

Examining the correlation between spatial distance and phenotypic  
similarity in ramets of the clonal plant  
*Lysimachia terrestris*

BY

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## Abstract

The Sackville Retention Pond was established in 2019, and currently hosts a wide range of native New Brunswick plants, including *Lysimachia terrestris*. *Lysimachia terrestris* is a highly clonal species which can reproduce through both bulbils and rhizomes. These two modes of cloning can have divergent effects on the distribution of clones within a population, ultimately affecting mating opportunities and leading to significant consequences for sexual reproduction. The population of *L. terrestris* at the Retention Pond offers a unique opportunity to explore how clonal dynamics (number and distribution of clones) and the spatial genetic structure of a newly founded clonal plant population develops over time. This study aims to (1) optimize DNA extraction techniques for *L. terrestris* and extract DNA for future genotyping and (2) to explore spatial and phenotypic correlations among potential clones. Leaf tissue and measurements of phenotypic traits were collected from 92 plants total, distributed across eight transects at the Retention Pond and a few geographically distant sites. For goal (1) DNA extractions were completed for 99 sampled plants and resulted in pure DNA with high concentrations that are suitable for subsequent genotyping. For goal (2) a principal component analysis was run on four phenotypic traits for 74 reproductive plants sampled from transects, and the two components were then used in ANOVA's and spatial autocorrelation tests such as Mantel's Test and Moran's I. The first principal component captured 43% of the total variation across the four traits, and represented a life history trade-off between reproductive effort and vegetative growth ("branchiness"). This PC axis exhibited significant spatial structure, both in the form of variation between transects, and across all spatial distances through the Moran's I test. The second principal component (capturing 34% of the total variation across the four traits) represented overall plant size, and was a weaker indicator of phenotypic spatial structure. In total, there appeared to be spatial structuring of some phenotypic traits into clumps within this population. Further research, including analysis of genotyped sequences from the DNA extracted in goal 1, is needed to determine whether the spatial phenotypic clustering observed in goal 2 is primarily attributable to spatial structuring of the environment, spatial structuring of clones (genotypes), or both.

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# 1 Introduction

## 1.1 Modes of clonal growth

Reproduction is a key process in an organism's life history and can be divided into two distinct modes, either sexual or asexual reproduction. Sexual reproduction is defined as the fusion of gametes from two individuals to form the zygote whereas asexual reproduction occurs when an individual can singularly create offspring that are genetically identical, except where mutations occur (Brawley & Johnson, 1992; de Meeûs et al., 2007). There are multiple different modes of asexual reproduction including budding, fragmentation, fission, sporogenesis, parthenogenesis and apomixis (Schmidt et al., 2015; Wright & Davis, 2006). The method of reproduction, either asexual or sexual, can vary greatly across species, and sometimes even within species. Some organisms, like humans and most other vertebrates, are capable of only sexual reproduction, and some, like archaea and bacteria, are known to reproduce asexually only. A large proportion of life on Earth can utilize both modes of reproduction, sometimes simultaneously (Vallejo-Marín et al., 2010). Using both modes of reproduction is especially common in plants, with flowering plants exhibiting a larger variety of reproductive structures than any other group of organisms (Barrett, 2015). The ability of angiosperms to use both sexual and asexual reproduction is extremely important to their life history and their ability to thrive in a wide range of environments worldwide.

The survival and evolution of plant species can be attributed in large part to their ability to use different modes of reproduction which can have differing benefits across environments. The ability of plants to alter their reproductive strategy between sexual or asexual modes is especially important in heterogeneous locations where sexual reproduction may be difficult to maintain. One of the most common modes of asexual reproduction is clonal growth which is used by an estimated 80% of angiosperms (Vallejo-Marín et al., 2010). Clonal growth occurs when a parent plant produces a nearly genetically identical offspring through the vegetative growth of stems, roots, or leaves (Franklin et al., 2021). Clonal growth strategies allow plants to forage for resources such as light, water and nutrients throughout inconsistent environments, lower the possibility of disease to take out the entire genotype, and allow for the division of

labour across multiple connected clones (Barrett, 2015; Vallejo-Marín et al., 2010). While there is a risk that disease can spread quickly through clonal connections, the connections between individuals within a clone also allows for the distribution of resources and acts as a mode to deliver important information on the suitability of the habitat (Bittebiere et al., 2020). Another important factor of clonality is the ability to pass genes onto offspring through different methods which can be beneficial by increasing fertility. With the many possible benefits that a plant gains from utilizing clonal growth, it is unsurprising that clonality is so common among different species. To fully understand how clonality can contribute to a population's overall fitness, the type of clonal growth and the extent it is used should be considered.

Various structures can enable clonal growth, and some species can even utilize more than one of these structures. The most common clonal structures include rhizomes (individuals produced along horizontal stems growing underground), stolons (individuals produced along horizontal stems growing above ground), bulbils (vagile propagules that replace the reproductive organs of the plant), bulbs and tubers (below ground clonal organs developed from modified leaves and stems, respectively) (Herben & Klimešová, 2020; Sosnová et al., 2010). Less familiar cloning strategies include the development of new individuals from the leaf tip (e.g., walking fern [*Asplenium rhizophyllum*] or mother spleenwort [*Asplenium bulbiferum*]) or through corms which allow for overwintering and buoyancy to float down waterways (e.g., broadleaf arrowhead [*Sagittaria latifolia*]) (Holt et al., 2020; Wright & Davis, 2006). Regardless of mode of cloning, each clonal individual derived from the original zygote is referred to as a ramet. All of the individual ramets that originate from a single genotype form the genet, i.e., the extended body that has developed from the unique genotype produced by sexual reproduction (Vallejo-Marín et al., 2010). While many possibilities exist for clonal structures, they will vary in their interactions with the environment and thus the benefits they provide for the genet in general.

## 1.2 Mode of cloning affects spatial genetic structure

Depending on the mode of cloning, ramets can remain physically connected to one another (e.g., rhizomes) or detach and become physically independent (e.g., bulbils). Ramets are able to grow, reproduce, and possibly disperse independently from the parent plant which

impacts the overall spatial genetic structure of the species population (Vallejo-Marín et al., 2010). For clonal species the spatial genetic structure of a population is determined by the distance that ramets are from each other and what other genets or species surround them (Loh et al., 2020). The mode of clonal growth used by a genet can impact the spatial genetic structure of a population and affect the probability of a ramet developing in a new population (Yakimowski & Barrett, 2014). Species that implement modes of clonal growth that remain physically connected to the parent plant often use the phalanx strategy. This strategy involves the close aggregation of ramets around the parental plant leading to clumping of clones and typically limited dispersal. On the other hand, clonal expansion that results in ramets developing from longer runners or becoming completely physically independent from the parent plant use the guerilla strategy, characterized by the intermingling of ramets from different clones (Barrett, 2015). For example, some physically separated propagules, often aquatic species, can be carried hundreds of metres before landing in suitable soil and growing into a full plant (Holt et al., 2020). Therefore, waterways, among other environmental features, can enhance the chance of a single ramet interacting with different genets, and even distant populations of plants. More often, these two strategies exist on a spectrum which can create clones in close proximity or spread over large geographic ranges (Barrett, 2015; Vallejo-Marín et al., 2010). Some species even will employ both phalanx and guerilla strategies simultaneously which may create complex spatial structures within a single genet. The utilization of both phalanx and guerilla strategies in many species creates interesting spatial genetic structures and can alter many characteristics of a population.

The spatial genetic structure of a population can impact key processes of plant populations such as gene flow, inbreeding, and may even be used to predict evolutionary developments in the population (Holt et al., 2020). The magnitude of the spatial genetic structure is determined by the likelihood that the plants surrounding a focal plant are related to it, wherein plants surrounded by close relatives represent a strong spatial genetic structure (Holt et al., 2020). Clonal plants often have a strong spatial genetic structure since common modes of clonal growth restrict dispersal capabilities of ramets and create genets that form in clumps. A strong spatial genetic structure may increase the possibility of self-fertilization occurring between flowers within the genet which consequently influences cross-pollen transfer (Yakimowski & Barrett, 2014). If pollinators are present, large clones may even experience more self-fertilization

than smaller clones because there will be a stronger spatial genetic structure (Albert et al., 2003). If clonal growth in a population results in an absence of mating opportunities from outcrossing, increased rates of self-fertilization may have detrimental effects on the population through inbreeding depression (Vallejo-Marín et al., 2010).

To combat the negative effects associated with inbreeding depression many clonal plants are self-incompatible, meaning pollen from a ramet is unable to fertilize any flowers within the genet (Barrett, 2015). In self-incompatible plants all the pollen transferred between ramets in the same genet is essentially wasted which can lead to pollen discounting. Pollen discounting occurs when pollen is transferred to a flower that cannot be successfully fertilized by that pollen which causes a reduction of pollen available for outcrossing. When pollen outcrossing is limited, the production of seeds and fruit in a population is reduced which is overall costly to male fitness (Vallejo-Marín et al., 2010). Ultimately, while self-incompatibility prevents inbred offspring, it may also reduce seed set causing lowered reproductive success in the population. Reduced fertility and even sterility resulting from limited pollen delivery has been noted in a number of clonal species with sexual polymorphisms including self-incompatibility (Barrett, 2015). Pollen discounting can potentially lead to the loss of sexuality completely and create a population which solely relies on clonal growth (Vallejo-Marín et al., 2010).

### 1.3 Molecular tools to study spatial genetic structure

Given the wide-ranging implications of spatial genetic structure for a population's ecology and evolution, understanding this structure and how it may impact a species is crucial. However, accurately identifying genets in the field to determine their size and spread can be very challenging. Recently the application of polymorphic molecular markers for studying clonal plants has allowed for the exploration into clonal phenotypes (Barrett, 2015). Polymorphic molecular markers are formed by analyzing the genome of an organism for polymorphisms specific to the species or individual. Common polymorphisms that are used in sequencing include single nucleotide polymorphisms (SNP's), restriction fragment length polymorphisms (RFLP's), or microsatellites (Kordrostami & Rahimi, 2015). Traditionally, studies looking to genotype clonal plant species have used microsatellites as the primary marker, but when little

genetic data is known for a species it may be more beneficial to look at SNP's from reduced representation fragments of the genome (Amor et al., 2020).

Molecular markers can be developed in multiple ways, though methods are typically divided into either whole genome sequencing or reduced representation sequencing. A reduced representation sequencing approach is more efficient, requires no previous information on the study organisms, enables genotyping of a larger sample within a certain budget and focuses on specific loci with enhanced coverage compared to whole genome sequencing (Peterson et al., 2012). Restriction site-Associated DNA Sequencing (RADseq) is a new way of genotyping that revolutionized ecological, evolutionary and conservation biology studies by providing a simple and cost effective way to study the genetics of non-model organisms (Andrews et al., 2016). RAD sequencing is done by adding a restriction enzyme into the genome that cuts the DNA at specific sequences creating fragments with known DNA sequences at either end. Adaptors, typically with barcodes that allow for the identification of individual samples when many are sequenced together, are ligated to both ends of each fragment. The adaptors will react with primers during the polymerase chain reaction to distinguish the unique parts of the genome important during marker development (Andrews et al., 2016). Currently, there are multiple different types of RADseq methods that each have different flexibility, bias and error margins, costs, or ability to answer certain questions (Andrews et al., 2016).

Double digest restriction enzyme associated DNA sequencing (ddRAD) is an adaption of RAD that uses two restriction enzymes to cut the DNA into fragments. Then for further processing, only sequences that were cut by both enzymes are included in the analysis (Andrews et al., 2016). This change alone substantially reduces the costs of sequencing and removes many steps which result in significant loss of DNA. Next, a specific size selection process is used to accurately adjust the DNA fragments to fit within the size range required for the type of research being conducted (Peterson et al., 2012). After the genome has been cut into specific fragments, these fragments can be studied to find any polymorphisms (e.g., SNPs), that will be able to distinguish an organism's genotype from others within the species thus allowing for the identification of clones (Yang et al., 2016). Plants that have nearly identical genomic polymorphisms can be considered clones, while the presence of additional polymorphisms

between ramets can indicate the level of relatedness among different genets within a population. (de Meeûs et al., 2007).

#### 1.4 Study system and goals

The spatial genetic structure of a population can change over time as the plant species and the surrounding habitat develop. Opportunities to study a single population from the beginning of its foundation throughout its establishment into an area can provide important information on plant characteristics like number and evenness of clones, spatial genetic structure, or the frequency of sexual reproduction (Holt et al., 2020; Ricono et al., 2020). However, these opportunities are scarce and so many aspects of clonal plant ecology are poorly understood. Often when these questions are tackled it is on invasive species, so gaps in knowledge persist for native clonal populations (Hierro et al., 2005; Yu et al., 2009). The Sackville Retention Pond, which was only recently developed in 2019 as a storm water retention pond, creates an ideal scenario to study the development of a native clonal plant population over time. At the Retention Pond the clonal plant *Lysimachia terrestris* is a newly established species that has already occupied a large area of the park. *Lysimachia terrestris* is a perennial native to New Brunswick and many other Canadian provinces (*Lysimachia Terrestris* - *Database of Vascular Plants of Canada (VASCAN)*, n.d.), though is invasive in some regions of Western Canada. *Lysimachia terrestris* is a hermaphroditic plant capable of reproducing through both sexual and asexual means. Asexual reproduction in this species occurs through clonal growth using two different forms – rhizomes (which produce closely spaced ramets) and bulbils (which allow for wide dispersal of ramets). *Lysimachia terrestris* is also an interesting study system because it produces an oil reward for pollination by specialist oil-collecting bees. The interaction of two modes of clonal growth, and sexual reproduction facilitated by a specialist pollinator, has the potential to make for interesting dynamics in the spatial genetic structure of *L. terrestris* population.

The goals of this research project were (1) to optimize tissue grinding and DNA extraction techniques for *Lysimachia terrestris* and extract DNA for sequencing; and (2) to explore the spatial distribution and phenotypic differences between clones in a localized population. The original plan of this project also involved a third goal, (3) to genotype a

population of *Lysimachia terrestris* from the Sackville Retention Pond to determine the number of genets present and their approximate size. This third goal would allow us to study the associations between genotype, phenotype, and distance but we were unable to fulfill this goal within the time constraints of the honour's thesis. DNA has, however, been extracted from all samples, and they will be sent for sequencing soon. Due to the genotypic data not being finished on time to be analysed, goal 2 was redesigned to only explore the relationship between spatial distance and phenotype. In the discussion, I consider how genetic data can offer insight on the observed associations between clonality and population structuring.

## 2 Methods

### 2.1 Study system

*Lysimachia terrestris* (Primulaceae) is a perennial plant native to New Brunswick, Nova Scotia and Ontario but is currently distributed throughout all provinces except Alberta and Saskatchewan (Antonio et al., 2021). *Lysimachia terrestris* is an erect plant reaching heights of 25 to 100 cm and inhabits wetland areas such as bogs, wet ditches, and lake and pond margins. The inflorescence is terminal, extends 10 to 30 cm in length, and forms a raceme which flowers from late June to September (Brown, 2017). Based on environmental factors, *Lysimachia terrestris* can display both sexual and asexual modes of reproduction. Clonal growth can be observed in *Lysimachia terrestris*' bulbils which allow for distant dispersal from the parent plant, or rhizomic growth which forms ramets which are physically linked together (Barrett, 2015).

### 2.2 Study site

Leaf tissue was largely collected from the Stormwater Mitigation Pond on Lorne St. in Sackville, New Brunswick (N 45°53'40.48", W 64°21'49.25"). The site was formed in 2019 to be used as flood control for the town of Sackville and is approximately 52,000 m<sup>2</sup> in size. The park

encompasses many different native plant species that thrive in marshy, wetland habitats, including *Lysimachia terrestris*. A dense population of *L. terrestris* has been observed in previous years at the northeastern side of the Retention Pond, covering a 5000 m<sup>2</sup> area. *L. terrestris* has also been found in lower densities in other areas of the Retention Pond.

We also collected leaf tissue from 5 haphazardly chosen plants along the south shore of Silver Lake, Sackville NB (N 45.928366, W -64.366401) for inclusion in genomic analyses. The Silver Lake site is roughly 3.8 km direct line distance from the Retention Pond site but there is no surface waterway connecting the two sites. We assumed plants at Silver Lake would be distantly related to plants at the Retention Pond site, and therefore included leaf tissue from Silver Lake in molecular analyses to provide a baseline for the expected genetic distance between distantly related genets.

## 2.2 Field sampling methods

Our experimental goals required spatially explicit sampling of plants at the Retention Pond to allow comparison of pairwise spatial, phenotypic, and genetic distances between individuals. Below, I describe the belt transect method we used to choose plants for inclusion in the study, and the methods we used for phenotypic data collection and leaf tissue collection for genetic analysis.

### 2.2.1 Belt transect sampling

Plant sampling at the Retention Pond took place from July 4<sup>th</sup> to 6<sup>th</sup>, 2022. Prior to the beginning of the sampling period, we investigated the population densities at the Retention Pond to determine approximate sampling locations. In total, we established eight belt transects (Parker et al., 2011) in the northeast area of the park, where the *L. terrestris* population has been noticeably dense since it established in 2019 (EJA personal observation). Transect length ranged from 4–14 m depending on the amount of *L. terrestris* present in the area. We marked the

beginning and end of each transect with labeled flags and recorded GPS coordinates of the flag's location to 10 cm accuracy using SXblue A90 GNSS receiver and an SXblue H6 Android data collector running Field Genius Android software. To create the 'belt', we placed 1 m<sup>2</sup> quadrats side by side along the length of the tape (Figure 1). We identified thirteen possible sampling positions within each quadrat prior to sampling (Yakimowski & Barrett, 2014). The center of the quadrat was assigned as P00, then the right corner of the quadrat touching the transect line was always designated as P01, with P02 – P08 following around the perimeter of the quadrat at 0.5m intervals counter-clockwise. The four remaining sampling points are located on a diagonal between P00 and each of the corner positions on the quadrat (Figure 1).

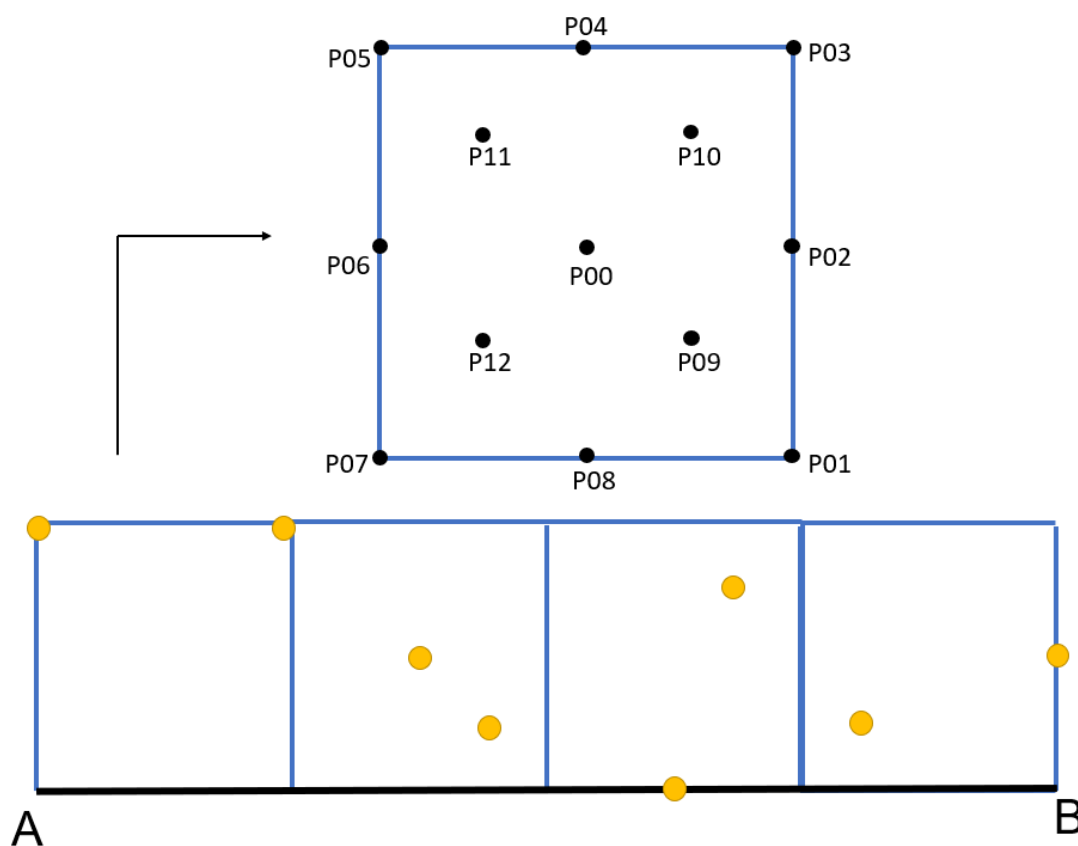


Figure 1. The thirteen predetermined positions that plants were randomly sampled from within each quadrat (top). Full belt transect including the measuring tape (black line), the quadrats (blue boxes), location of flags (A & B for either side), and an example of how sampling occurred (yellow circles represent plants sampled).

Up to two of the thirteen positions within each quadrat were randomly selected for plant sampling (Figure 1). Random sampling was accomplished using a randomized list of positions (P00 – P13) generated in R (R Core Team, 2022). The list told us which order of positions to sample, and then the *L. terrestris* plant closest to the center of the position point (with a 10 cm radius) was the plant that was used for sampling. If *L. terrestris* was only present in a few positions within a quadrat, non-random sampling was performed. Where possible, the position containing a *Lysimachia* ramet that was closest to the next randomized position was used. For example, if the random position was P03 then whichever position close to P03 that contained *L. terrestris* was used instead, like P04 or P10. Otherwise, if *Lysimachia* was only found in one or two positions, those positions were specifically chosen to be sampled without any use of the randomized list. If *Lysimachia* was only present in one position, then only one plant was sampled from the quadrat. Quadrats that completely lacked any *L. terrestris* were not sampled and sampling efforts continued with the next suitable quadrat along the transect.

In total, 88 plants were sampled from eight transects ( $11 \pm 2.45$  per transect). We sampled leaf tissue from an additional two plants at each of two points at the Retention Pond that were 68 m and 178 m, respectively, from the main study population, with few *L. terrestris* plants in between (Figure 2). Each sampled plant was marked on the primary axis with a uniquely labelled piece of coloured tape containing the transect number, quadrant number, and position ID. Phenotypic data and leaf tissue were collected from each sampled plant, as described below.

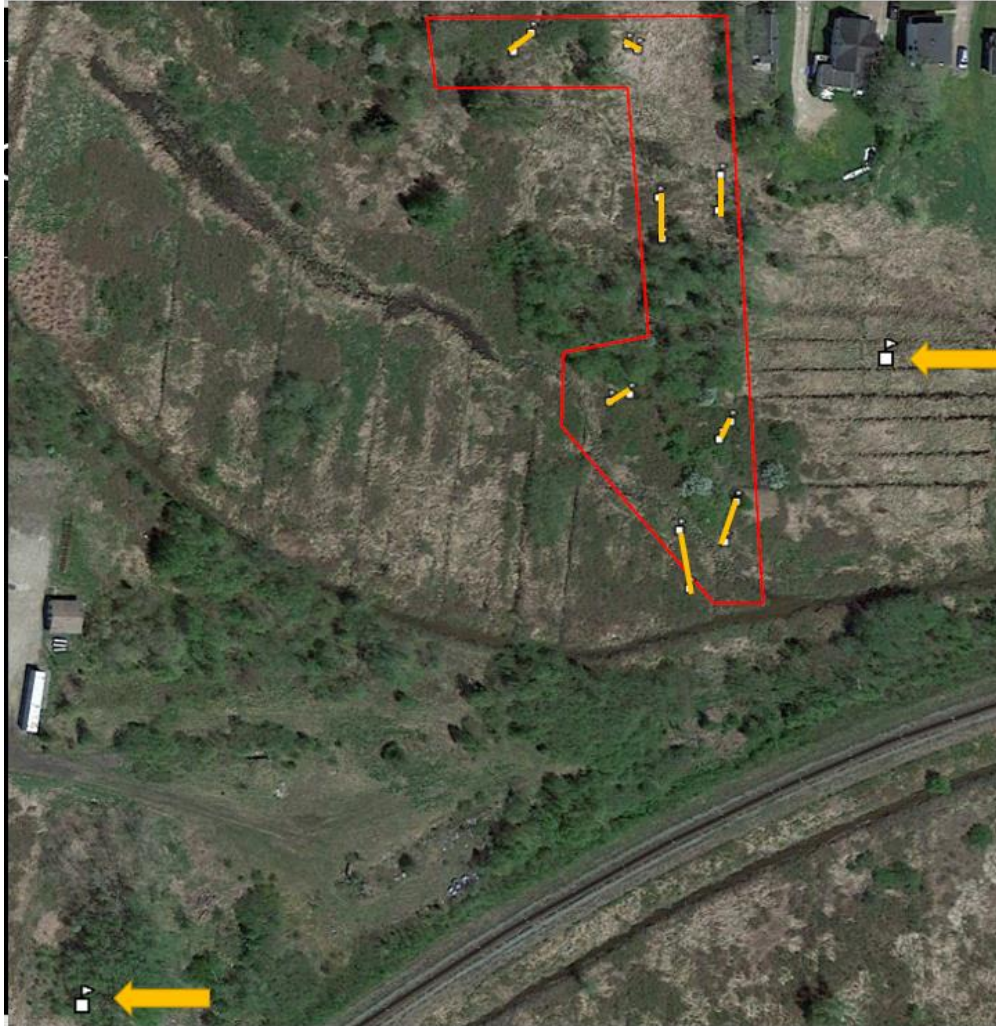


Figure 2. Main population of *Lysimachia terrestris* occurred within the red outline where eight belt transects were placed (represented by yellow lines). Additional plants were sampled from further distances shown by the white boxes that the yellow arrows are pointing to. Map of the Retention Pond was taken from Google Earth Pro.

### 2.2.2 Phenotypic data collection

We recorded several plant-level phenotypic traits during the initial sampling: (1) plant height on the primary axis while holding the primary axis tall; (2) reproductive status (1 if buds, flowers, or fruit present, 0 if non-reproductive); (3) flowering start (1 if flowering started before sampling, 0 if not yet flowering but buds were present, NA if non-reproductive); and (4) flowering progress on primary inflorescence (proportion of primary inflorescence over which

flowers have already opened (determined by measuring height to the lowest flowering position and to upper most open flower), or NA if not yet flowering or non-reproductive). Other plant-level traits were recorded by collecting plants at the end of the growing season and processing them in the lab: (6) proportion of flowers setting fruit; (7) plant above-ground dry mass (excluding bulbils); (8) number of secondary inflorescences; and (9) total mass of bulbils. Finally, we took a photograph of at least one sampled plant from each transect to provide a record of its general phenotype. For the purpose of this research, I refined the traits so only four main plant-level traits were included in the statistical analyses for this study. These traits were: height to the bottom flower on the inflorescence, total dried biomass, branchiness (calculated as: mass of branches/total biomass), and reproductive effort (calculated as: mass of flowers/total biomass).

To allow future analysis of floral traits and within-plant floral variation, we also repeatedly collected flowers from reproductive individuals. We visited the site roughly once a week until all flowers had opened on most plants. At each visit, 1–4 flowers per flowering individual were collected and stored separately in microtubes containing 70% EtOH. If multiple flowers were gathered, the flowers being removed were evenly distributed over the whole flowering portion of the inflorescence. We recorded the date each flower was collected, and whether it was collected from the primary or secondary inflorescence. To approximate flower position within the plant, we recorded the height of flowers collected from the primary inflorescence (measured from ground while holding the plant straight). Processing these flowers is beyond the scope of this honour's thesis, but they will eventually be dissected to measure floral traits including flower size and trichome density.

### 2.2.3 Leaf tissue collection

Leaf tissue was collected from each sampled plant and placed in a paper envelope labeled with a unique code and initials. The paper envelope was then placed in a plastic bag filled with flower drying silica gel. Leaves were collected from near the growing tips where the leaves are the newest, and multiple leaves were collected from each plant to ensure enough fresh tissue was gathered for subsequent genomic DNA extractions. Additional plant tissue was collected from a

random plant within each transect and was both dried with silica drying gel and freeze dried with liquid nitrogen (see Appendix for more details). The purpose of extracting DNA from these samples was to compare the two tissue drying methods, so this DNA was not used for further analysis.

We returned to Sackville Stormwater Retention Pond in mid-September to collect each plant that was sampled in this experiment. The plant was cut at the base of the stem using garden shears and folded up to fit inside a medium sized paper bag. The paper bag was labelled in the bottom corner with the same unique code that was given to each plant at the beginning of the sampling period. All the paper bags containing plants from a single transect were placed inside a larger grocery bag to keep the samples in order.

The plants we collected from the Retention Pond were brought back to the Austen Lab for further phenotypic data collection. First, any seeds present in the plant were removed, counted, weighed, and placed in a small paper envelope with the unique plant ID written on it. The rest of the plant tissue was then left in a drying oven for over 24 hours. After 24 hours, the flowers were removed from the plant and counted, then the branches were removed from the primary axis and the branches and primary axis were then weighed separately to obtain dry mass of each. Any underdeveloped fruit or flowers were not included in the data collected for this study.

## 2.3 Molecular methods

### 2.3.1 Genomic DNA extraction

Once the collected leaf tissue had dried to a brittle stage (approximately 24 hours in silica drying gel), the leaf tissue was then ground into a fine powder using a modified version of the QUIAGEN DNeasy Plant Mini Kit protocol. Dry mass of the leaf tissue was removed from the envelope and weighed to be approximately 19 to 20 mg for each plant to be used in DNA extractions. The weight of the tissue, the microtube label, and the envelope number were all recorded, and the tissue was placed in its respectively labelled microtube, along with two 2.4 mm

metal balls. The set of microtubes with tissue were then evenly distributed in both grind blocks of the QUAIGEN TissueLyser II and the blocks were fixed into place. The TissueLyser was run at 25 Hz for 1 minute, then samples were rearranged within the grind block, and run through the TissueLyser again for 30 seconds. Samples were observed after grinding to ensure the tissue was pulverized into a uniform powder. DNA extractions using the QUAIGEN DNeasy Plant Mini Kit, immediately proceeded the tissue grinding. The DNA extractions were performed according to the QUAIGEN kit protocol with a few adjustments because of equipment limitations, and to enhance DNA yield. These changes are as follows: for step 12 disruption of the pellet was avoided even if additional lysate (including dissolved DNA) could not be collected. For step 14, the solution was pipetted directly onto the membrane rather than the sides of the tube, and the height of the liquid once in the tube was visually inspected. During step 16, the pipette was angled to wash the walls of the tube above where the liquid was observed to reach in step 14. After centrifuging at step 17, the tubes were rotated 180 degrees while in the centrifuge and the centrifuge was run again to insure complete drying of the membrane.

### 2.3.2 Quality control

After genomic DNA extractions were performed for all sampled plants, including duplicate extractions of 5 samples, the products were put onto a nano spectrophotometer to determine the DNA purity and concentration (ng/ $\mu$ L). Prior to use, the nano spectrophotometer was wiped down with Milli Q water, prepared with 2  $\mu$ L of Milli Q water and then calibrated with 2  $\mu$ L of Buffer AE. To measure the samples, 2  $\mu$ L of DNA was placed on the nano spectrophotometer sensor using a pipette tip, careful not to probe the sensor. The nano spectrophotometer machine was then closed, and the measurement was initiated. The results of each sample were recorded, and a picture of the graph visualized on the computer was taken.

Once the nano spectrophotometer readings were collected, mock digests of each sample were made as a quality control. The quality control ensures that the DNA samples that will be used for sequencing are not contaminated with restriction enzymes since this would interfere with the genotyping process. The typical mock digest uses a 50  $\mu$ L solution, but we halved the process to a 25  $\mu$ L solution to preserve a large volume of pure DNA. The volume of pure DNA

used in the mock digest for each sample was calculated on Microsoft Excel based on the concentration readings from the nano spectrophotometer so that approximately 500 ng of DNA was used. This solution included a constant amount of 2.5  $\mu$ L of 10xNEBuffer, the calculated volume of pure DNA, and Milli Q water was added when needed to bring the final volume of the solution to 25  $\mu$ L. The components were all added to a labelled microtube, were flicked and inverted to mix, and then centrifuged for 10 seconds. The mock digest solutions were left in a 37 °C water bath for 2 hours, and once finished were immediately put into the freezer to preserve them for future use.

The mock digests and their respective pure DNA samples were run on a 0.8% agarose gel to verify that DNA was not cut into smaller fragments during the mock digest. Three wells evenly distributed on both the top and the bottom row were loaded with BioBasic DNA 100 bp Marker. The pure DNA was loaded in the remaining available top wells, with the volume adjusted based on the concentration of the sample to ensure approximately 500 ng of DNA was added. The full volume (25  $\mu$ L) of the mock digests were added in the bottom row in the available wells, ensuring the sample on the top and bottom wells were from the same plant. The location of the well and the respective solution added was then recorded in a lab notebook. The gels were run for approximately 30 minutes at 100 V. Samples were considered to be of good quality if the mock digest and the pure DNA developed from the same plant did not differ in appearance when run on the gel (i.e., there was no cuts in the DNA).

### 2.3.3 DArTseq

Ninety-four samples, including the 5 replicates, will be selected to be sent to Diversity Arrays Technology (DArT) Pty. Ltd. in Australia. The samples are to be processed using DArTseq technologies which use a form of Restriction site Associated DNA Sequencing (RADseq) used to reduce genome complexity in plants to develop genetic markers and allow for genotyping.

Restricted site Associated DNA Sequencing (RADseq) is a technology where the genomic DNA of an organism is cut into fragments by a restriction enzyme. The restriction

enzyme used can be changed to adjust the frequency of cuts along the DNA sequence, and thus the length of the fragments formed. After the fragments have been cut, adapters containing a unique barcode corresponding to the individual sample are ligated onto the sticky ends of the fragment. This process allows for multiple samples of DNA to be pooled and sequenced simultaneously, and then separated during library formation. Fragments of certain sizes may be removed through electrophoresis (Parchman et al., 2018). RADseq allows for the fragments to be analyzed for SNPs or other polymorphisms in the sequence to determine genetic markers (Davey & Blaxter, 2010).

DArTseq technologies select parts of the genome which largely encompass active genes which are suitable for marker discovery and genotyping. The data sent back from DArTseq will allow us to identify which of our sampled plants are ramets from the same genet, enabling us to examine the spatial genetic structure of the population. The DArTseq data will also enable development of PCR-based genetic markers for *Lysimachia terrestris* clones, which could be used in future population studies.

## 2.4 Statistical Analysis

All statistical analyses were done using R statistical programming software version 4.2.2. (R core team, 2022) with R Studio interface (RStudio team, 2022). Standard deviation was used as an error metric for all statistical calculations.

### 2.4.1 Plant coordinate calculation

We calculated the coordinates of each individual plant sampled using triangulation from the coordinates from both ends of each transect. Determining precise position of plants in this manner was possible because plants were always sampled from specific positions within the quadrats, and quadrats were positioned at specific locations within transects.

## 2.4.2 Principal component analysis and ANOVA's

I began the data analysis by using a principal component analysis in the R package 'psych' (v.2.2.9; Revelle, 2022) to reduce the four phenotypic traits into principal components that can be used to represent the major axes of phenotypic variation found in this study. As an initial examination of spatial genetic structure, I ran two simple analyses of variance (ANOVA) using transect as the independent variable and the two principal components as the dependent variable in their own respective analysis. The ANOVA which was run on transect and PC1 values was significant, so I used Tukey's HSD to determine where significant differences occurred. Box and whisker plots of each of the principal components against the transects were also developed.

## 2.4.3 Spatial autocorrelation

In addition to the crude estimate of phenotypic spatial structure via ANOVA, I undertook two additional refined analyses (Mantel test and Moran's I).

The Mantel test (from R package 'vegan' [v.2.6-4; Oksanen et al., 2022]) begins with a pairwise matrix of spatial distance, and pairwise matrix of phenotypic dissimilarity ("distance"). Using the coordinates calculated for each plant sampled, we created a pairwise spatial (Euclidean) distance matrix for all plants in the experiment. Then, using the first two principal components, I created pairwise phenotypic difference (Euclidean) matrices (one matrix for each of the two principal component axes). To test for any correlation between the pairwise spatial distance and pairwise phenotypic trait distances we used multiple Mantel Tests. The Mantel Test is a spatial autocorrelation analysis which determines the correlation between two distance matrices, here, a spatial matrix and a phenotypic matrix. This analysis returns a Mantel's statistic which ranges from 1, which represents a strong positive correlation, to -1, which represents a strong negative correlation, where 0 is no correlation.

Since we expected clumping of clones in the population rather than a continuous range of changing phenotype, I used Moran's I from the R package 'spdep' (v.1.2-8; Pebesma & Bivand,

2023) to determine whether nearby plants are more similar to each other than distant plants. In this study, Moran's I was also completed the principal components gathered from the PCA. To calculate Moran's I, I first created a distance matrix using the coordinates calculated for every plant, then created an inverse matrix and assigned 0 to the diagonal. The inverse distance matrix creates the spatial weights that are used to calculate the Moran's I statistic for PC1 and PC2. To do this, an average trait value is calculated for all the sampled plants. When calculating the average trait value of a focal plant, the contribution of the neighbouring plants to this average is weighted by the distance from that plant to the focal plant. This creates an average neighbour phenotypic value for every sampled plant. Similarly, to the Mantel Test, the analysis returns a value which ranges from +1, representing perfect clustering of similarities, to - 1 representing perfect dispersion, with 0 also indicating no spatial correlation.

### 3 Results

#### 3.1 Goal 1: Optimizing DNA extraction for analysis

DNA was extracted from each plant sampled in this study to be used in future research. In total, an average of 19.49 mg per sample of dry leaf tissue from 99 sampled plants (92 plants from the Retention Pond, 2 plants from Silver Lake, and 5 replicates) were used in the molecular analysis for *Lysimachia terrestris*. I found that DNA yield was more concentrated when using leaf tissue dried in silica drying gel rather than leaf tissue that had been freeze dried with liquid nitrogen (see Appendix). The DNA was extracted into a 100  $\mu\text{L}$  solution, and a nano spectrometer was used to analyse the concentration and purity of the DNA extractions. The mean absorbance at wavelengths of 260 and 280 nm for the DNA samples was 1.466 and 0.797, respectively. The ratio of these absorbance levels is indicative of the purity of the DNA and was on average 1.84. The solutions had approximately  $73.3 \text{ ng}/\mu\text{L} \pm 22.5$  of pure DNA ranging from 5.7 to 157.6  $\text{ng}/\mu\text{L}$  of DNA.

The pure DNA was run alongside mock digests on a gel electrophoresis to test for the quality of the samples. Figure 3 clearly shows that there was no difference between pure DNA and the mock digests suggesting no contamination is present.

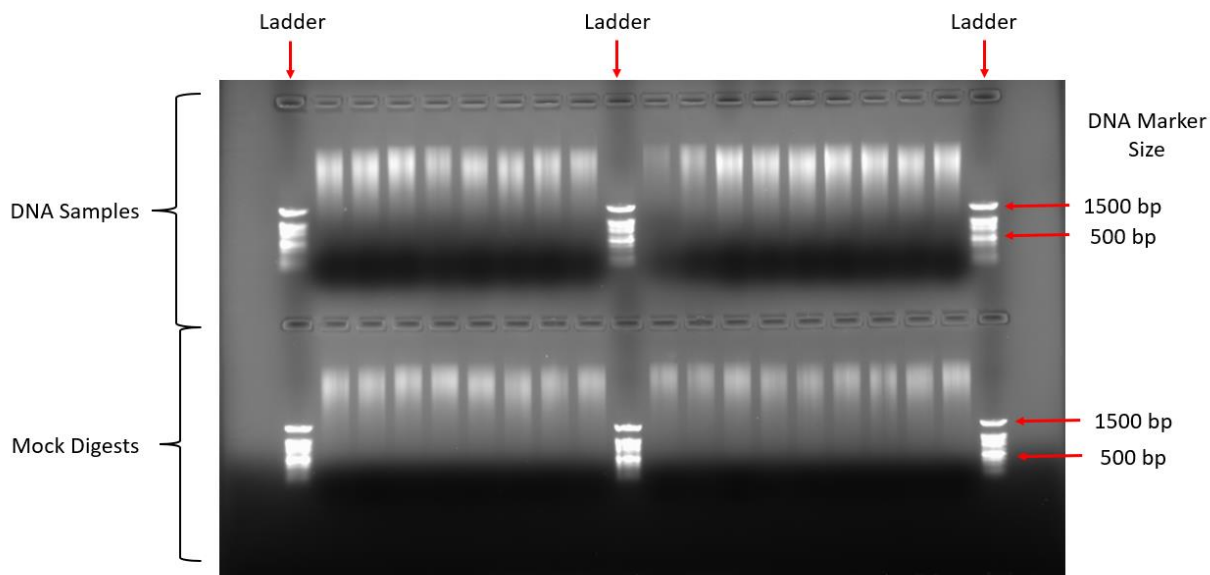


Figure 3. Gel electrophoresis image of pure DNA (top row of wells) and mock digests (bottom row of wells). The wells of both the pure DNA and mock digests are aligned so that the top and bottom rows both have the same plant sample in the corresponding well. Red arrows show where the ladders were placed (top of image), and their DNA marker size (right of image).

## 3.2 Goal 2: Explore the spatial distribution and phenotypic differences between clones

### 3.2.1 Plant phenotypes

In total, I sampled 92 plants at the Retention Pond across 8 transects and 2 more distant locations, and additional leaf tissue was collected from 5 plants at Silver Lake. Phenotypic data were collected solely from plants sampled at the Retention Pond. The mean height of all the plants sampled was 43 cm and ranged from 12 to 69 cm in height with a normal distribution. The mean mass of the dried primary axis was  $0.89 \text{ g} \pm 0.53$ , the mass of the dried branches was

0.75 g  $\pm$  0.62, and the average total biomass of all the sampled plants once dried was 1.64 g  $\pm$  1.00. Of the plants sampled 87% of the plants were at reproductive status (N = 80), and of those 73% were flowering during the sampling period (N = 58). Of these reproductive flowering plants only 30 produced any fruit with 20 of these plants producing 3 or less fruits.

During the field sampling period we observed that different phenotypes appeared to occur in different transects within the population. For example, plants sampled from the northernmost side of the Retention Pond had noticeable kinks in the primary axis which were not observed in transects placed to the south (personal observation). We also noticed some transects had considerably branchier plants than other transects. Other apparent phenotypic differences among floral traits were observed as well (ex. the size and shape of petals) although we did not analyze these traits in this study (Figure 4).



Figure 4. Floral trait phenotypic differences between transects sampled at the Retention Pond. Each picture was taken from different transects, and the arrows point towards differences in the petals of these flowers. Photographs by Gwen Deadman-Wylie

### 3.2.2 Major axes of phenotypic variation

Statistical analysis of associations between phenotypic difference and spatial distance was restricted to the 74 reproductive plants sampled within the 8 transects at the retention pond site (11 non-reproductive plants were excluded because some of the traits included in the analysis can be measured on reproductive plants only and an additional 3 plants had insufficient data collected).

The correlation matrix done on four of the phenotypic traits (total biomass, height to bottom flower, reproductive effort, and branchiness) determined that there are both positive and negative correlations among these traits. The largest positive correlations reside between the total biomass with both height to bottom flower and branchiness, with correlations of 0.37 and 0.38, respectively. There was also a negative correlation between branchiness and reproductive effort of -0.44. The rest of the correlations between traits ranged from -0.25 to 0.21.

These correlations are large enough to continue with a principal component analysis to reduce the dimensionality of the data and identify underlying patterns of variation. Of the four initial components which represented each trait, only two were determined to be statistically useful with Eigen values larger than 1. These two components explain a total of 77% of the variability observed in the data, indicating that they capture a substantial amount of the underlying structure in the dataset. The first principal component (PC1) is representative of the traits branchiness and reproductive effort and accounted for 42% of the variability in the data. The second principal component (PC2) represented the traits height to bottom flower and total biomass and accounted for 34% of the variability in the dataset (Table 1). The traits represented in PC1 suggest a life history trade-off due to the negative correlation, whereas the traits represented by PC2 are both positively correlated and can be interpreted as the overall size of the plant (Figure 5).

Table 1. Principal component analysis loadings of each phenotypic trait for the two principal components used for further analysis

Phenotypic Trait	PC1	PC2
Height to Bottom Flower	-0.11	0.92
Total Biomass	0.65	0.64
Reproductive Effort	-0.77	0.27
Branchiness	0.84	-0.13

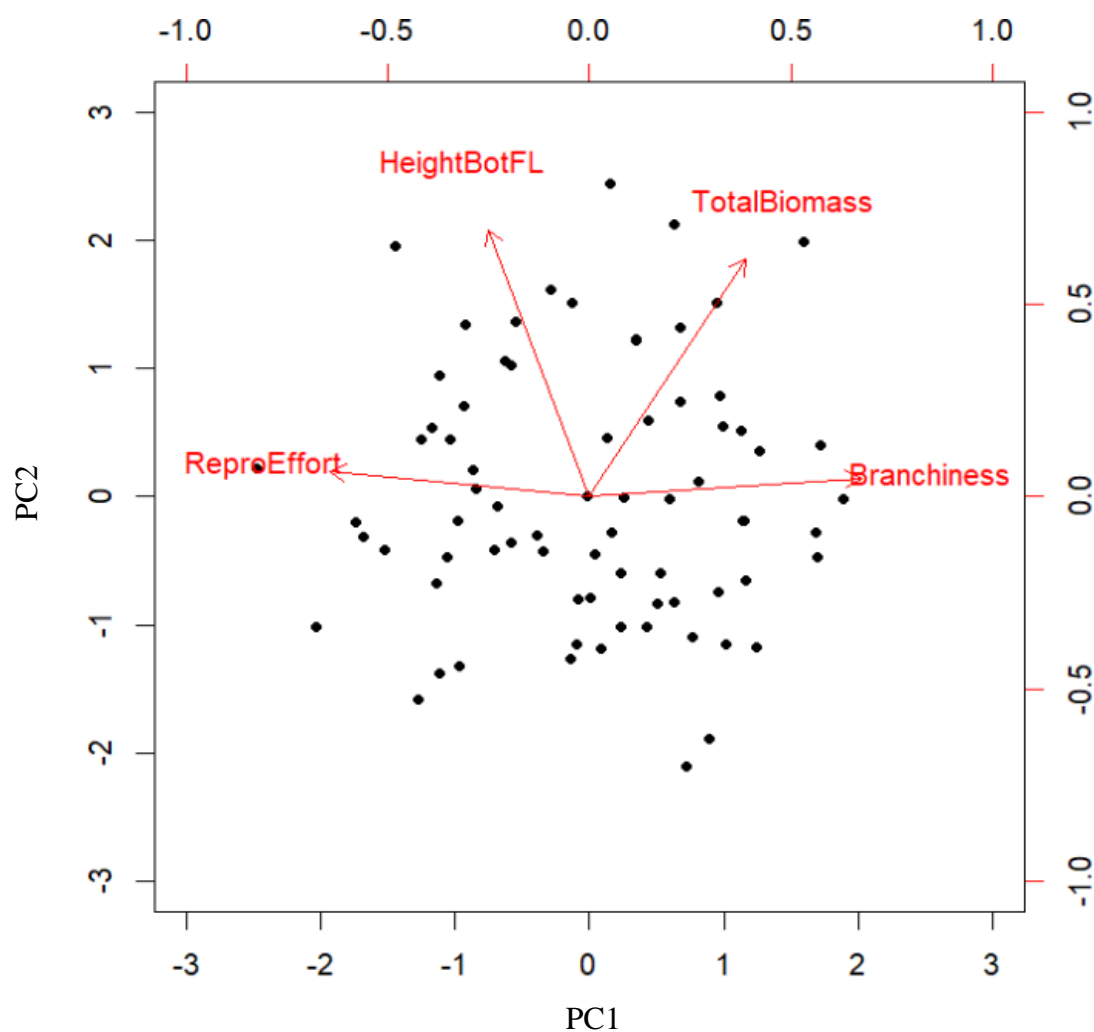


Figure 5. Plot of the principal component analysis (PCA) showing the two principal components which represent the largest amount of variance in the phenotypic traits of sampled plants. Axis PC1 correlates strongly with reproductive effort (ReprEffort) and branchiness, and axis PC2 correlates with height to bottom flower (HeightBotFL) and total biomass.

### 3.2.3 Associations between phenotypic dissimilarity and spatial distance

The analysis of variance tests determined there were significant differences ( $p = 0.0001$ ) of PC1 phenotypic traits between the transects (Table 1). A follow up Tukey HSD post hoc testing determined significant differences between transect 2 with transects 5 and 7, and transect 6 with transects 5, 7 and 8 (Figure 6). The ANOVA completed with PC2 phenotypic values did not detect statistically significant differences between transects (Table 2).

Table 2. Analysis of Variance (ANOVA) table on the first principal component traits and transects that plants were sampled from.  $\alpha = 0.05$ , significance codes (from R Studio): 0 = '\*\*\*\*', 0.001 = '\*\*', 0.01 = '\*', 0.05 = '.', 0.1 = ' '.

	<b>Df</b>	<b>Sum Squares</b>	<b>Mean Squares</b>	<b>F</b>	<b>p</b>
PC1	7	25.79	3.684	5.151	0.0001****
Residuals	66	47.21	0.715		

Table 3. Analysis of Variance (ANOVA) table on the second principal component traits and transects that plants were sampled from.  $\alpha = 0.05$ , significance codes (from R Studio): 0 = '\*\*\*\*', 0.001 = '\*\*', 0.01 = '\*', 0.05 = '.', 0.1 = ' '.

	<b>Df</b>	<b>Sum Squares</b>	<b>Mean Squares</b>	<b>F</b>	<b>p</b>
PC2	7	10.04	1.4347	1.504	0.181
Residuals	66	62.96	0.9539		

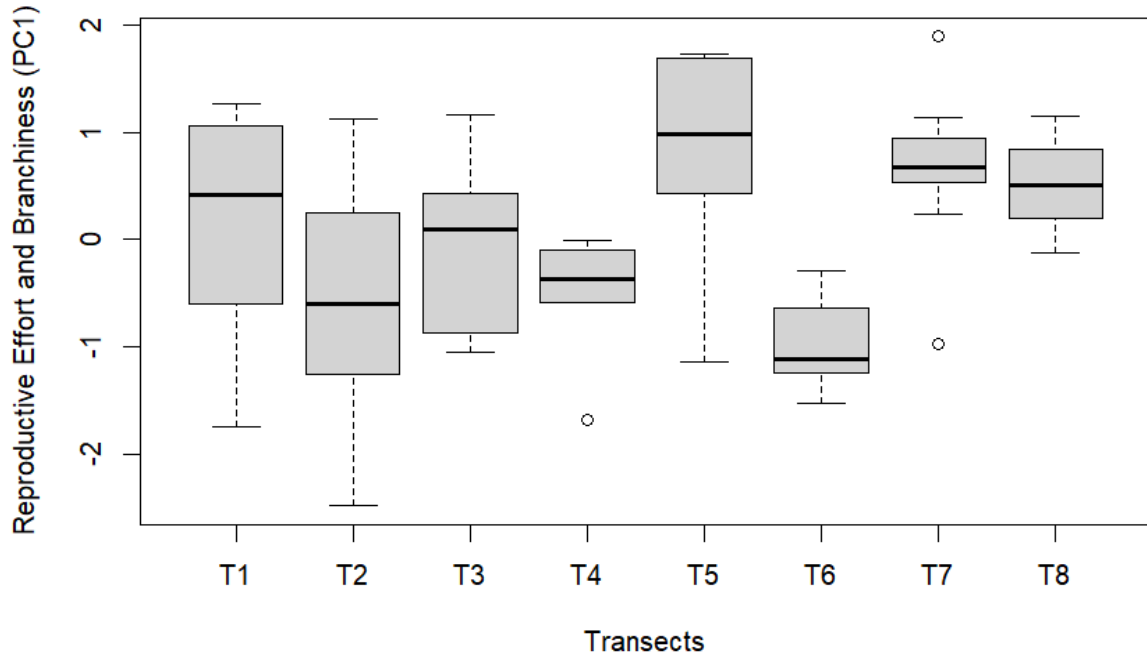


Figure 6. Box and whisker plot of phenotypic traits represented by principal component 1 (PC1 – branchiness and reproductive effort) across different transects.

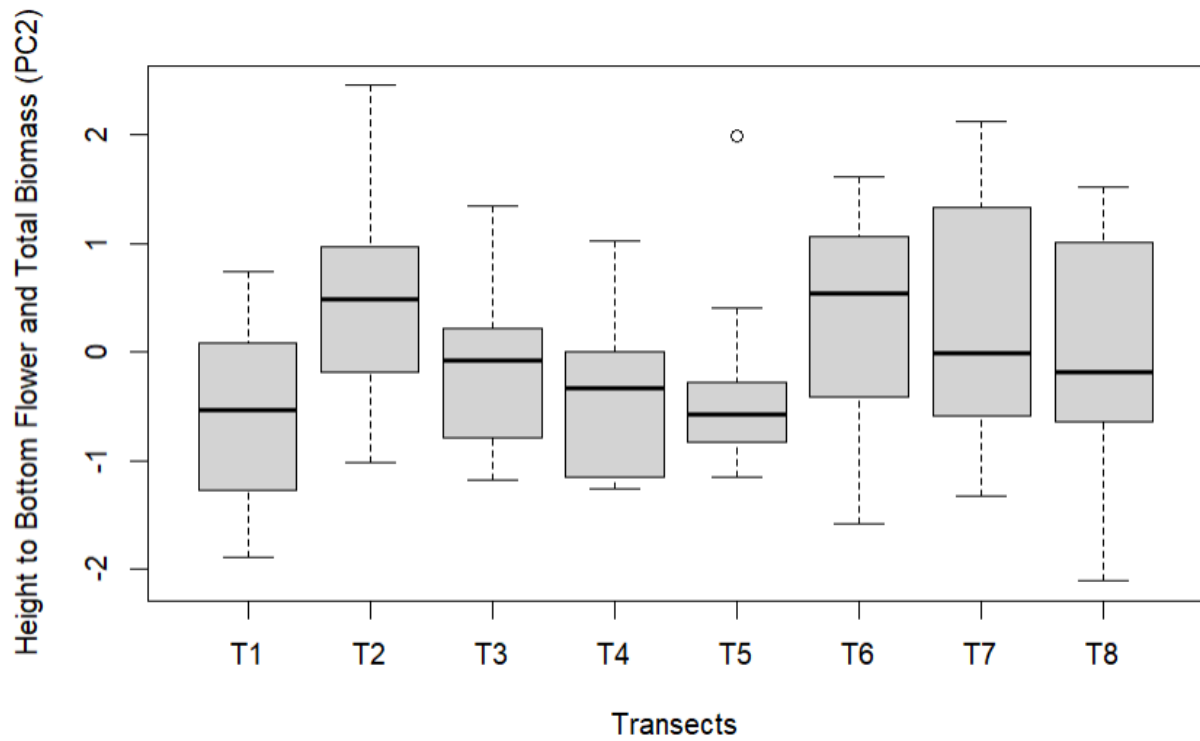


Figure 7. Box and whisker plot of phenotypic traits represented by principal component 2 (PC2 – height to bottom flower and total biomass) across different transects.

The two principal components developed from the principal component analysis were each used in a Mantel Test along with a spatial distance matrix to explore whether a relationship was present between spatial and phenotypic distance. No relationship was detected for either axis (Figure 8). The Mantel test statistic for PC1 was 0.04995 ( $p = 0.07$ ). The Mantel Test using PC2 returned similar results, with a Mantel statistic of 0.01728 ( $p = 0.28$ ). Therefore, both tests fail to reject the null hypothesis that there is no correlation between spatial distance and phenotypic distance.

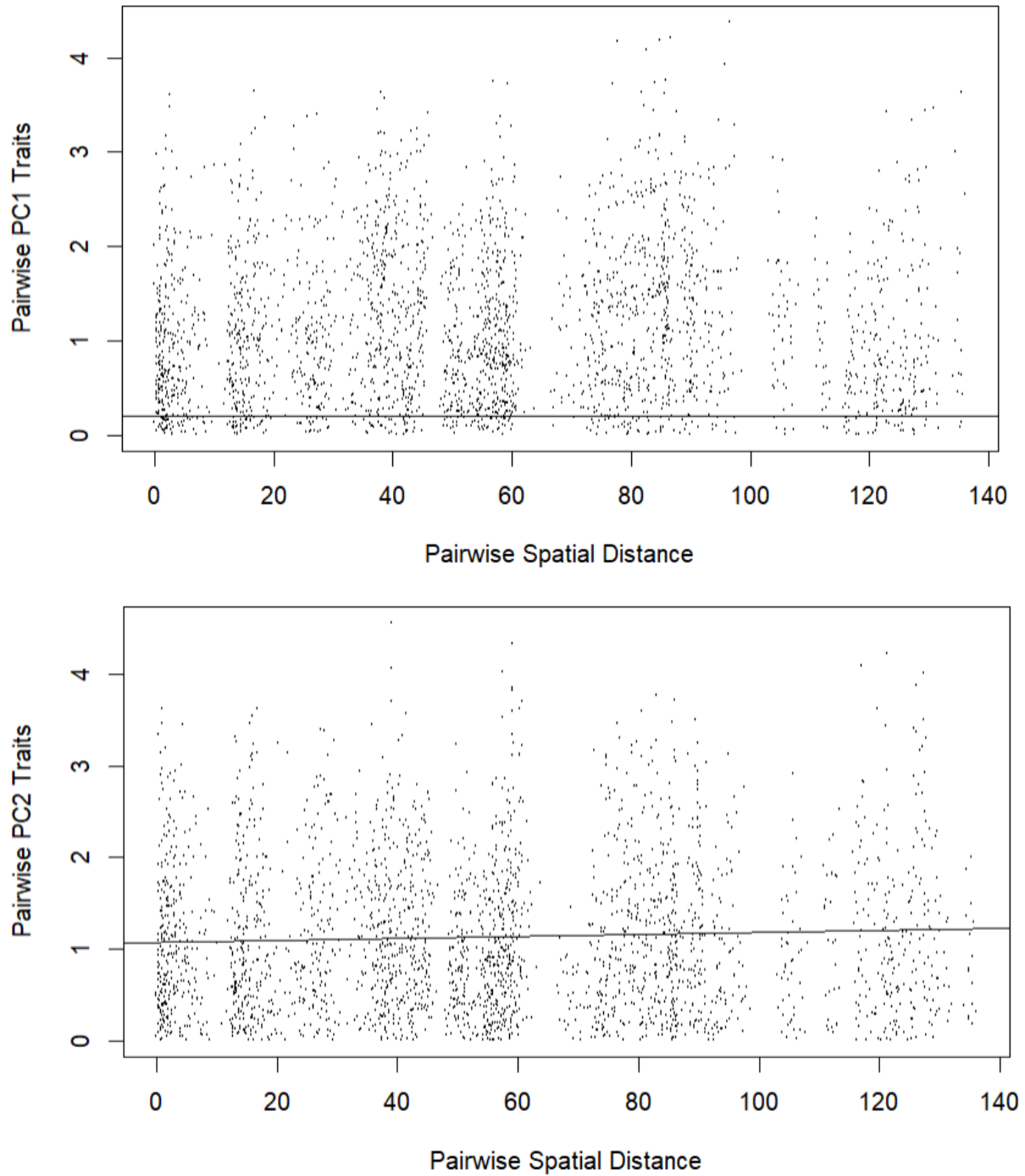


Figure 6. Mantel Test pairwise spatial distance matrices against phenotypic trait matrices for PC1 (top) and PC2 (bottom) traits.

The two principal components developed from the PCA were then used in a Moran's I test to explore whether the population was spatially structured by clustering of similar phenotypes. The correlation between spatial distances and phenotypic traits represented by the PC1 were statistically significant ( $p < 0.0001$ ). This relationship of distance and phenotype returned a Moran's I statistic of 0.381 which suggests a positive correlation (Figure 9). This indicates that neighbouring plants are likely to have more similar phenotypic traits represented by the PC1 than would be seen if there was no correlation between distance and phenotype. When Moran's I was done using PC2, the Mantel I statistic was 0.128, and the test was statistically significant ( $p = 0.05027$ ) (Figure 10). The Moran's I analysis also returned an expected value for both tests of -0.013, indicating the value that would be returned if there was no correlation between spatial distance and phenotype.

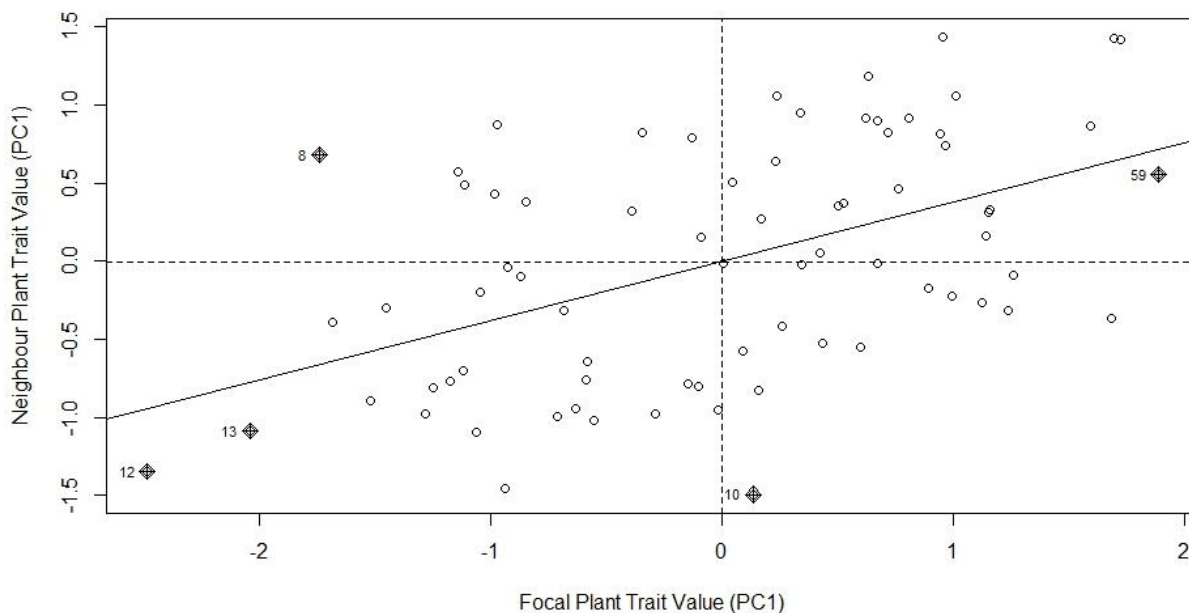


Figure 7. Moran's scatterplot of PC1 values plotted against spatially lagged PC1 values. Each point represents a focal plant trait value and its neighbour's trait value. The upper right and lower left quadrats represent positive correlations while the upper left and lower right represent negative correlations. The slope of the line is the Moran's I statistic (0.381). The diamond points represent potential outliers in the data.

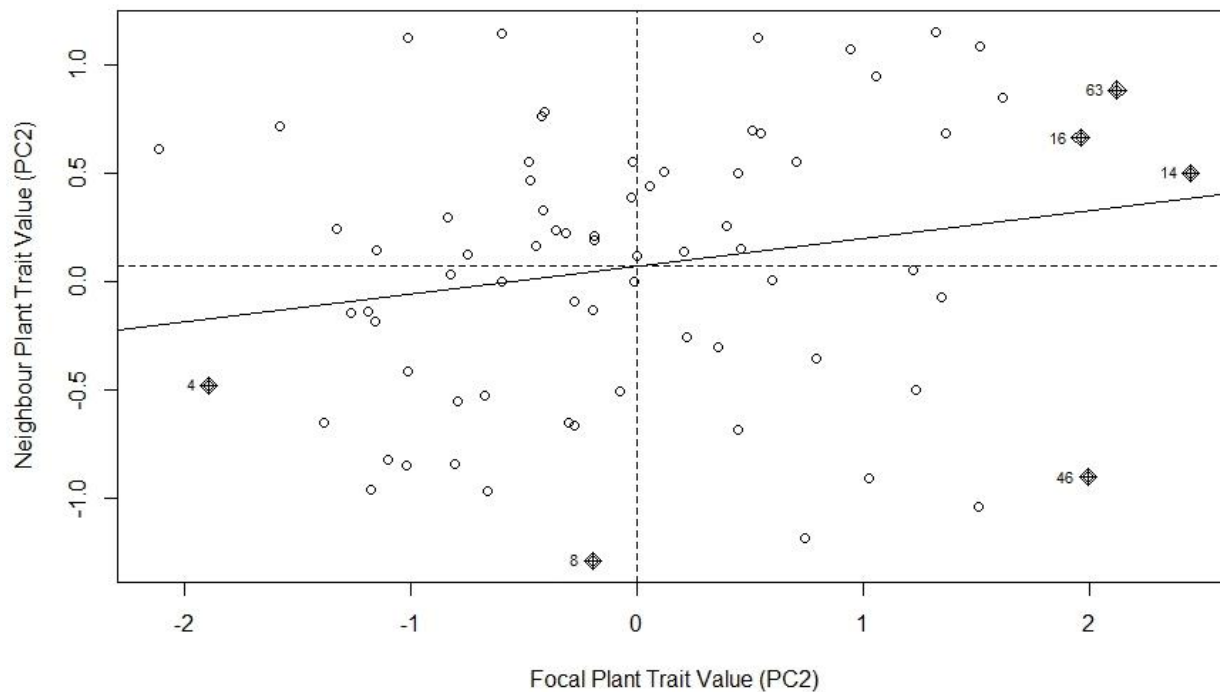


Figure 8. Moran's scatterplot of PC2 values plotted against spatially lagged PC2 values. Each point represents a focal plant trait value and its neighbour's trait value. The upper right and lower left quadrats represent positive correlations while the upper left and lower right represent negative correlations. The slope of the line is the Moran's I statistic (0.128). The diamond points represent potential outliers in the data.

## 4 Discussion

This project aims to describe the spatial structure of a newly established population of *Lysimachia terrestris*, and how this structure may develop over time. To this end, I sampled leaf tissue and phenotypic traits from 92 *L. terrestris* plants using a spatially explicit sampling design. I fulfilled two main goals, (1) to optimize tissue grinding and DNA extraction techniques for *Lysimachia terrestris* and complete extractions from the sampled plants; and (2) to explore the spatial distribution and phenotypic differences between clones in a localized population.

During laboratory practices I was able to develop a method for tissue grinding and subsequent DNA extractions for *L. terrestris* which consistently returned concentrations of DNA considered to be acceptable for downstream sequencing. For goal 2, I used statistical analyses to

determine whether there was any spatial autocorrelation of plants phenotypes in the population. I found significant support that neighbouring plants were more likely to have similar phenotypic traits than plants at farther distances. This was most pronounced in phenotypic traits represented by PC1 (branchiness and reproductive effort), which also exhibited significant differences between transects. This finding suggests that *Lysimachia terrestris* phenotypes are spatially structured in clumps throughout the Retention Pond, but it is unknown if this phenotypic structure is due to clonality, spatial variation in the environment, or a combination of both. Below, I discuss how DNA extractions from this study will allow for future sequencing to occur, the connections between clonality and life history trade-offs, and the implications of clonal growth on spatial structuring. I conclude by highlighting the future directions which will allow for a more comprehensive understanding of the interactions between genotype, phenotype, and the environment.

#### 4.1 High quality DNA extractions will support genotyping of *Lysimachia terrestris*

The first goal of this research was to optimize tissue grinding and DNA extraction techniques for *Lysimachia terrestris*. The ratio of 260/280 nm absorbance of wavelengths of *L. terrestris* was on average 1.84, and DNA is generally considered to be pure if values above 1.80 (Oh et al., 2010). These methods yielded a mean DNA concentration of 72 ng/ $\mu$ L (N = 100 samples), with only 1 extraction considered to have failed (concentration = 5.7 ng/ $\mu$ L). The mock digests used to assess the quality of the samples determined that the DNA extracted is of good quality and is not contaminated. The quantity and quality of DNA extracted ideal is for genotyping by DArTseq since this company requires DNA samples to be between 50 and 100 ng/ $\mu$ L. Follow up research will be able to utilize the DNA extractions from this study to complete the genotyping of the population and then attempt to determine the abundance and distribution of clones.

#### 4.2 Trade-off between branchiness and reproductive effort

In using PCA to describe plant phenotypes, I found that, at least for the traits I measured, a large proportion (43%) of the phenotypic variation in my plant sample was attributable to a trade-off between investment in vegetative growth (branches) and investment in sexual reproduction (flowers). This trade-off suggests that a plant can either invest energy into growing many branches but will produce few flowers or, instead can invest in producing many flowers but will consequently have relatively few branches. Trade-offs such as this one are common in plant species since life history traits are energetically demanding and resources are limited (Bajcz & Drummond, 2017). Typically, traits like reproduction and growth cannot both be optimized in an environment especially because reproduction is particularly costly, so a compromise must occur (Bajcz & Drummond, 2017; Obeso, 2002).

Clonal plants thrive in heterogenous environments, but environments with poor or scarce resources are likely to magnify the extent of trade-offs throughout a plant population (Bajcz & Drummond, 2017). Multiple studies found by removing flowers (i.e., a reduction in energy allocated towards reproductive effort), the growth or condition of other traits improved compared to control groups. In the clonal shrub *Vaccinium angustifolium* (lowbush blueberries) for example, removing flowers leads to an overall increase in total vegetative mass and leaf size (Bajcz & Drummond, 2017), and in *Rhododendron lapponicum* (another clonal shrub), flower removal similarly lead to increased branch number and overall branch mass (Karlsson et al., 2006). Large clonal growth organs, such as rhizomes, are also energetically costly to produce and may contribute to life history trade-offs against sexual reproduction (Herben et al., 2015). Investing large amounts of energy into sexual reproduction is most important when the risk of mortality is high in the individual plant. Since clonal plants are able to share resources through connections and can also reduce the chance of disease destroying an entire genet, the risk of complete genet mortality is low. To improve overall fitness, clonal plants can instead withhold reproductive efforts if optimal conditions are not met (Herben et al., 2015). In long living clonal species like *L. terrestris* there may be purposeful trade-offs such as the ones I found in this study that allow for overall higher fitness in the population.

#### 4.3 Discrepancy of results between Mantel and Moran's I

Since *Lysimachia terrestris* can use multiple modes of clonal growth this can create complex spatial genetic structures of clones, and possibly of phenotypes. Spatial autocorrelation analyses allow this structure to be explored.

This study explored the use of two different spatial autocorrelation analyses to understand how the structure of the population has differing phenotypes. Analyzing the phenotypic data collected using the Mantel Test strongly showed no correlation between the spatial distance and phenotypic traits of ramets in this population. For this research, the Mantel Test is used to detect whether phenotypic dissimilarities increase linearly with increasing spatial distances, or in other words whether larger spatial differences coincide with larger phenotypic differences. The Mantel's statistic for both principal components was close to 0 which suggests no correlation between spatial distance and phenotype. However, previous studies have found that clonality often strongly impacts the spatial genetic structure of a population through the aggregation of clonal genotypes (Alberto et al., 2005). If *L. terrestris* at the Retention Pond exhibits a phalanx clonal growth strategy, typical of rhizomatic structures, it is likely that ramets of a clone were structured in clumps across the study area (Alberto et al., 2005). So, potential clustering of clones throughout the population could have resulted in ramets of the same genet being sampled from different transects at various regions of the Retention Pond. In this case it would be expected that linearly increasing spatial distance does not necessarily mean increased phenotypic dissimilarities.

Based on the Moran's I results, there is strong support that plants within the population were structured in clusters of phenotypically similar traits. *Lysimachia terrestris* is capable of clonal growth through both bulbils and rhizomes but the former is primarily used for long distance dispersal (Herben & Klimešová, 2020). Rhizomes however, have long term physical connections between ramets which often exhibit slow lateral spreading capabilities (Sosnová et al., 2010). Based on the drastically different structures associated with these clonal growth organs, it is highly likely that rhizomes are the primary clonal structure used by *Lysimachia terrestris* at the Retention Pond population.

#### 4.4 Underlying drivers of association between phenotype and spatial distance

Clustering of similar traits may indicate a strong spatial genetic structure of the *Lysimachia terrestris* population at the Retention Pond if phenotype is representative of genotype. However, it is also possible that environmental factors are instead driving phenotypes (Ricono et al., 2020). Since sampling was done in multiple transects that were placed throughout the population, there may be environmental heterogeneity which would cause different levels of nutrients, water, and sunlight in the transects. The Moran's I test can not distinguish where the clumping of phenotype occurs within the population, just that it is present. Without knowing where clumps of phenotypic similarities occurred, and without research on environmental conditions, we are unable to fully understand the populations' genotypic structure. Furthermore, the ANOVAs used in this study which examined phenotypic differences between transects found significant support that phenotypic traits varied among transects located in multiple areas of the population. Transects without significant differences may also indicate multiple clones were sampled in a single transect and so different phenotypes were expressed in that area. Thus, the ANOVAs cannot be accurately used to determine where clumping occurred.

In clonal populations of *Asclepias syriaca* (common milkweed), the phenotypic differences within transects were based on transect location, not the genotype of the ramet. While ramets of a clone were physically closer in space, they were not more phenotypically similar to each other than ramets from other genets (Ricono et al., 2020). However, even if phenotypes are more similar among individuals with the same genotype, it still does not necessarily mean that clonality is the driving force. The location of clones and their surrounding environmental conditions are important to consider when making conclusions on what is most strongly impacting phenotype. Common garden experiments are a useful experimental tool which can help determine the reason behind phenotypic differences in a population by looking at genotype-environment interactions (de Villedenreuil et al., 2016). A few ramets from multiple genets in the population should be planted into controlled environments to monitor if phenotypic differences between the genets still arise. If phenotypic traits are consistently similar between ramets when in a controlled environment, and different between genets, then it can be concluded that genetic similarity drives phenotypic similarity.

The spatial genetic structure of a population can also have implications on the sexual reproductive success through plant and pollinator interactions. This study found low fruit set in the *Lysimachia terrestris* population at the Retention Pond with most plants producing no fruit at all. Reproductive output in clonal plants has been quantified to be relatively lower than non-clonal species in the past (Herben et al., 2015). As clones grow in size, the number of flowering ramets increases which in turn increases the chance of within-genet self-pollination (Yakimowski & Barrett, 2014). Populations of clones with longer rhizomes have been found to have lower reproductive outputs than those using other clonal growth modes because the structure of these ramets reduces cross-fertilization success (Herben et al., 2015). The Austen Lab has detected a specialist pollinator, *Macropis nuda*, to be abundant at the Retention Pond and these bees are known to frequently visit *L. terrestris* (Brooks, 2023). Moreover, past research in the lab (Crosby, 2022) found that hand pollinations between flowers of the same ramet did not set fruit, suggesting that this *L. terrestris* population is self-incompatible. Surprisingly, low fruit set, which was noticed in this current study, was also found when outcrossing was simulated using pollen from another ramet within the Retention Pond site (Crosby, 2022). The apparent presence of a strong spatial structure of phenotypes in this study suggests that methods used by Crosby (2022) likely involved ramets from the same genet being used in crosses which resulted in the low fruit set. When pollinating clonal plants, bumblebees have been found to spend a large amount of time foraging within a single genet and more so within a single ramet of that genet (Hu et al., 2015). Despite high visitation rates of *Macropis* bees at the Retention Pond, pollination may be ineffective if clones are aggregated and bees minimize inter-ramet distances while foraging (Ne'eman et al., 2006). Thus, low reproductive output in this population may be influenced by the spatial genetic structure and self incompatibility of the species. To follow up on Crosby's study, crosses of ramets from the population could be completed using ramets that appear phenotypically different to examine whether fruit set improves.

Many of the aforementioned traits of clonal plants also makes them incredibly successful invasive species. A single clonal propagule has the ability to spread rapidly through a population and can do so without pollinators present making invasions much easier than for species which require sexual reproduction (Keser et al., 2014). The properties of clonal growth allow resources to be shared throughout the whole genet which is particularly valuable for dominating heterogenous habitats or competitive environments (You et al., 2014). Most research exploring

how clonal spatial genetic structure changes over time is done on invasive species which have no natural competitors (Hierro et al., 2005). Understanding how the spatial genetic structure develops in a native species, such as *Lysimachia terrestris* in New Brunswick, allows more insight into clonal populations, and their evolutionary and ecological relationships. A study done by You et al. attempted to classify the differing success of native and invasive clonal species in a population and found that invasive species reap more benefits in the same habitat (You et al., 2014). This study done compared two different species occupying the same environment but general differences between these species were not necessarily accounted for. Interestingly, *Lysimachia terrestris* is an invasive species in regions of British Columbia. Future studies done on populations *L. terrestris* in British Columbia would allow research to understand how clonal traits and expansion capabilities differ in native and invasive regions within the same species.

#### 4.5 Future Directions and Conclusions

There are several directions to pursue with this research which would help to better understand the interactions of phenotype, genotype, and environment in this population, and how interactions change over time. An obvious first step is to send the DNA that was extracted in this project to DArTseq to have the samples sequenced. By sequencing the samples gathered from this study, a future student would be able to explore the spatial genetic structure of the population and determine the number and evenness of *Lysimachia terrestris* clones at the Retention Pond. Understanding how clones are spatially structured in the population could provide intel on pollinator impacts on reproductive success, and potentially hint towards the mode of clonal growth being used by this population. We would expect clonal plants using rhizomes to be geographically limited in their dispersal thus would likely be clustered together, while plants using bulbils may create ramets from a single genet to be dispersed in various areas of the park. If analyses of the spatial genetic structure determine that clumping of clones is occurring in the population then conducting common garden experiments could provide an explanation on whether environmental heterogeneity or clonality is the driving force for phenotypic traits.

The ability to distinguish possible groups of clones in the Retention Pond based on phenotypic traits was highly reliant on the traits analyzed in this study. In a plant there are multiple different phenotypic traits that can be quantified or measured to understand overall phenotype. However, it is likely that not every measurable trait will have considerable differences between clones, especially between closely related clones. It is possible that the traits used in this study to represent the phenotype of the sampled plants were not strong indicators of genotypic differences. For example, it is possible that floral attributes (ex. width or length of a petal) are more representative of genotypic differences between clones. Multiple flowers were obtained and preserved from every plant sampled in this study which allows this limitation to be explored in the future.

Understanding how a native clonal plant population develops spatially over time is a vastly understudied topic which provides essential information to better understand the mechanisms of clonal growth. *Lysimachia terrestris* is a particularly valuable plant to study because it is highly clonal, using both bulbils and rhizomes, is self-incompatible, and has both native and invasive populations throughout Canada. I found that *L. terrestris* is spatially structured at the Retention Pond based on some phenotypic traits that represent life history trade-offs (branchiness and reproductive effort). The scope of this study was unable to determine whether this structuring in the population was a result of clonal differences, or instead was influenced by environmental heterogeneity. However, I was able to optimize methodology which was used to extract the DNA from every plant sampled in this study. The DNA is ready to be sequenced in the future which will provide insight on the spatial genetic structure and allow for the exploration into the relationships between genotype and phenotype. Studying native clonal populations can provide valuable insights into the ecology of a species and inform future conservation efforts.

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## Appendix

### Liquid Nitrogen/Dry Ice Sampling Method:

Sampling using the flash-freeze method took place at the Sackville Stormwater Retention Pond on July 26, 2022. Leaf tissue was collected from one marked plant in each of the previously laid out eight transects (i.e., eight plants were sampled). Leaf tissue was collected and separated into two piles with approximately the same mass of tissue. The first pile of tissue was placed in a labelled paper envelope, which was then put in a plastic bag containing flower drying silica gel, as per the methodology used in previous sampling efforts. The other pile of leaf tissue was placed in the center of a labelled rectangular piece of aluminum foil (approximately 16 cm by 24 cm). The aluminum foil was folded into thirds both length and width wise to create an enclosed pocket for the tissue. The folded aluminum containing the leaf tissue was then placed inside a Styrofoam cooler containing dry ice. The samples were pushed into and covered with the dry ice. Once the leaf tissue collection was completed the samples were brought back to the Austen Lab. Liquid nitrogen was poured into a small Dewar flask and the aluminum foil samples were placed into the flask and pushed down with metal tongs. The samples were left in the Dewar to freeze for about one minute. While the samples froze, a labelled 9x9 microtube box with the dividers removed was filled with dry ice and placed inside the Styrofoam container with dry ice. The samples were removed from the liquid nitrogen with tongs and placed inside the microtube box, with a bit of dry ice added to the box as well. The microtube box was then stored inside a -80 °C freezer until the samples were used for DNA extractions.

DNA extractions were completed for these samples, followed by nano spectrophotometer readings. The values returned from the nano spectrophotometer was compared to DNA extractions done using silica drying gel of the same sampled plants to determine which method yielded higher concentrations of pure DNA. The DNA extracted from liquid nitrogen and silica drying gel had similar purities with values of 1.78 and 1.79 260/280 nm, respectively. However, the liquid nitrogen dried tissue had an average concentration of  $24.8 \pm 10.17$ , while the silica dried tissue  $33.5 \pm 4.66$ . Since samples dried in silica drying gel had a higher concentration of DNA, this method of drying was used for the DNA extractions to be considered for genotyping.