, 2009). Most orchids are pollinated by just one, or few closely related species of insects (Weston et al., 2005). A study conducted by Kolanowska and Jakubska-Busse (2020) assessed whether the likelihood of a type of orchid, *C. calceolus*, would become extinct due to habitat destruction. In this study, the researchers looked at the availability of *C. calceolus* pollinators, and found that most pollinators would have their habitats destroyed in the near future. Previous insect pollinators of *C. calceolus* would no longer be found in the same environmental niches due to climate change (Kolanoskwa and Jakubska-Busse, 2020). It can be extrapolated that the loss of these orchids to pollinate may cause pollinator populations to suffer. Therefore, the extinction of orchids may create subsequent extinction events for species involved in a symbiotic relationship with orchids, such as fungi and insects (Swarts and Dixon, 2009). The loss of these relationships with orchids may have minor or detrimental effects on the ecosystem through trophic cascades (Eklöf and Ebenman, 2006). Hence, it is crucial to prevent trophic cascades from occurring by conserving orchid populations.

Species within the orchid subfamily, Cypripedioideae, are at risk of extinction, with 90% of the species in this family considered at high risk (Gale et al., 2018). *Cypripedium passerinum* is an orchid native to Alberta and have highly specific habitat requirements which limits its distribution to North America (Gale et al., 2018). Populations are currently declining, leading to its categorization as vulnerable on the IUCN red list (IUCN, 2020). Threats to this type of orchid include residential and commercial development, agriculture and aquaculture, human disturbances through recreational use, and transportation and service corridors that increase the environmental pressure on these orchids (IUCN, 2020). Conservation actions such as site protection, habitat restoration, *ex-situ* conservation, and research regarding population size, distribution, and trends are urgently needed to conserve this species (IUCN, 2020). Hence, *C. passerinum* was chosen as the study organism for this project due to its conservation status.

One of the locations where *C. passerinum* can be found is the study site of this project, Wagner Natural Area (WNA), Edmonton, Alberta. WNA is known for housing highly diverse populations of flora and fauna, including rare and unusual species (Vujnovic et al., 2000). There are 26 species of orchids found naturally in Alberta, of which 16 native species are found in WNA. There are several plant species that are placed on watch lists, and the conservation status of these species are being tracked due to increased vulnerability to environmental pressures (Vujnovic et al., 2000). The location of WNA increases the pressures on species residing in WNA due to urbanization and recreational use as it is flanked by highway 16, a railroad, urban centres such as Edmonton, St. Albert, and Spruce Grove, and industrial areas like Acheson industrial area.

The objective of this project was to isolate microsatellite markers from *C. passerinum* using Fast Isolation by AFLP of Sequences Containing Repeats (FIASCO) and to develop primers using the DNA sequences that flank isolated microsatellites. Such microsatellite primers would have the capacity to assess the genetic diversity of *C. passerinum* populations both within and outside the WNA. The project will contribute to the integrated conservation efforts of the Orchid Species Preservation Foundation (OSPF) to preserve the species found in WNA.

Project Design

Sample collection and DNA extraction

Leaf tissue samples were collected from *C. passerinum* genets in WNA (53.5702° N, 113.8273° W). A genet was defined as a distinct group of ramets separated by at least 1m. A single leaf from multiple ramets within a genet was collected using scissors sterilized with 70% ethanol. The leaf tissues were stored in a sealable plastic bag and transported from the field on ice. Leaf tissue was processed using an ethanol (70%) sterilized hole punch to create uniform leaf discs with a diameter of 7mm before storing at -80°C. Total genomic DNA was extracted from 10 uniform leaf discs using the Zymo Universal Genomic DNA Extraction Kit according to the manufacturer's instructions (Zymo), eluted in a final volume of 32 µL, and stored at -20°C. The concentration and purity of isolated DNA was determined spectrophotometrically using a NanoPhotometer (Implen).

FIASCO – Digestion-Ligation

Microsatellites were isolated by FIASCO as described by Zane et al. (2002) using genomic DNA of a single *C. passerinum* individual. The *Mse*I adaptor was prepared by mixing 24.5 µL of 100 µM of *Mse*I-1: 5'- GACGATGAGTCCTGAG-3' and *Mse*I-2: 5'- TACTCAGGACTCAT-3' in 100 mM NaCl (Zane et al., 2002). This mixture was heated to 95°C for 5 minutes and slowly cooled to room temperature to allow the sequences to anneal together and stored at -20 °C until needed (Zhang et al., 2008). The total genomic DNA was simultaneously digested with restriction enzyme *Mse*I and ligated to the prepared *Mse*I AFLP adaptor (Zane et al., 2002). This was done by mixing 1X CutSmart Buffer (NEB), 1.0 Mm ATP (NEB), 6.25 units of *Mse*I restriction enzyme (NEB), 1 µM *Mse*I adaptor, and 400 units of T4 Ligase (NEB), for a final volume of 25 µL.

FIASCO - PCR Amplification of Digested-Ligated Sample

The adaptor-ligated digested products were diluted (1:10), and directly amplified in a 20 uL PCR reaction using primer *Mse*I-N (5'-GATGAGTCCTGAGTAAN-3') (Zane et al., 2002). The PCR reaction was performed with a final volume of 50 µL which consisted of 0.4 µM of *Mse*I-N primer, 1X PrimeSTAR GXL Buffer (TaKaRa), 200 µM dNTP mix (TaKaRa), 0.8 units of PrimeSTAR

GXL DNA Polymerase (TaKaRa), and 5 µL of the diluted adaptor-ligated products. The PCR conditions were as follows: an initial denaturation at 94°C for 2 minutes, 14-26 cycles of 94°C for 30 seconds, 53°C for 1 minute, 68°C for 1 minute, and a final extension of 68°C for 5 minutes were performed (Zane et al., 2002). Successful amplification was scored by resolving 10 µL of PCR products on a 1.2% (w/v) agarose gel run in 0.5x TBE and demonstrated a smear with no distinct or prominent bands. PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) as per manufacturer's directions, and was eluted in 32 µL of elution buffer. 2 µL of the eluted products were used to determine the concentration and purity by spectrophotometry (Implen).

FIASCO – Hybridization

Fragments containing microsatellites were selected by hybridization using a biotinylated oligonucleotide with the desired AT, AC, and AAG repeat motif with the amplified adaptor-ligated DNA according to Glenn and Schabel (2005). The oligonucleotides used were $(AT)_{11}$, $(AC)_{11}$, and $(AAG)_7$ (Table 2). These probes were prepared in a final volume of 100 µL containing 500 ng of the purified PCR amplified adaptor-ligated DNA and 50 pmol of the biotinylated oligonucleotides in 6X SSE/0.1% SDS. This mixture was then heated to 95°C for 5 minutes to allow the probe to anneal to the DNA and cooled to room temperature for 15 minutes.

In order to recover fragments containing microsatellites, streptavidin coated metal magnetic beads were used. These beads were prepared washing 1 mg of streptavidin coated magnetic beads (4 mg/mL) (NEB) with equal volumes of (10 mM Tris-HCl, 1 mM EDTA, and 0.1 M NaCl; hereafter called TEN_{100}), twice. The beads were then washed twice with 1X Hybridization buffer (Hyb Buffer) which consisted of 6X SSC and 0.1% SDS. The beads were then suspended in 250 µL of 1X Hyb Buffer. The DNA probe mixture and 2.5 volumes of the prepared beads were then mixed together and incubated at room temperature for 30 minutes on an orbital shaker at slow speed. The fragments containing microsatellites within the DNA-probe-bead-complexes were then harvested in a magnetic particle concentrator (MPC) (NEB). The collected DNA-probe-bead-complexes were then washed with low stringency using 400 µL of 2X SSC/0.1% SDS twice and high stringency using 1X SSC/0.1% SDS twice, and then finally 1X

SSC/0.1% SDS at 40°C twice. The DNA-probe-bead-complexes were then resuspended in 100 µL of TE (10 mM Tris-HCl and 1mM EDTA) and heated to 95°C for 5 minutes. This mixture was then loaded onto the MPC and the supernatant was quickly recovered and purified using PCR Clean-up Gel Extraction Kit (Macherey-Nagel) and eluted in a final volume of 52 µL. 2 µL of the purified product was used to determine the concentration spectrophotometrically (Implen).

FIASCO – Selective PCR Amplification

Microsatellite enriched fragments were amplified independently in 20 µL PCR reactions using a cocktail of 3 of the 4 possible *Mse*I-N (5'-GATGAGTCCTGAGTAAN-3') primers according to Zane et al. (2002). The PCR reactions were prepared by adding 2 µL of purified DNA template, 1X PrimeSTAR GXL Buffer (TaKaRa), 200 µM dNTP mix (TaKaRa), 0.8 units of PrimeSTAR GXL DNA Polymerase (TaKaRa), and 0.4 µM of three of the four *Mse*I-N primers (Table 2). The PCR conditions were as follows: an initial denaturation at 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 53°C for 1 minute, 68°C for 1 minute, and a final extension of 68°C for 5 minutes. 10 µL of PCR products were resolved on a 1.2% (w/v) agarose gel run in 0.5x TBE. Those reactions that displayed a smear of fragments without the presence of a selectively amplified PCR product were chosen for library construction. The reactions chosen to create the AT, AC, and AAG libraries were the reactions that lacked *Mse*I-A, *Mse*I-A, and *Mse*I-C, respectively.

Genomic Library Creation

A microsatellite enriched genomic DNA library was generated by cloning the selected PCR products into the pMiniT 2.0 vector (NEB) using the PCR cloning kit (NEB) according to the manufacturers' instructions. Transformants using 1 µL of the selectively amplified DNA were used in the ligation reaction and plated on pre-warmed Luria Broth agar plates containing 100 µg/mL ampicillin and incubated at 37°C for 24 hours. Transformants were picked using a toothpick and was transferred to a master plate with a grid system. The clones were named according to the probe used during the FIASCO procedure and in numerical order (ex. The first transformant picked from the AT library would be AT#1). LB broth with 100 µg/mL ampicillin was prepared in a 50 mL conical centrifuge screw cap tube and was dispended into culture

tubes. All of the clones on the master plates were used to inoculate 1.5 mL of the LB broth and grown at 37°C for 24 hours with shaking at 250 rpm. Glycerol stocks were prepared by adding 500 µL of liquid culture with 500 µL of 50% glycerol and stored at -80°C.

Clones from the library were subjected to colony PCR using cloning analysis primers provided with the NEB PCR cloning kit according to the supplier's instructions. Cell suspensions were prepared by touching a toothpick to the colony on the master plate and left in 50 µL of sterile water for at least 10 minutes. The colony PCR reactions were prepared in a final volume of 50 µL containing: 1X PrimeSTAR GXL Buffer (TaKaRa), 200 µM dNTP mix (TaKaRa), 1 unit of PrimeSTAR GXL DNA Polymerase (TaKaRa), 0.4 µM of forward and reverse cloning primers (NEB; Table 3), and 2 µL of DNA cell suspension. The PCR was carried out with an initial denaturation of 94°C for 2 minutes, 30 cycles of 94°C for 15 seconds, 55°C for 15 seconds, and 68°C for 1 minute, and a final extension at 68°C for 5 minutes. 10 µL of the PCR products were resolved on a 1.2% agarose gel (w/v) with 0.5x TBE buffer. The PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) as per manufacturer's directions, and eluted in 32uL of elution buffer. 2 µL of the eluted sample was used to determine the concentration spectrophotometrically (Implen).

Sequencing & Sequencing Analysis

Purified PCR products was bidirectionally sequenced in a 10 µL reaction containing 225 ng of PCR products and 0.25uM of T7 promoter primer, or SP6 upstream primer whose binding sites flank the multiple cloning sites of the pMiniT 2.0 vector (Table 3). Sanger sequencing was performed by the Molecular Biology Services Unit (MBSU) in the Department of Biological Sciences at the University of Alberta. The T7 promoter sequences was reverse complemented and used to align with the SP6 promoter sequences to generate a consensus sequence in CLC Genomics Work Bench (2021). The consensus sequences for each clone were examined for the presence of microsatellites using the web tool, microsatellite finder (http://insilico.ehu.es/mini_tools/microsatellites/). For each of the microsatellites found, the types of microsatellites were identified. A perfect microsatellite was defined by a single motif uninterrupted within the repeating unit, an imperfect microsatellite was defined by a single

motif interrupted by up to 12 bases within the repeating unit, and compound microsatellites were defined by multiple repeating motifs adjacent to one another. The unique sequences flanking identified microsatellite loci were used to design primers using the web tool Primer3 (https://bioinfo.ut.ee/primer3-0.4.0/).

Primer Development & Testing

These designed primers were then used in PCR with genomic DNA from *C. passerinum* to determine whether any of the identified microsatellite loci are polymorphic. PCR was performed in a 50uL volume containing: 1x PrimeSTAR GXL Buffer, 0.2 mM dNTPs, 0.5 uM of each forward and reverse primers (Table 4), 1.25 U PrimeSTAR GXL DNA Polymerase, and 100 ng of *C. passerinum* genomic DNA. The PCR conditions for the amplification consisted of an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 15 seconds, 53-57°C for 15 seconds, then 68°C for 60 seconds per kilobases of sequence with a final elongation of 5 minutes at 68°C. The amplified PCR products was resolved on a 1.2% (w/v) agarose gel run in 0.5x TBE to determine amplification success.

Results

The microsatellites in this study were isolated by FIASCO. The genomic DNA of a *C. passerinum* individual was extracted, fragmented by *Mse*I digestion, ligated to *Mse*I adaptor, and then amplified by PCR (Table 1). The concentrations of the extracted genomic DNA and PCR amplified digested-ligated samples are shown in Table 1. The concentrations for the samples were 52.0 ng/µL and 116.45 ng/µL, respectively. The DNA samples were of high purity and contained no protein or RNA contamination. The amplified digested-ligated sample was resolved on a gel which produced a smear (Fig. 1).

In order to select for fragments containing microsatellites of the desired AT, AC, and AAG motif, biotinylated oligonucleotides were used (Table 2). Fragments containing microsatellites were selected using streptavidin coated metal magnetic beads and harvested in a magnetic field. These samples were then subjected to a selective PCR amplification using a 3-

primer cocktail (a combination of three of *Mse*I-A, *Mse*I-C, *Mse*I-T, or *Mse*I-G) (Fig. 2). The PCR products that used the primer cocktail lacking *Mse*I-A was selected for library creation as the smear was the dimmest and had no prominent bands.

The amplified products without the *Mse*I-A primer were ligated into a vector and transformed into *Escherichia coli.* The volume chosen for the insert was previously optimized for best single colony isolation (Brooks, n.d.). A total of 207, 216, and 264 clones were isolated for the AT, AAG, and AC-enriched libraries, respectively. Colony PCR was performed for the first 127 clones in the AC library and first 80 clones in both AAG and AC libraries (Fig. 3; Table 3). Of the 80 AC clones, 2 clones showed non-specific amplification (Fig. 3B). One AAG clone showed two prominent bands and were not studied further (data not shown). A clone from the AT library did not amplify (Fig. 3H). The concentration for the purified DNA for all clones were between 42.100 ng/µL and 173.350 ng/µL. These purified DNA were then sent for sequencing (Table 3).

A total of 284 clones were sent for sequencing, and 158 microsatellites were identified. 111 of these microsatellites were dinucleotides, while 46 of them were trinucleotides. There was only one tetranucleotide found. In the AC library, 85% of the dinucleotide microsatellites found were perfect microsatellites while 8.8% and 5.8% of the microsatellites found were imperfect and compound microsatellites, respectively (Fig. 4). All trinucleotides found were perfect microsatellites. In the AT library, most of the dinucleotides and trinucleotides were perfect microsatellites (74.4% and 76.9%, respectively). 18.6% of the dinucleotide repeats and 19.2% of the trinucleotide repeats were compounds microsatellites and the remaining were imperfect microsatellites. The only tetranucleotide found was a perfect microsatellite.

To test the ability of designed primers to amplify *C. passerinum* genomic DNA, previously designed primers were used on *C. passerinum* DNA in a PCR reaction (Fig. 4). The PCR reaction successfully generated PCR products of sizes between 105 bp and 181 bp (Table 4). Other designed primers (GN-AT1 – GN-AT20) also successfully generated PCR products (data not shown).

Discussion

The development of microsatellite markers for the endangered orchid, *C. passerinum*, can help to conserve this species by providing researchers information regarding the genetic diversity of the population at WNA. This information can then be used to plan conservation strategies more efficiently for these at-risk orchids.

In this study, three microsatellite-enriched genomic libraries were generated using $(AT)_{11}$, $(AC)_{11}$, and $(AAG)_7$ biotinylated oligonucleotides by FIASCO. These microsatellites were of interest as these motifs are highly represented in plants (Kalia et al., 2011). During the initial amplification of the digested-ligated products, a smear was observed in the gel (Fig. 1). This result suggests that the genomic DNA of the *C. passerinum* individual was fragmented by *Mse*I into many different sizes. The intensity of the different digested-ligated samples in the gel may indicate that there was improper loading of the samples during agarose gel electrophoresis as an equal amount of sample was loaded into each lane.

Following the microsatellite enrichment process by streptavidin-probe-DNA hybridization, a selective PCR amplification was performed (Fig. 2). The *Mse*I primer cocktail lacking *Mse*I-A, *Mse*I-A, and *Mse*I-C was used to generate the AT, AC, and AAG libraries, respectively. This is because the products generated by that combination of primer cocktail had the least prominent bands in the gel which suggests that no fragments were preferentially amplified. Fragments that were preferentially amplified can lead to bias in cloning and decrease the chances of isolating unique clones and microsatellite loci.

Colony PCR was performed for transformants from the genomic library for a total of 303 clones (Fig. 3). Of those clones, 2 clones from the AC library showed non-specific amplification and varying annealing temperatures (55°C and 57°C) did not fix the non-specific banding, so further optimizing is needed for these clones. One clone each from the AT and AAG library showed two prominent bands which were a result of a mixed colony during cell suspension preparation. One of the clones from the AT library showed no amplification which may suggest that this clone did not contain an insert. These samples were then sent for sequencing. Following sequencing, the microsatellite loci were analyzed. A total of 158 microsatellite loci

was identified and 85% of these were perfect microsatellites. However, it is important to note that the microsatellite loci identified are not unique and there are replicates of each microsatellite loci. In the near future, unique sequences must be identified for each library to determine the accurate number of microsatellite loci and the types of microsatellites identified.

The sequences flanking the identified microsatellite loci were then used to design primers, some of which have been verified to be able to amplify genomic DNA from *C. passerinum*. The designed primers that have not been tested should be tested to verify their ability in amplifying genomic DNA. Verified primers will be used to detect polymorphisms and assess genetic diversity of the *C. passerinum* population at the WNA by capillary electrophoresis using the QIAxcel system (Qiagen) and scoring allele sizes.

Upon completion of this project, microsatellite markers and primers developed in this project can be used to assess genetic diversity of the *C. passerinum* population at the WNA. The WNA was established as a natural reserve to conserve species diversity *in-situ.* As *in-situ* approaches are rarely effective on their own, further work to conserve genetic diversity *ex-situ* is needed (Cecco et al., 2020). As many native Alberta orchids have not been successfully propagated before, the Orchid Species Preservation Foundation (OSPF) started the propagation project to successfully culture, propagate and reintroduce orchids back into the wild (Orchid Species Preservation Foundation, 2021). This project aimed to provide genetic information about the native orchid, *C. passerinum,* in order to select individuals that should be targeted for *ex-situ* conservation efforts. The selection of individuals that represent a fit population would increase the success of propagation and recovery of the vulnerable *C. passerinum* in WNA and elsewhere in its geographical locations. Geographically, *C. passerinum* is distributed throughout North America: Montana and Alaska in the States, and Alberta, British Columbia, Manitoba, Northwest Territories, Ontario, Quebec, Saskatchewan, and Yukon in Canada (Williams, 1990). As the geographical location of *C. passerinum* is limited, culturing these orchids *ex-situ* is crucial.

Assessing population genetic diversity is an important component in *ex-situ* conservation. The microsatellites isolated in this project will elucidate the genetic variation in *C.*

passerinum orchids in WNA, thus allowing researchers to determine other factors such as adaptability to environmental pressures. Microsatellites have been used as a genetic marker to assess genetic diversity in many different researches. Researchers were able to utilize microsatellites to determine the adaptability of different species to natural evolutionary mechanisms such as bottlenecks (Amos, 2016; Jehle and Arntzen, n.d.). Microsatellites were also used to study how diverse a sorghum population was and to perform the best combinations for a parental cross to produce the best fit offspring, increasing their survivability (Mutegi et al., 2011). Similarly in this study, microsatellites isolated in this study can be used to determine the genetic diversity of *C. passerinum* orchids in the wild. The genetic diversity information generated by this project can be used to find the best fit *C. passerinum* individuals to propagate and reintroduce to the wild. This propagation project will hopefully be able to reduce the risk of extinction in orchids.

Furthermore, this project can provide other information for other integrated conservation strategies. This project further solidifies the efficacy of the FIASCO technique in the context of terrestrial orchids so that other studies can use this technique to isolate microsatellites and use microsatellites to examine genetic diversity in organisms. Designed primers in this project that are able to amplify polymorphic microsatellite loci can be used in future large-scale population and conservation studies for the *C. passerinum* species. In similar studies, designed primers were able to isolate novel microsatellites in closely and distantly related species (Almeida et al., 2013; Arnold et al., 2002). Almeida et al. (2013) isolated microsatellites in another type of orchid, *C. labitata.* The researchers used the designed primers amplifying microsatellite loci to isolate novel microsatellites in a closely related species, *C. warneri.* This suggests that the designed primers from this project may be used on a closely related orchid species found in WNA, *C. pubescens.* The ability to cross amplify microsatellites in closely related orchid species may be beneficial to researchers that aim to gather large amounts of genetic diversity information at one time. Other work could include using other common motifs represented in plants such as AG, CCG, GC, CCT, and GA to generate microsatellite-enriched genomic libraries by FIASCO to further elucidate the genetic diversity of the *C. passerinum* population.

In conclusion, the results from this project can be used to provide genetic information of *C. passerinum* in WNA, such that these orchids may be propagated and reintroduced to the wild to reduce the risk of extinction. This research project may also contribute to other studies in related species and other *ex-situ* conservation strategies.

Figure Legends

Figure 1. **PCR Amplification of Digested-Ligated *C. passerinum* Genomic DNA.** The genomic DNA of a *C. passerinum* individual was simultaneously digested and ligated and amplified in a PCR reaction which was then resolved on a 1.2% agarose gel (w/v). **Lane 1:** 100 bp ladder (Truin Science). **Lane 2:** Negative control with no template DNA. **Lanes 3-7:** Replicate samples of PCR amplified digested-ligated DNA products.

Figure 2. **Selective PCR Amplification of Digestive-Ligated *C. passerinum* Genomic DNA.** The digested-ligated *C. passerinum* samples for the AC, AT, and AAG libraries were selectively amplified using a primer cocktail and resolved on a 1.2% agarose gel. **Panel A; Lanes 1-6:** AT library, **Panel A; Lanes 9-14:** AAG library, **Panel B; Lanes 1-6:** AC library. **Panel A; Lane 1:** 100 bp ladder (Truin Science). **Panel A; Lanes 2-5:** *Mse*I-A removed, *Mse*I-T removed, *Mse*I-C removed, and *Mse*I-G removed. **Panel A; Lane 6:** Negative control with no template DNA with all four primers. **Panel A; Lanes 7&8:** Empty. **Panel A; Lane 9:** 100 bp ladder (Truin Science) **Panel A; Lane 10:** Negative control with no template DNA with all four primers. **Panel A; Lanes 11-14:** *Mse*I-A removed, *Mse*I-T removed, *Mse*I-C removed, and *Mse*I-G removed. **Panel B; Lane 1:** 100 bp ladder (Truin Science). **Panel B; Lane 2:** Negative control with no template DNA with all four primers. **Panel B; Lanes 3-6:** *Mse*I-A removed, *Mse*I-T removed, *Mse*I-G removed, and *Mse*I-C removed.

Figure 3. **Colony PCR of Transformants Selected from the Genomic Library Enriched for AT, AC, and AAG Microsatellites.** Transformants from the genomic library enriched for AT, AC, and AAG microsatellites were subjected to colony PCR and resolved on a 1.2% agarose gel (w/v). **Panel A; Lane 1:** 1 kbp ladder (New England Biolabs). **Panel A; Lane 2:** Negative control with no template DNA. **Panel A; Lanes 3-18:** AC#85-AC#100. **Panel B; Lane 1:** 1 kbp ladder (New England Biolabs). **Panel B; Lane 2:** Negative control with no template DNA. **Panel B; Lanes 3-10:** AC#101-AC#108. **Panel B; Lane 11:** Negative control with no template DNA. **Pangel B; Lanes 12-19:** AC#109-AC#116. **Panel C; Lane 1:** 1 kbp ladder (New England Biolabs). **Panel C; Lane 2:** Negative control

with no template DNA. **Panel C; Lanes 3-18:** AC#117-AC#132. **Panel D; Lane 1:** 1 kbp ladder (New England Biolabs). **Panel D; Lane 2:** Negative control with no template DNA. **Panel D; Lanes 3-18:** AC#133-AC#148. **Panel E; Lane 1:** 1 kbp ladder (New England Biolabs). **Panel E; Lane 2:** Negative control with no template DNA. **Panel E; Lanes 3-18:** AC#149-AC#164. **Panel F; Lane 1:** 100 bp ladder (Truin Science). **Panel F; Lane 2:** Negative control with no template DNA. **Panel F; Lanes 3-18:** AAG#1-AAG#16. **Panel G; Lane 1:** 100 bp ladder (Truin Science). **Panel G; Lane 2:** Negative control with no template DNA. **Panel G; Lanes 3-18:** AAG#33-AAG#48. **Panel H, Lane 1:** 1 kbp ladder (New England Biolabs). **Panel H; Lane 2:** Negative control with no template DNA. **Panel H; Lane 3:** Empty. **Panel H; Lanes 4-13:** AT#31-AT#40.

Figure 4. **The Types of Microsatellites and Motif Patterns Found in Isolated Microsatellites.** Microsatellites were identified and categorized based on the sequencing results. **Panel A:** Types and motif patterns of identified microsatellites from the AC library. **Panel B:** Types and motif patterns of identified microsatellites from the AT library. The blue, red, and green bars are perfect, imperfect, and compound microsatellites, respectively.

Figure 5. **PCR Amplification of Microsatellites in *C. passerinum* Genomic DNA.** Designed primers were used to amplify *C. passerinum* individuals and resolved on a 1.2% agarose gel (w/v). **Lane 1:** 1kbp ladder (New England Biolabs). **Lane 2:** Negative control with no template DNA. **Lanes 3-11:** AT1, AT2, AT5, AT6, AT9, AT9-2, AT9-3, AT10, and AT10-2, respectively.

Figures

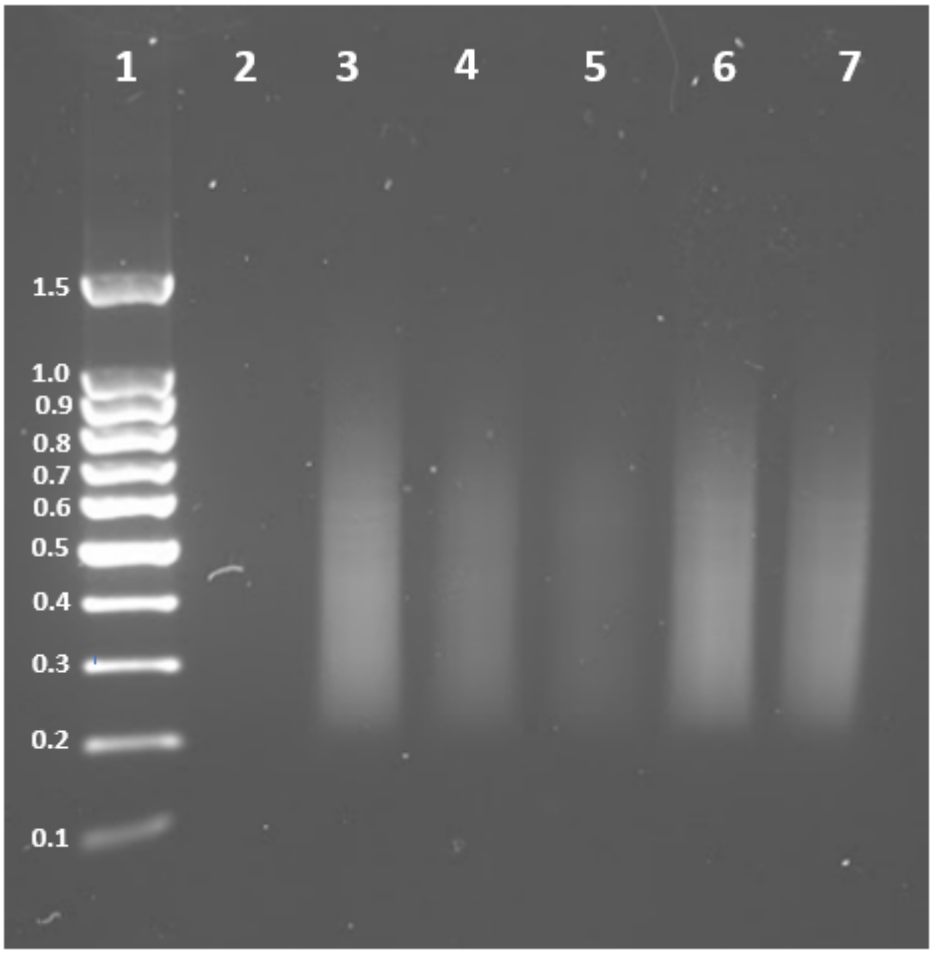


Figure 1. **PCR Amplification of Digested-Ligated *C. passerinum* Genomic DNA.**

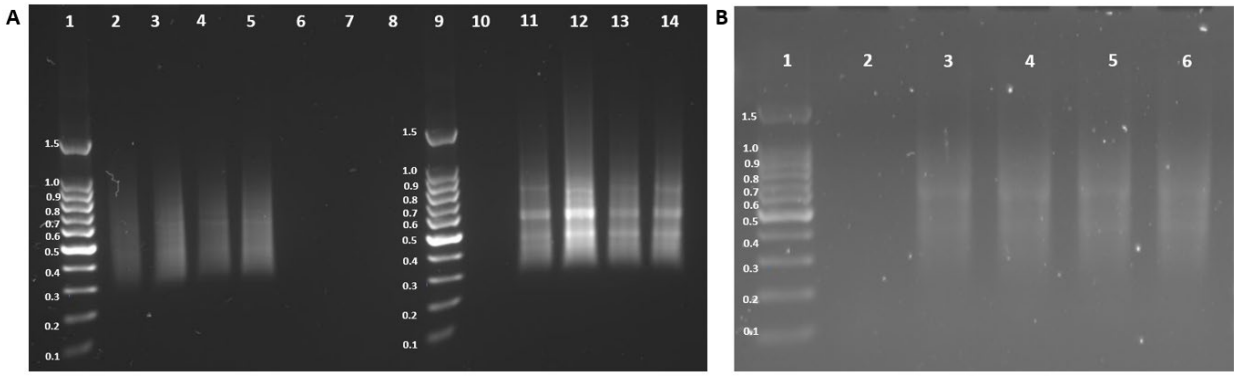
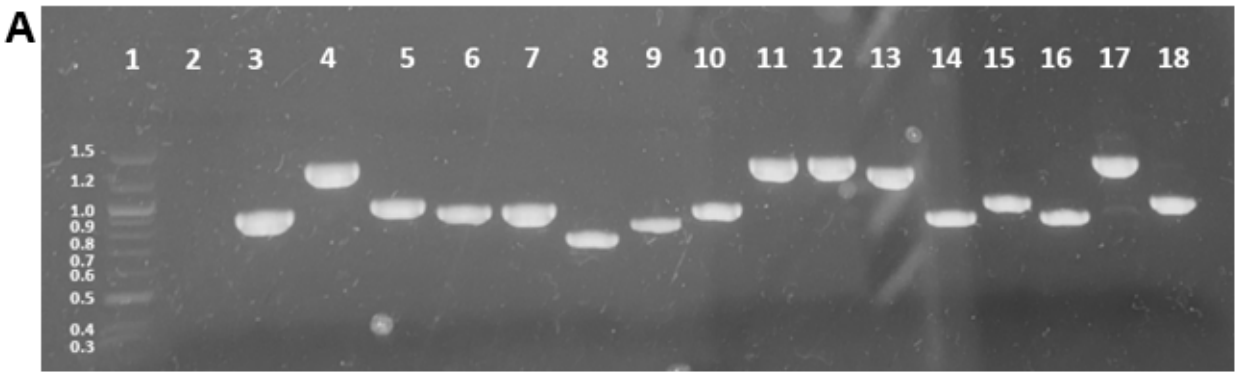


Figure 2. **Selective PCR Amplification of Digestive-Ligated *C. passerinum* Genomic DNA.**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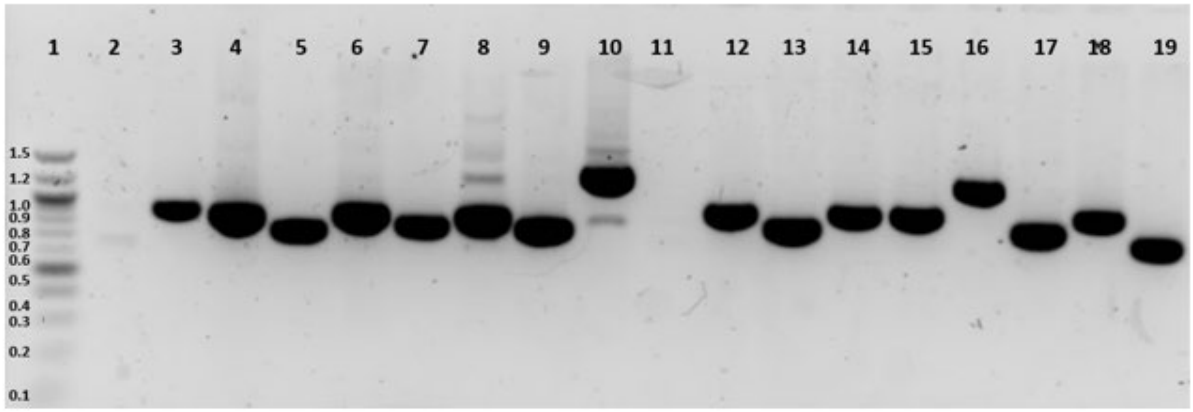
B
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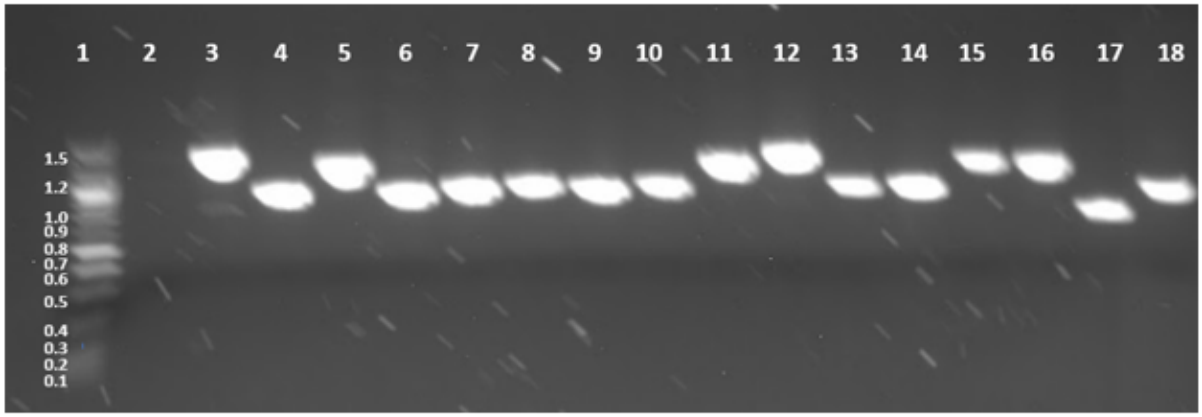
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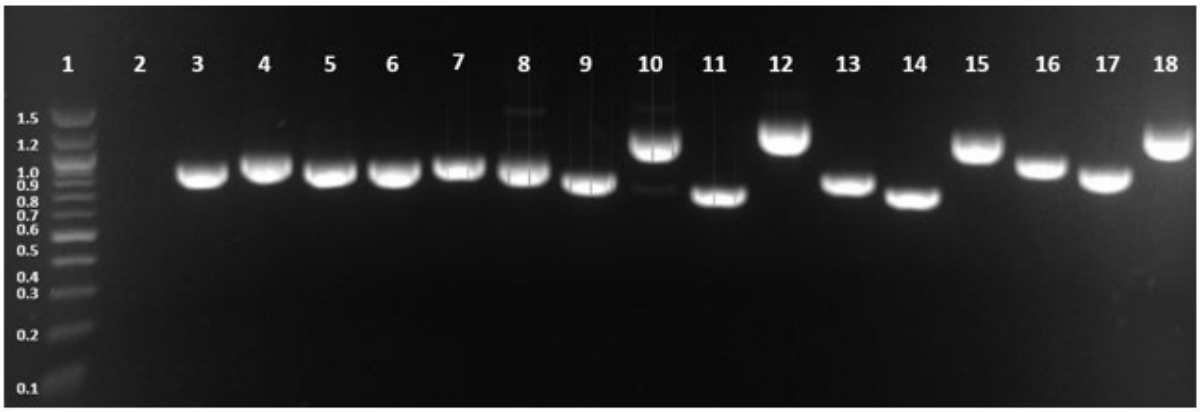
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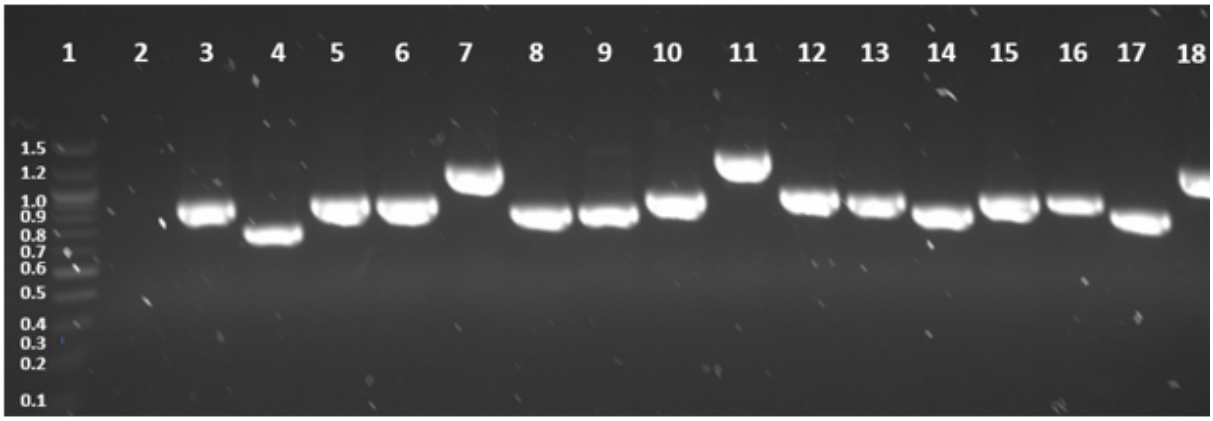
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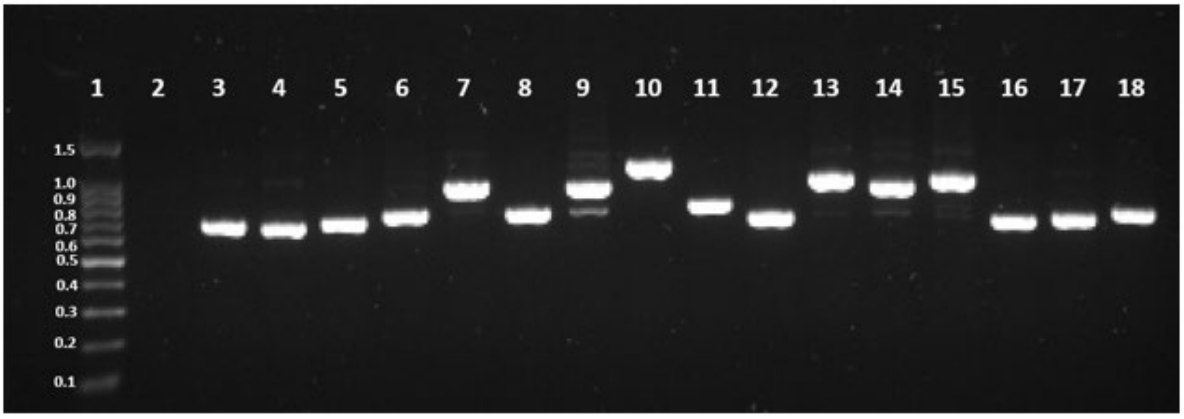
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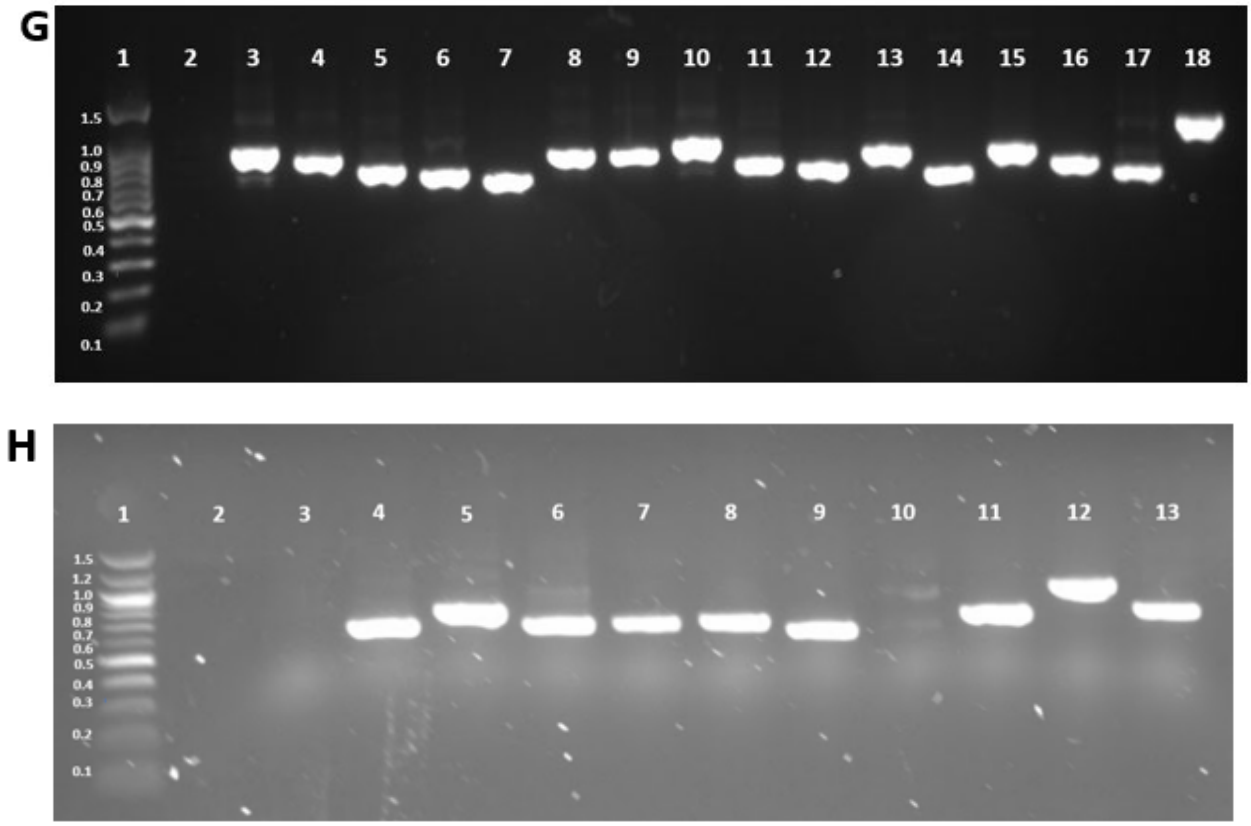

Figure 3. **Colony PCR of Transformants Selected from the Genomic Library Enriched for AT, AC, and AAG Microsatellites.**

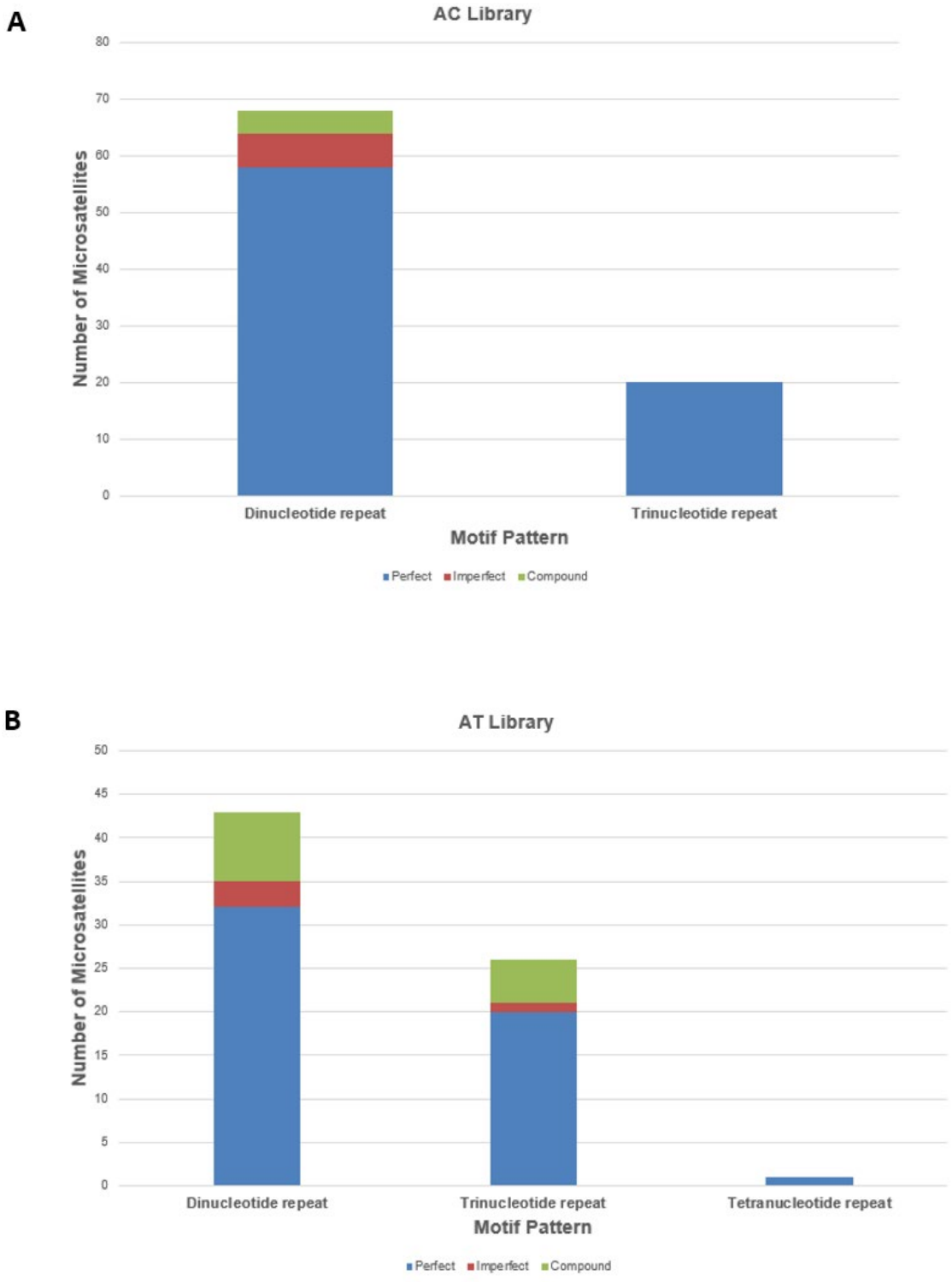

Figure 4. **The Types of Microsatellites and Motif Patterns Found in Isolated Microsatellites.**

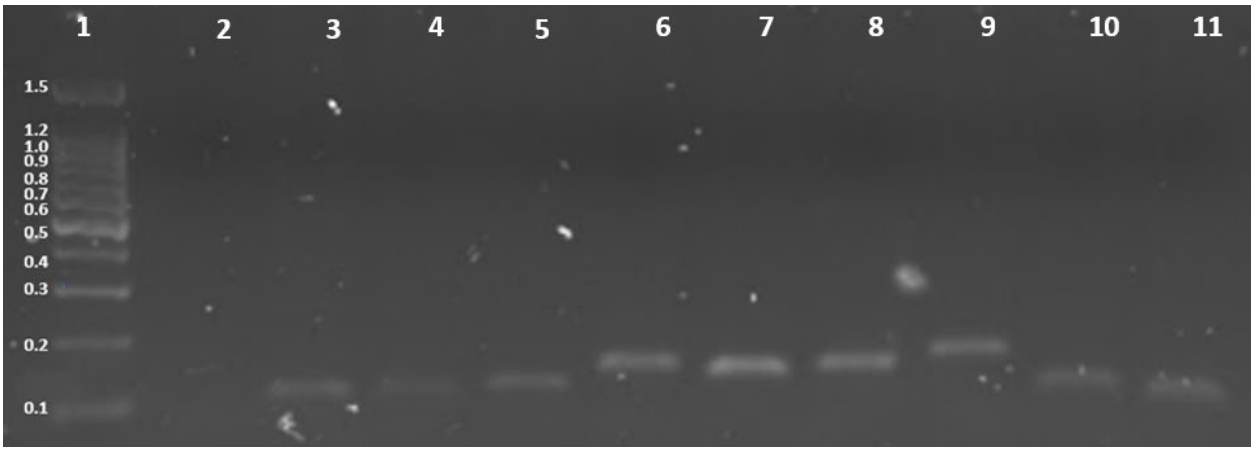

Figure 5. **PCR Amplification of Microsatellites in *C. passerinum* Genomic DNA.**

Tables

Table 1. **Concentration of *C. passerinum* Genomic DNA.** DNA from a *C. passerinum* individual was extracted and simultaneously digested and ligated with *Mse*I adaptor. The concentration and purity for both DNA samples are shown below.

	DNA Extraction Sample	Digested-Ligated Sample
Concentration (ng/µL)	52.0	116.45
A_{260}/A_{280}	1.398	1.914
A_{260}/A_{230}	0.640	0.918
$A_{260(nm)}$	1.276	2.328

Table 2. **Sequences of Adaptors and Primers Used During the FIASCO Procedure to Generate the AT, AC, and AAG Microsatellite-Enriched Genomic Libraries.**

Name	**Sequence (5'-3')**	**Tm (°C)**
AC Probe	BioSg/ACACACACACACACACACAC	58.2
AT Probe	BioSg/ATATATATATATATATATAT	29.0
AAG Probe	BioSg/AAGAAGAAGAAGAAGAAGAAG	47.4
***Mse*I-1 Adaptor**	GACGATGAGTCCTGAG	48.4
***Mse*I-2 Adaptor**	TACTCAGGACTCAT	39.7
***Mse*I-1-FWD**	GATGAGTCCTGAGTAAN	45.0
***Mse*I-A**	GATGAGTCCTGAGTAAA	44.6
***Mse*I-C**	GATGAGTCCTGAGTAAC	45.7
***Mse*I-T**	GATGAGTCCTGAGTAAT	44.3
***Mse*I-G**	GATGAGTCCTGAGTAAG	45.4

Table 3. **Primers Used to Characterize Microsatellites.**

Primer Pair	**Sequence (5'-3')**
Colony PCR -Forward	ACCTGCCAACCAAAGCGAGAAC
Colony PCR - Reverse	TCAGGGTTATTGTCTCATGAGCG
T7 Promoter	GATATCACTCAGCATAAT
SP6 Promoter	ATTAGGTGACACTATAG

Table 4. **Sequences of Designed Primers Using Sequences Flanking Identified Microsatellites.**

	Forward Primer					Reverse Primers			
Primer Pair	PCR Product	Sequence (5'-3')	Position	Length	Tm (°C)	Sequence (5'-3')	Position	Length	Tm (°C)
AT1	108	AAGAGTGGGAGGCTGGATTG	232	20	59.38	TGACTAGCAGCACTTCCCTA	339	20	57.77
AT2	112	GCATGTGGTAGTGGGCGA	109	18	59.73	TACCCGGATCCACTCAGACT	220	20	59.37
AT5	124	GCCAAGTGAAGGTGATGCAT	131	20	58.82	GGCACAAACAACCCCAGAC	254	19	59.26
AT6	158	GGATGGCCAATGGATAGCTTC	300	21	58.83	TGTTTAGTTGTCTCATCCTGAAGG	457	24	58.45
AT9	146	AACACCTCAAGCTCCCAGAG	42	30	59.31	CCAAGGGAAGAAGAAGATTGTGT	187	23	58.60
AT9-2	154	CGAGCGATGAAGAGGAACAAA	100	21	58.66	TCTCTCACACTTCTCCTCGT	253	20	57.43
AT9-3	181	GGAGAGTTACACGAGGAGAAGT	224	22	58.92	TACGGAGGAAAGGGAACGAC	404	20	59.1
AT10	117	AGTTGGAGGGTAGTATTCAGTCA	145	23	58.32	GCTGATTAGGCATTGTTCTTGA	261	22	57.03
AT10-2	105	AATCAATACACCCAGAGACCC	486	21	57.07	CGAGGCATTTGGAAGTAAGAGG	590	22	59.06
GN-AT1		CATCACGAGAGGATGAAGCA			59.94	GGATCCGAATTCAGGAGGTA			58.93
GN-AT2		TGGTGACATCCAGCCATCT			60.06	AACTCCAACCCTCCAATTCC			60.17
GN-AT3		TTACCCGGATCCACTCAGAC			59.93	TGCAAGTAGCACGTGTAGGC			60.08
GN-AT4		GACAGGAGATCCGTGGTGAT			59.93	CGAATCGACCGATTGTTAGG			60.46
GN-AT5		CCACACCATTGGGTTTAGAA			58.32	ATGCAGTCGGTAGGTTTTGG			59.99
GN-AT6		GTGTGTATCGCTCGAGGG			57.29	CCATGAGGCCGAATGTTGAT			58.32
GN-AT7		CAGCAAACCGTTGACAGATG			60.30	CGAATCGACCGATTGTTAGG			60.46
GN-AT9		CCCAGGACTTTCAGAGTTGG			59.69	CGAATCGACCGATTGTTAGG			59.97
GN-AT10		TCTACCATTGCAAGCCACTG			59.86	CCAGTTAGGGGCTCTGATACC			60.46
GN-AT11		TGTTGAGACGCTTACTCAGGAC			58.54	CGAATCGACCGATTGTTAGG			60.46
GN-AT12		GATGGATTCCGCAAGTTGTC			60.47	TGTTCCTCCAAGTTCTCGAT			57.29
GN-AT13		CTGTTCCTGTGCTGCAGTGT			60.10	GGCCAAAATTTCAGGCCTAC			60.80
GN-AT14		AGCCGTAGGTGCTCATGAAT			59.72	TGTGCTCATCAGAGATGATAGG			57.92
GN-AT15		CCCAGGACTTTCAGAGTTGG			59.69	CGAATCGACCGATTGTTAGG			60.45
GN-AT16		GGTTTAGCTTGGTGGTCGAA			60.11	TCTTACTGCCCCACACTTCC			60.11
GN-AT17		TGTTGAGACGCTTACTCAGGAC			56.54	CGAATCGACCGATTGTTAGG			60.46
GN-AT18		TTCAATGAATGATGCTGAAACA			59.16	AAACTGCTTGTGGGATGCTT			59.74
GN-AT19		TGGTGACATCCAGCCATCT			60.06	AACTCCAACCCTCCAATTCC			60.17
GN-AT20		TTGGAGGCTGATTAGGCATT			59.67	GGCAGATGAGTTGGAGGGTA			60.07

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