Determining the emergence timing, morphological characteristics, and species composition of *Galium* populations in western Canada

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By

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Abstract

Three species of *Galium* are commonly believed to thrive in western Canada; *Galium aparine* L., *Galium spurium* L. and *Galium boreale* L. Prairie weed surveys indicate that 'cleavers' (*Galium aparine* and *Galium spurium*) have increased in relative abundance since the 1970's, resulting in contaminated canola seed and harvest difficulties. The ability to identify and distinguish between species is important to understand their competitive ability or potential to outcross, potentially spreading traits such as herbicide resistance between species. The objectives of this thesis were to: (1) identify variation in the ITS1-5.8S-ITS2 that could be used for species identification, (2) verify the species composition of *Galium* populations in western Canada, and (3) evaluate emergence timing in spring and fall and morphological traits impacting cleavers biology. The target ITS1-5.8S-ITS2 complex was isolated from the ribosomal DNA of ten cleavers populations (including reference *Galium aparine* and *Galium spurium* populations), and was then cloned and sequenced to identify single nucleotide polymorphisms that could be used to differentiate species. The results identified a sequence variation that consistently differentiates between *Galium* species. In addition to several variable nucleotides in the ITS regions, one variable loci was identified within the highly conserved 5.8S gene. Sequence analysis of the ITS1-5.8S-ITS2 complex of *Galium* field collections from western Canada indicated that all samples were *G. spurium.* To address objectives 2 and 3, a common garden experiment of six cleavers populations with different geographical origins in western Canada were planted and their emergence monitored for a two-week period. Various other traits were also measured for each population. Field emergence studies showed differences between populations with regard to start of emergence (~150-250 GDD) and time to 50% emergence (~275-470 GDD) in spring. Fall emergence among populations was very low (1-9%) in comparison to spring emergence (2-

17%). Plant traits measured in the study did not differ between populations, supporting the results of the molecular work and leading to the conclusion that all populations were derived from a single species.

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Dedication

I would like to dedicate this thesis to my father, Wayne De Roo, and my mother, Carol De Roo, for their love, inspiration, guidance, and support. In addition, I would like to dedicate this thesis to my late Aunt Dree who always encouraged me to pursue my academic, professional, and personal goals.

Table of Contents

List of Tables

List of Figures

List of Equations

List of Abbreviations

AB	Alberta
AFLP	Amplified Fragment Length Polymorphism
AIC	Akaike's Information Criterion
ALS	Acetolactate Synthase
ANOVA	Analysis of Variance
BBN	Base Branch Number
CPWC	Critical Period of Weed Control
CTAB	Cetyl Trimethylammonium Bromide
D	Day
FED	Fecundity
FLW5	Flowering 5%
FLW50	Flowering 50%
FLW95	Flowering 95%
GDD	Growing Degree Days
HR	Hour
HSD	Honest Significant Difference
ITS	Internal Transcribed Spacer
LAI	Leaf Area Index
LW	Leaf Weight
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
SK	Saskatchewan
SNP	Single Nucleotide Polymorphism
TSW	Thousand Seed Weight
TW	Total Weight

1.0 Introduction

Galium species are a member of the *Rubiaceae,* or Madder family, which has flourished in a variety of crops (Malik and Vanden Born 1988, Moore 1975). Three species, *Galium aparine* L., *Galium spurium* L., and *Galium boreale* L. are present on the Canadian prairies. The increased presence of two species, *G. aparine* and *G. spurium* (collectively called 'cleavers'), has been recorded in field surveys (Leeson et al. 2005; Leeson 2012). Recently, the increased frequency of cleavers in western Canada has made it vital to understand why they have become so successful in crops and how to better utilize control strategies. Cleavers can have a significant impact on crop yield and quality as they are similar in size to canola (*Brassica napus* L.), making it difficult to separate out of samples for processing (Malik and Vanden Born 1988). Its climbing and tangling nature increases lodging in crops, especially wheat (*Triticum aestivum* L.) and flax (*Linum usitatissimum* L.), and can make harvesting operations difficult (Malik and Vanden Born 1987a). Greater control of cleavers in western Canada is needed, but is further complicated by the development of resistance to Group 2 herbicides (Acetolactate Synthase (ALS) inhibitors) and Group 4 (synthetic auxins; quinclorac) herbicides (Hall et al. 1998). Cleavers are also predicted to be at a high risk of developing resistance to glyphosate (Beckie et al. 2013).

Herbicide applications are the major method of weed control in western Canada, and spray timing is critical to ensure adequate control of weeds such as cleavers. Emergence timing studies and advances in emergence modelling are providing better information of when producers should implement a method of control. However, environmental conditions can have a significant impact on cleavers emergence (Royo et al. 2010b; Royo et al. 2012), but little has been done to determine if genetic differences exist among populations. Additional information, such as the evaluation of vegetative traits, could aid in the implementation of more accurate and

effective control measures for each species through more appropriate application timing (Bryson et al. 2008).

G. boreale is easy to distinguish from its *Galium* counterparts, as it has a perennial lifecycle, four narrow leaves per whorl, and slender, smooth stems (Pratt et al. 2015). Additionally, it often is not found in crop fields, but around them in ditches, pastures, or forested habitats. *G. aparine* and *G. spurium* have been found to compete for resources with all types of crops (Malik and Vanden Born 1988). Both species have stems and leaves covered with trichome hairs that make the plant feel sticky and allow them to adhere to and climb on surrounding structures. Minor differences in flower color and size, leaf shape and size, and seed size can be used in the field to differentiate species, but proper identification is logistically challenging because it requires a highly trained eye and substantial time. Chromosome counts (*G. spurium* n=10; *G. aparine* n=11) could also potentially be used to differentiate species, but is also time consuming and very tedious (Malik and Vanden Born 1988; Moore 1975).

Research in Canada has referred to cleavers populations as *G. spurium* or *G. aparine* (Malik and VandenBorn 1978a,b,c; Sapsford et al. 2011). European researchers identify their cleavers as predominately *G. aparine* (Hübner et al. 2003; Mennan and Ngouajio 2006; Mennan and Zandstra 2005), even though appropriate measures were not used to correctly identify the *Galium* species. Recent advancements in molecular biology could potentially be used to increase the efficiency in weed species identification, which would aid management efforts. The increased use of molecular biology in weed science, such as markers in the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA), are aiding in proper species identification, detecting competitive traits, and monitoring the effects of selection pressures on weed populations (Hübner et al. 2003; Vander Stappen et al. 1998). Cleavers species have been a

growing problem in western Canada with limited information to explain the increase in abundance. Harnessing molecular biology to correctly identify *Galium* species, combined with an evaluation of field characteristics, such as emergence timing and plant traits of different populations, could help to explain the increase in abundance and possibly offer biological knowledge with which to improve cleavers management.

This project focused on evaluating the *Galium* species complex and species characteristics that may have contributed to increased cleavers abundance. It was hypothesized that mixed species populations of *G. aparine* and *G. spurium*, variable rates of emergence and time to median emergence, and competitive trait differences between populations have influenced cleavers abundance in western Canada. The objective of the first study was to detect variation in the ITS1-5.8S-ITS2 rDNA region that could be used to differentiate *G. aparine* from *G. spurium*. The second objective was to develop a marker from variation found in the rDNA region to determine the species composition and relatedness of cleavers populations. The second study aimed to determine the emergence timing of cleavers populations in western Canada and to evaluate the morphological characteristics that influence competitive ability. The results from these studies will aid in the understanding of cleavers biology and may help to explain the increased abundance on the prairies, as well as provide insight for improved control of populations in the future. Evaluating the emergence timing of populations should allow growers to plan more efficient control strategies to minimize crop damage.

2.0 Literature Review

2.1 Studying Genetic Diversity

Organisms differ in genome size, ploidy level, chromosome number, and the nature and number of functional genes, all of which contribute to variation that can be used for genetic analysis. Molecular biology can be used to derive genetic and physical maps, for DNA sequencing, and for genomic informatics. Markers from molecular techniques can be derived from any gene with known or unknown function and detectable variation (Liu 1998). Morphological and cytological markers, like flower color and chromosomal banding patterns, were the first to be developed in the 1800-1900's to evaluate Mendelian traits.

The 1950's saw the evolution of allozyme markers that corresponded to different alleles of enzymatic activity. Allozyme markers are often visualized on polyacrylamide gels with electrophoresis, where different bands correspond to different alleles of a gene (Hunter and Markert 1957). Recombinant DNA technology and restriction fragment length polymorphisms (RFLP) was developed in the early 1980's and shortly after that, DNA marker and PCR technology was introduced. This led to a rapid development of techniques such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellites (Jasieniuk and Maxwell 2001). These techniques have both advantages and disadvantages that depend on sources of material and objectives of the experiment. Since the development of DNA markers, computers, robotic automation, and data communication have been developed to reduce the cost and increase the speed of results. The ideal characteristics for a molecular marker are high polymorphism, simple genetic interpretation, short time constraints, high repeatability, and easy automation (Liu 1998). All of these techniques have been utilized in crop breeding and have also shown promise in weed science. Information measuring variation

between weed populations, identifying genetic codes behind desirable competitive traits, and studying the movement of weed species are essential to designing long-term weed control strategies.

The importance of correctly identifying weed species is important when trying to determine how to control them. For example, *Amaranthus powellii* S. Wats. and *Amaranthus hybridus* L. were misidentified as *Amaranthus retroflexus* L. in southern Ontario, which led to early confusion on the severity of atrazine resistance in *Amaranthus retroflexus* L. (Warwick and Weaver 1980). The use of morphological traits to identify closely related species may not always be adequate and in some cases, molecular identification is warranted. Like the *Amaranthus* species, *Echinochloa* species are also sometimes difficult to differentiate morphologically. RFLP allowed Mennan and Kaya-Altop (2012) to distinguish late watergrass (*Echinochloa oryzicola* L.) from early watergrass (*Echinochloa oryzoides* Ard.) by using a non-coding region within chloroplast DNA. They concluded that late watergrass is not the same species as early watergrass in Turkish accessions, and could be successfully differentiated using RFLP (Mennan and Kaya-Altop 2012). Additional techniques have been used to differentiate barnyard grass (*Echinochloa crusgalli* L.) and early watergrass using differences in isozyme and RAPD banding patterns. In an experiment to develop single-nucleotide polymorphisms (SNPs) in *Picea* species, multiple SNPs were identified in the chloroplast and nuclear DNA (Germano and Klein 1999). The SNPs can now be used to identify samples of *Picea* trees from a single pine needle. The further use of SNPs for species identification will be important to understanding the biology of weed species that compete with field crops.

While there has been limited research into the molecular differentiation of *G. aparine* and *G. spurium*, variation of *Galium* populations in Europe has been detected through sequencing the

ribosomal DNA (Hübner et al. 2003). The use of the internal transcribed region in ribosomal DNA of *G. aparine* plants showed that there was little variation between plants from the same population, but different populations did contain several insertions and single nucleotide polymorphisms. Based on this, there may be potential for the variation found within the internal transcribed region to allow for the development of a molecular marker to differentiate *G. aparine* from *G. spurium*. However, such research has not yet been conducted.

2.2 Variation in the Internal Transcribed Spacer Regions

Regions of the 18S-5.8S-26S rDNA complex (shown in Figure 2.1) can be readily amplified by PCR and sequenced using universal primers (White et al. 1990). The universal primers developed by White et al. (1990) are available for use in amplifying the ITS1 and ITS2 regions in all eukaryotic species (Table 2.1).

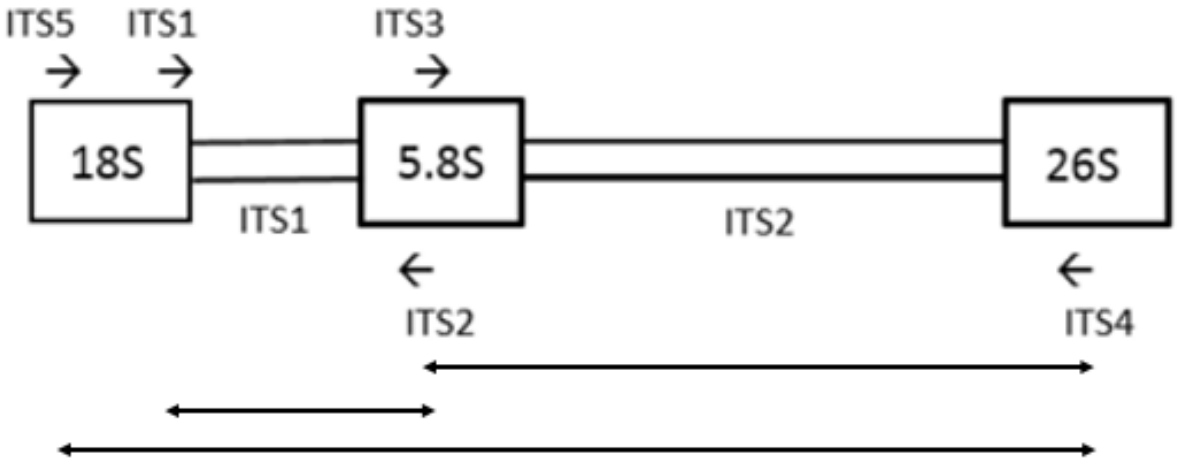

Figure 2.1. The ribosomal DNA region of biological organisms. Long arrows identify fragments that can be amplified using PCR. Short arrow indicate locations of primers use for PCR amplification.

Table 2.1. Universal primers for the ITS complex

Primer Name	Primer Sequence (5' → 3')
ITS1	TCCGTAGGTGAACCTGCGG
ITS2	GCTGCGTTCTTCATCGATGC
ITS3	GCATCGATGAAGAACGCAGC
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG

Several features of the rDNA complex make it a desirable area in the genome to look for variation. First, it is highly repeated in the nuclear genome, which promotes detection, amplification, cloning, and sequencing (Baldwin et al. 1995). Since it is highly repeated in the genome, the process of PCR and nucleotide sequencing of amplified products needs only a small amount of DNA (0.1-10 ng), which is easy to obtain using crude DNA preparations (White et al. 1990). Second, this area undergoes concerted evolution, meaning there is uniformity through the repeated copies. Third, it is small in size (~700bp) and highly conserved within species (Baldwin et al. 1995). Other benefits to using the ITS regions for distinguishing species includes biparental inheritance, universality, simplicity, intragenomic uniformity, intergenomic variability, and low functional constraint (Álvarez and Wendel 2003). Alternatively, chloroplast DNA or mitochondrial DNA sequence variations can also be useful in evaluating interspecific relationships between angiosperms, but such DNA is typically less conserved (Taberlet et al. 1991). The benefits and simplicity of the rDNA complex make it an ideal candidate when investigating markers for species identification.

The use of the internal transcribed spacer (ITS) region has been successful in distinguishing many angiosperm species from each other. A summary of plant family ITS regions by Baldwin et al. (1995) showed considerable variation in the ITS1 and ITS2 regions of different species. Spacers between the rRNA genes are poorly conserved at deeper levels and the high degree of variation is ideal for differentiating plant species. Length variation of each ITS region has been identified; ITS1: 187-298 bp and ITS2: 187-252 bp (Baldwin et al. 1995). Identifying variation at the molecular level is ideal when studying species with similar taxonomy. Vander Stappen et al. (1998) used variation in the ITS1 region to evaluate the *Stylosanthes* species. Eleven DNA sequence types were distinguished from insertions/deletion

events and 15 single base pair substitutions in ITS1 were found in *Stylosanthes* species. From this variation markers were developed to separate *Stylosanthes guianensis* from other species in the genus using RFLP and selective PCR (Vander Stappen et al. 1998). Variation in the ITS region of different species has also been well documented in the Compositae (Baldwin 1993), Winteraceae (Suh et al. 1993) and Fabaceae (Wojciechowski et al. 1993) families. Small-subunit nuclear rDNA sequences that are separated by the ITS regions evolve relatively slow, and are useful when studying closely-related organisms such as *G. aparine* and *G. spurium*. The rDNA genes located between ITS1 and ITS2 are not often used for species differentiation, as they are highly conserved and contain little to no variation. Some rDNA genes between species appear to contain phylogenetic information at deep basal branches in plant families. Additionally, the accuracy of new sequences can be determined from aligning the 5.8S gene as an internal check (Cullings and Vogler 1998). The only study of the ITS1-5.8S-ITS2 complex in *G. aparine* did show variation in the 5.8S gene, which could be an indication of incorrectly identified species (Hübner et al. 2003). However, no known species populations were used in that study, and such reference populations are necessary to accurately distinguish *G. aparine* from *G. spurium* using sections of the rDNA complex.

2.3 *Galium* spp. in Western Canada

Several *Galium* species exist across Canada, but the two *Galium* species of greatest concern to agriculture are *Galium aparine* L. and *Galium spurium* L. Both are annual species that have the ability to overwinter in a vegetative state (Malik and Vanden Born 1988). Morphological similarity between these species has resulted in difficulties visually distinguishing them from each other. Both have ovate, petiolate cotyledons that are notched at the apex, pubescent leaves, square stems, whorled, sessile leaves, and a schizocarp fruit with two carpels

per flower (Malik and Vanden Born 1988). All flowers of *G. aparine* and *G. spurium* are also self-fertile, although little is known about their potential to form hybrids (Moore 1975). Figure 2.2 presents a key that can be used to differentiate between *Galium* species (Mersereau and DiTommaso 2003).

The overlapping vegetative and reproductive characteristics of *G. aparine* and *G. spurium* means observers need to consider multiple traits for proper identification. Moore (1975) developed the key in Figure 2.3 describing various characteristics that can be used when distinguishing these species. Consequently, the only current measure that most effectively distinguishes these troublesome species is chromosome counts. Although the chromosomes of each species have a similar karyotype, the number of chromosomes in each species (*G. aparine* n=11 and *G. spurium* n=10) can differ greatly depending on polyploidy (Malik and Vanden Born 1988). However, chromosome counts can be difficult to perform, prone to human error, and are very tedious to perform. Furthermore, the similar size and shape of *G. aparine* and *G. spurium* chromosomes has been shown to cause misidentification from poor preparations (Homeyer 1935; Moore 1975).

Key 1. Key for separation of the 15 species of *Galium* found in Canada and the Northern United States and which occur in regions occupied by *G. mollugo*

A. Fruit smooth to granular, with rounded to small sharp projections, but without hairs or bristles
- B. Stems erect or nearly so
  - C. Principal leaves 3-nerved, in whorls of 4; flowers white ..... *G. boreale*
  - C. Principal leaves 1-nerved, in whorls of 5 or more; flowers white, greenish or yellow
    - D. Leaves flat or slightly revolute; flowers white or greenish ..... *G. mollugo*
    - D. Leaves strongly revolute; flowers bright yellow ..... *G. verum*
- B. Stems weak and ± matted, ascending or reclining or scrambling
  - E. Leaves sharply pointed, the main ones in whorls of (5-)6 ..... *G. asprellum*
  - E. Leaves blunt or rounded to almost pointed, mostly in whorls of 4–6
    - F. Corollas 2-4 mm wide, mostly 4-lobed, the lobes longer than wide
      - G. Cymes repeatedly branched, bearing 5-many flowers; nodes glabrous ..... *G. palustre*
      - G. Cymes once or twice branched, bearing 2-4 flowers; nodes pubescent
        - H. Leaves ascending or loosely spreading, usually 2.5–6 mm wide; mature fruit 2–2.8 mm ..... *G. obtusum*
        - H. Leaves ± recurved or reflexed when mature, mostly 1.0–2.5 mm wide; mature fruit 1–1.8 mm ..... *G. labradoricum*
    - F. Corollas less than 2 mm wide, mostly 3-lobed, the lobes about as wide as or wider than long
      - I. Pedicels flexuous, curved at the tip, (6)8–20 mm long, densely minutely roughly hairy with hairs hooked backwards; leaves mostly in whorls of 4; flowers solitary (or 2) on peduncles; peduncles flexible ..... *G. trifidum*
      - I. Pedicels stiff, not curved at the tip, 3–8 mm long, glabrous; leaves mostly in whorls 4–6 (usually at least some 5-6 leaves); flowers mostly 2–3 on each peduncle; peduncles stiff ..... *G. tinctorium*

A. Fruit bristly or hairy
- J. Main leaves in whorls of 4; stems erect or ascending, not roughly hairy.
  - K. Flowers (and fruits), mostly sessile or subsessile, lateral on inflorescence branches
    - L. Leaves oval or elliptic, widest near the middle, apex obtuse ..... *G. circaezans*
    - L. Leaves lanceolate, widest below the middle, acute or acuminate. ..... *G. lanceolatum*
  - K. Flowers (and fruits) all on stalks, terminal on inflorescence branches
    - M. Leaves broadly obovate to broadly ovate-elliptic, margins glabrous; inflorescence diffuse, few flowered; pedicels (4)5–15 mm long; hairs of fruit hooked ..... *G. kamtschaticum*
    - M. Leaves lance-linear; margins ciliate; inflorescence dense, many flowered; pedicels 1-4 mm long; hairs of the fruit not hooked ..... *G. boreale*
- J. Main leaves in whorls of 6 or 8; stems prostrate or ascending; roughly hairy with hairs -hooked backwards
  - N. Perennial; stems glabrous or slightly pubescent; flowers 2–3 mm wide; leaves roughly hairy on the margins with hairs directed towards the tip, other hairs hooked backwards on the midvein beneath ..... *G. triflorum*
  - N. Annual; stems roughly hairy; flowers 1–2 mm wide; leaves roughly hairy with hairs hooked backwards on the margins and often the midvein beneath
    - O. Stem nodes glabrous to slightly pubescent; flowers 1.0–1.5 mm wide, greenish yellow; fruits 1.5–2.8 mm long (excluding spines) ..... *G. spurium*
    - O. Stem nodes usually covered with tangled or matted, woolly hairs; flowers 2 mm wide, white; fruits mostly 2.8–4.0 mm long (excluding spines) ..... *G. aparine*

Figure 2.2. A key to separate and identify the *Galium* species in regions of Canada and the United States where *Galium mollugo* is also found. Adapted from Mersereau and DiTommaso (2003).

KEY TO THE TAXA

Flowers greenish-yellow, 1–1.5 mm in diameter; pollen grains to 24 microns (μ) in polar diameter; fruits 1.5–2.8 mm long (excluding spines), fruits spiny or smooth; stem nodes glabrous or lightly pubescent, leaves narrow, linear-lanceolate, mucronate, firm textured, to 40 mm long, 3 mm broad

Fruits smooth..... *G. spurium* f. *spurium*
Fruits spiny..... *G. spurium* f. *vaillantii*

Flowers white, 2 mm in diameter, pollen grains 25–30 μ in polar diameter; fruits usually 2.8–4 mm long (excluding spines) but occasionally smaller, fruits spiny, very rarely smooth; stem nodes usually tomentose but sometimes almost glabrous; leaves linear-oblanceolate, lax, to 50 mm long, 5 mm broad

Fruits smooth or tuberculate, without spiny hairs..... *G. aparine* f. *intermedium*
Fruits spiny..... *G. aparine* f. *aparine*

Figure 2.3. Key to properly identify *Galium aparine* from *Galium spurium* using multiple morphological characteristics. Adapted from Moore (1975).

2.3.1 Galium aparine

The name *G. aparine,* or catchweed bedstraw, has traditionally been applied to all annual *Galium* plants with eight leaves per whorl (Moore 1975). While these plants do contain whorls of eight, other *Galium* species, such as *G. spurium,* can also produce eight leaves per whorl. Plants of this species are dark green, often highly branched, and have weak stems up to 1.2 m tall. Leaves are linear to oblanceolate in shape and are found in whorls of 6-8. The seed size of *G. aparine* is often larger than its congeners (>3 mm), and it has greenish-white flowers that are 2 mm in diameter, with pollen grains that average 25-31 microns; seed color can range from grey to dark brown, as seen in Figure 2.4 (Moore 1975). Four levels of ploidy have been identified in *G. aparine,* with hexaploids having 2N=6X=66 chromosomes being the most common (Kliphuis 1962).

G. aparine is distributed around the world, from temperate zones in the northern and southern hemisphere to higher altitudes in tropical regions (Holm et al. 1977). In Canada, woodland populations of *G. aparine* are classified as native plants, while more aggressive *G. aparine* populations found in crop fields were introduced from Eurasia. This species is very plastic, and can be found in woods, thickets, prairies, seashores, and wastelands (Correll and Correll 1972; Moore 1975).

Figure 2.4. Vegetative and reproductive characteristics of *Galium spurium* (A-D) and *Galium aparine* (E-H) grown in controlled greenhouse conditions. Photo credit to Dr. Sara Martin, AAFC.

2.3.2 Galium spurium

G. spurium, or false cleavers, differs from *G. aparine* primarily by its smaller seed size. Seeds of this species are often < 3mm in diameter. Compared with *G. aparine*, *G. spurium* is typically a greener, stiffer plant, with smaller (1-1.5 mm), greenish-yellow flowers (Figure 2.4). Leaves are often more linear in shape, possess small cotyledons, and are 'stickier' than *G. aparine* (Malik and Vanden Born 1988; Moore 1975). The phenotypic plasticity of this species allows for many of its characteristics to overlap with *G. aparine*, which makes it very difficult to separate them based on morphological characters (Moore 1975). Unlike the majority of the *Galium* species, *G. spurium* has a base number of 10 chromosomes (n=10) instead of 11 (n=11). The diploid number 2N=2X=20 is the only chromosome number reported for *G. spurium*. There is also no reported polyploidy in *G. spurium* plants, which is unlike *G. aparine* (Podlech and Dieterle 1969). The significant difference in chromosome number also influences pollen size. Pollen grains of *G. spurium* average 18-22 microns in diameter (Moore 1975).

G. spurium is not native to Canada, and was likely introduced from Eurasia into eastern Canada via contaminated seedlots (Moore 1975). It is widely distributed throughout Europe, but is not common in northern and western Asia (Hanf 1983). Plants of this species prefer open, lighted habitats with high moisture and fertility. They are also better adapted to dry, sunny habitats than *G. aparine*, which is why it is prevalent in crop production in Canada (Moore 1975; Malik and Vanden Born 1984). Further illustrating this, *G. spurium* is typically found on dark brown, black, gray-black, and gray wooden soils in Canada that are recognized for high fertility and adequate moisture (Malik and Vanden Born 1988).

2.3.3 Galium boreale

G. boreale L., also known as Northern Bedstraw, is a perennial plant species native to North America. It is commonly found in and around forested habitats, but can be found on a variety of other moist habitats such as rock ledges, stony ground, stream banks, and roadside ditches (Pratt et al. 2015). *G. boreale* is relatively easy to distinguish from other *Galium* species. It has numerous upright, smooth stems up to 76 cm tall, and narrow leaves two inches long that are often in whorls of four. Three prominent veins can be found on the leaves, and leaves lack the trichomes found on *G. aparine* and *G. spurium*. *G. boreale* spreads by a creeping rhizome and self-seeding of diaspores. Clusters of small white flowers are borne at the top of each growing point, and each flower produces a pair of schizocarps approximately 2 mm in length. These seeds are covered in soft hairs, but are not hooked like *G. aparine* and *G. spurium* (Pratt et al. 2015).

Northern Bedstraw is not normally considered a problematic species in crop production as it prefers shaded environments. It also is not a species that is typically consumed by livestock and other wildlife like moose and elk, but can be an early food source for black bears. Nevertheless, it often co-occurs with important ungulates (Pratt et al. 2015).

2.3.4 Galium mollugo

Introduced as an ornamental plant from Europe, *G. mollugo* (smooth bedstraw) has since adapted as a weedy species of pastures and meadows, but not annual cropping fields (Darbyshire et al. 2000). This species prefers moist, cool temperate habitats, but can tolerate drought. It is a very widespread plant and can be found from mountain ranges to prairie grasslands. Since its introduction to North America, *G. mollugo* has proven to be an effective invader of established

meadows and pastures used for animal production. It is unknown why animals will selectively graze around these plants, and such selective browsing further increases invasion by *G. mollugo* populations. *G. mollugo* is a long-lived perennial and like *G. boreale*, *G. mollugo* reproduces by vegetative rhizomes and seed. The stems are smooth in comparison to other *Galium* species and can grow up to 120 cm tall. Leaves formed from each node are in whorls of 5-8 (Mersereau and DiTommaso 2003).

In some cases, control of *G. mollugo* is warranted in pastures and meadows. Furthermore, *G. mollugo* and *Galium verum* L., another introduced species in Canada, often co-exist in close proximity and can naturally undergo introgressive hybridization that results in a very aggressive weed (Batra 1984). The rhizome root allows for the plant to survive contact herbicides like other vegetative reproductive weedy species, although these herbicides will reduce plant biomass. Some systemic herbicides, such as glyphosate, will provide a more permanent control solution (Parakh and Schreiber 1960). The most effective control measure is to use tillage or competitive forage species such as alfalfa to outcompete *G. mollugo* plants (Kinne 1955).

2.4 Ecology of Cleavers

2.4.1 Growth traits associated with competitive ability

There are several physiological and morphological traits that can influence the ability of weeds to compete with crops. The ability of weeds to germinate rapidly, emerge early with rapid leaf expansion, and the resulting subsequent canopy development give them a competitive advantage (Kropff et al. 1993). Plants that appear first and which can establish themselves quickly realize a significant advantage, as competition for many resources is asymmetric. Nevertheless, cropping systems have yet to take full advantage of this principle (Weiner et al.

2001). In addition to these physiological traits, research has shown that annual weeds associated with certain growth parameters are positively correlated with competitiveness. Roush and Radosevich (1985) evaluated *Amaranthus retroflexus* L., *Chenopodium album* L., *Echinochloa crus-galli* L, and *Solanum nodiflorum* Mill. in competitive replacement series experiments and found total plant biomass, leaf area, and leaf area ratio were key parameters associated with competiveness.

Differences in plant traits between species could be a possible explanation as to why some are more competitive or can escape control measures better than others. Structural plant characteristics of *Ipomoea* and *Jacquemontia* populations in the United States exhibited significant differences between accessions, which could be used to differentiate species (Bryson et al. 2008). Stems of hybrid, ivy-leaf, sharp-pod, and small-flower *Ipomoea* and *Jacquemontia* species are pubescent, which could inhibit pesticide uptake into the plant. Leaf characteristics of different species can also influence plant competitiveness. Leaf area in various *Ipomoea* and *Jacquemontia* species ranged from 5.8 to 155.2 cm^2, and leaf pubescence varied between 0, 50, and 90% (Bryson et al. 2008). The variation in plants traits between the *Ipomoea* and *Jacquemontia* accessions provides an explanation as to why some of these species become more prevalent than others. Other key weedy traits influencing plant competitiveness include high seed fecundity, broad germination requirements, dispersal over distances and into several habitats, rapid vegetative growth and flowering, and tolerance to unfavorable conditions, including competition (Bagavathiannan and Van Acker 2008). For example, feral rye is an escaped domestic plant that shows little seed dormancy at seed shed and typically germinates in a single cohort (Stump and Westra 2000). While dormancy and seedbank persistence is low, feral rye

populations can still reduce winter wheat yields by 50% (Westra and D'Amato 1989). It also persists as a serious weed in wheat-fallow rotations, where rye had not been planted for years.

Populations of *Galium* species in Europe have been shown to possess significant differences in morphological traits. Measurements of cotyledon size and shape, plant height, number of whorls per main shoot, number of leaflets per whorl, time to flowering, number of branches, dry matter production, and seed weight between populations all differed among various European populations (Hübner et al. 2003). In addition to morphological traits, minor variation was also detected in herbicide sensitivity, but none of the measured traits provided a reasonable explanation as to why cleavers were less problematic in Norway than in other European countries. Similar to Europe, cleavers populations in some areas of Canada are more prevalent than in others. Western Canadian maps showing the increase in cleavers abundance from the 1970's to the 2000's indicate that populations are more dominant in the northern parts of Alberta, Saskatchewan, and Manitoba (Beckie 2011). Over those thirty years, cleavers populations were rarely documented in southeast Alberta and southern Saskatchewan. Although the reasons for this are unknown, the competitive ability of various populations, differential herbicide susceptibility among populations, or variation in environmental conditions, could explain some of the differences in the abundance of this weed across Canada, especially the Prairies.

2.4.2 Dormancy and germination characteristics

The timing of weed emergence makes a significant contribution to the potential success of weedy species. Annual weeds are often more susceptible to herbicides at early growth stages; hence, understanding their germination and emergence patterns is important for management (Kusdk and Streibig 2003). Germination and emergence of plants is ultimately determined by the

inherent dormancy factors of the individual seed, such as seed coat permeability, embryo maturity, and presence of germination inhibitors within the seed. This allows weeds to adapt to their environment and ensure they can complete their lifecycle by providing temporal variation. For long-term weed control, it is important to understand and study these processes (Baskin and Baskin 1985).

Galium species in western Canada are believed to act as winter annuals or summer annuals (Malik and Vanden Born 1988). While lifecycles of winter and summer annuals are very different, there is still much research needed to determine if different emergence timings influence dormancy states, microsite requirements, phenology, plant vigor, or competitive ability (Cici and Van Acker 2009). Many traits including dormancy, emergence patterns, recruitment characteristics, and fecundity, are important to consider when developing long-term weed management strategies, but gaps in our understanding remain with regard to cleavers. Mennan and Ngouajio (2006) also note that long term weed management studies need more information on seed dormancy, persistence, seasonal germination, seedling emergence, and variation among species, populations, and communities. Emergence timing is very important in determining if a plant can compete with its neighbors, if it is consumed by herbivores or infected by diseases, and if it will complete its lifecycle.

Dormancy is the main characteristic influencing emergence under a range of environmental conditions. However, once dormancy is broken, final emergence is also dependent on microsite conditions; the environmental conditions surrounding the immediate area of the seed. The main conditions influencing emergence are soil temperature, water potential, light quality, and air quality (Forcella et al. 2000). The effect of temperature on plant emergence has been well-documented. Additionally, it has been concluded that temperature can be used directly

as a predictor of plant emergence via thermal time or growing degree days (Angus et al. 1981). Fluctuating temperatures can also have an effect on breaking seed dormancy. For example, seeds of *Sorghum halapense* L. remain relatively dormant unless they undergo 1-3 diurnal cycles of temperature fluctuations with an amplitude of 15°C (Benech Arnold et al. 1990). The effects of light availability on emergence are far less understood. Only 10-20% of seed in populations of *Amaranthus*, *Ambrosia*, and *Chenopodium* may require light during the emergence period (Gallagher and Cardina 1998). Light quality and air quality have not been fully investigated in regards to emergence. In general, when seed is stored in under 3% seed moisture, oxygen and water do not affect seed viability, but at moisture levels above 15%, seed longevity declined in anaerobic environments (Roberts and Ellis 1989).

Like many species, studies evaluating the dormancy, germination, and emergence timing of *G. aparine* and *G. spurium* are relatively limited. Early studies of *G. aparine* reported that seed could readily germinate in the dark, particularly if freshly harvested (Lauer 1953; Sjöstedt 1959). Seed that was at least a year old could tolerate weak light intensities, but intensities as low as 20% of full daylight still inhibited germination. The addition of nitrates enhanced germination in light-dormant seeds, but alternating temperatures between 10-30°C failed to increase germination (Sjöstedt 1959). Minimum and maximum temperatures for germination of *G. aparine* were believed to be 2°C and 20°C, respectively, with the optimum temperature for germination between 7-13°C (Lauer 1953). Oxygen levels of 6-8% are also required for germination of *G. aparine* seeds, with germination percentages increasing with increased oxygen content (Malik and Vanden Born 1988). Increasing oxygen concentrations up to 21% resulted in increased cleavers germination under both light and dark conditions (Boyd and Van Acker

2004). *G. aparine* germinates best in soils at a depth of 2-5 cm with 40-60% water holding capacity (Hirinda 1959).

In contrast to *G. aparine*, germination of *G. spurium* improves at a slightly deeper depth of 3-6 cm and in soils at 50-80% water holding capacity. The optimum temperature for germination of *G. spurium* is a constant 22°C (Malik and Vanden Born 1988), but alternating temperatures of 10-20°C, 15-20°C or 14-24°C were also conducive to germination. *G. spurium*'s response to light is similar to *G. aparine* such that inhibition of *G. spurium* germination can occur as a function of light intensity and duration. For example, Malik and Vanden Born (1987c) observed germination inhibition at low light intensities for a week and in full light exposure for 36 hrs. The authors noted that mature *G. spurium* seeds on the soil surface do not germinate unless covered with a thin layer of soil (Malik and Vanden Born 1987c). Other factors, such as nitrates, will also induce germination in photodormant seed if stored under dark conditions for at least 7 days. Germination of *G. spurium* was also enhanced by the green light effect, while red, blue, and far-red light were all inhibitory (Malik and Vanden Born 1987c).

The germination of a closely related species, *Galium tricornutum*, is also inhibited by light and can be stimulated by the addition of low concentrations of nitrates and gibberellic acid. The difference between *G. tricornutum* and *Galium* spp. is in the range in temperatures that allows for germination. *G. tricornutum* prefers cooler temperatures than its congeners, with a 13/7 °C day/night temperature regime (Chauhan et al. 2006).

2.4.3 Emergence and recruitment characteristics

Many agricultural practices, such as tillage, alter microsite availability thereby impacting seed germination and emergence (Spandl et al. 1998). Research on tilled and untilled plots found

that even shallow tillage can have a positive effect on cleavers seedling recruitment via vertical redistribution of the seed in the soil profile (Reid and Van Acker 2005). A light tillage operation caused the microsites to become slightly cooler, but there was no effect on soil moisture or soil bulk density. Their results suggest that vertical seed distribution from tillage has a greater effect on cleavers recruitment than a change in microsite conditions. Similarly, Boyd and Van Acker (2003) also showed that vertical seed distribution had a significant effect on cleavers emergence. Surface germination of cleavers was 20-27 times lower than buried seed (between 1-4 cm), regardless of constant or fluctuating moisture levels (Boyd and Van Acker 2003). Burying the seed, however, aids in cleavers recruitment by redistributing seed into dark environments, frequently exposing the seed to light with multiple tillage events may inhibit germination.

Plants of the *Galium* genus exhibit a high degree of plasticity in the timing of seed germination and emergence (Defelice 2002). Moreover, there appears to be a strong genotype by environment interaction with regard to the emergence of *Galium* seed (Royo-Esnal et al. 2012). Royo-Esnal et al. (2010a, 2010b, 2012) reported that the emergence percentage and timing of cleavers populations were related to temperature and rainfall, but the response of each population varied between years. Modelling of three *Galium* species showed lower temperatures (~ 3°C) had a positive effect on the emergence of *G. aparine*, *G. spurium* and *G. tricornutum*. Higher temperatures and low rainfall also induced more variation in species emergence (Royo-Esnal et al. 2010b). Rainfall and soil moisture were particularly important to the successful emergence of cleavers; populations of *G. spurium* were more sensitive to drought that *G. aparine* (Royo-Esnal et al. 2010b). Similarly, cold temperatures followed by a wet springs resulted in higher emergence of spring and autumn cohorts (Royo-Esnal et al. 2010a).

In contrast to European populations, two distinct ecotypes of *G. spurium* in Japan exhibited differences in germination requirements of seed (Masuda and Washitani 1992). The dormancy of spring emerging populations was broken by cold temperatures of 4°C, while secondary dormancy in autumn emerging populations was broken by temperatures of 25°C. These observations may also be true for cleavers populations in western Canada where winters are particularly harsh, and the moisture from snow melt provides abundant moisture in the spring. The increased observations of fall and spring emerging cleavers populations on the prairies suggests there could be multiple times of peak emergence that are not yet fully understood.

Many weed species in Canada emerge in spring and fall, including cleavers. Growers across Canada have observed emergence of cleavers in the fall, but limited research has been done to evaluate emergence timing and plant characteristics of fall emerging *Galium* species. Freshly shed cleavers seed can readily germinate and successfully overwinter to compete with spring-sown crops (Cici and Van Acker 2009). For example, fall emerging populations in Turkey produced seeds with two germination peaks each year, ranging from March to May and September to November (Mennan and Ngouajio 2006). In contrast, spring emerging populations in that study only had one period of peak emergence, while fall emerging populations were multimodal. The fall populations also had more unique emergence periods in that they germinated earlier, quicker, and more frequently than spring populations, suggesting they could have an increase in competitive ability with crops. Cleavers are also a problem in fall-seeded crops in Germany, with populations typically germinating in mid-September to mid-October (Taylor 1999). Fall emerging cohorts of many species, such as bluebur (*Lappula echinata)* and prickly lettuce (*Lactuca serriola* L.), produce significantly greater seed yields than spring

emerging plants (Malik and Vanden Born 1988). However, spring emerging cleavers have relatively lower seed production (300-1500 seeds plant^{-1}) in comparison to bluebur and prickly lettuce, and fall emerging population yields have not been documented.

2.5 Management of Cleavers

Cleavers affect 19 different crops in 31 countries (Holm et al. 1977). Recent weed surveys show that cleavers is increasing in abundance across western Canada and within individual fields. Cleavers are now the 9th most abundant weed in Prairie fields, up from number 30 in the 1980s (Leeson et al. 2005). A similar trend was observed in Saskatchewan canola fields, where cleavers are ranked number 6, up from 31 in the 1970s (Leeson 2012). Cleavers in canola are particularly troublesome for producers and canola crushers, as the similar size and shape of the seed make them nearly inseparable from canola seed. Cleavers contaminated canola seed can even cause depressions in the steel rollers during the crushing process, likely due to cleavers strong seed strength. The high abundance of cleavers, especially in canola, needs to be addressed to ensure contamination does not influence Canada's reputation for high quality canola products.

While most studies focus on the competitiveness of *G. aparine*, one study by Malik and Vanden Born (1987a) examined *G. spurium* infestations in rapeseed (*B. napus)* fields. Contamination of *G. spurium* in rapeseed fields led to reduced oil quality, harvesting difficulties, and yield losses of 18-38% (Malik and Vanden Born 1987a). *G. spurium* infestations that were present beyond 6 weeks after crop emergence reduced yield most significantly, thereby preventing weed emergence for 4 weeks after crop emergence. However, weed escapes still produced enough seed to contaminate the crop (Malik and Vanden Born 1987a). Seed that is not

collected by the combine is thrown out the back of the combine with chaff, which then further perpetuates the cleavers problem in later years.

Yield losses from *G. aparine* vary by crop type, weed density, and location. In England, winter wheat yields were reduced by 12-57% when *G. aparine* competed with wheat (Wright and Wilson 1987). Early research by Mennan (1998) showed it took only 10 plants m^{-2} of *G. aparine* to reduce wheat yields by 18% in Turkey. Similarly, 10% of wheat fields in Pakistan are infested by *G. aparine*, and densities of 18-72 plants m^{-2} reduced wheat yields by 4-32% (Aziz et al. 2009). Some cultural control measures can be implemented to increase crop yields by giving the crop an early advantage through asymmetric competition and increased weed suppression. Klem et al. (2014) recently studied this phenomenon between wheat and cleavers, where earlier and increased crop competition significantly decreased the maximum size and shape of the *G. aparine* growth curve. Cultural control practices, such as early seeding date, increased wheat yields in Pakistan (Aziz et al. 2009). Additionally, the use of competitive wheat cultivars and increased seeding rates also enable crops to better compete with cleavers (Mennan and Zandstra 2005). The Bezostaja wheat cultivar in Turkey was more competitive than other cultivars and reduced cleavers biomass and seed yield. All cultivars exhibited decreased height as weed density increased, although wheat yield losses were lower for more competitive cultivars.

Modern conventional cropping systems rely heavily on the use of herbicides to control difficult to control weeds, such as cleavers. Any non-selective herbicides, such as glyphosate, can be used to control cleavers in a pre-emergent or post-harvest burn application (Brenzil 2012). Aside from glyphosate- (RoundUp®) and glufosinate- (Liberty-Link®) resistant canola, the only in-crop chemicals registered for use are acetolactate synthase (ALS) inhibitors and auxin-type herbicides (Saskatchewan Ministry of Agriculture 2015). Timing of herbicides application is also

critical for efficacious control of cleavers. If a herbicide is applied too early, weeds can emerge after the crop. However, if the herbicide is applied too late, the weeds could compete with the crop early in its life cycle, and may be past the stage of optimal control for herbicides.

The critical period of weed control (CPWC) is defined as the period during the crop's life cycle that needs to be kept weed-free to prevent a specific level of yield loss (Nieto et al. 1968). The CPWC in canola is believed to be between the 4- to 6- leaf stages (Martin et al. 2001), and in peas weed removal should take place during the first two weeks of pea emergence (Harker et al. 2001). However, the emergence of cleavers populations has not been well documented in Canada and therefore, understanding the periodicity of emergence of this species would aid in more effective implementations of control strategies. This was shown by Klem et al. (2014), who noted that *G. aparine* that emerged 9 and 14 days after wheat produced 40 and 60% less biomass, respectively. The weed seedbank exhibits unpredictable and variable emergence periodicity that influences weed populations and densities in the field (Forcella 1992). In most cases, emergence is not normally distributed around the time it takes to reach 50% emergence (Forcella et al. 2000). With irregular emergence there is a narrow window to achieve optimal control with herbicides when targeting cleavers; often this is between 1-4 whorls but this depends on the herbicide product (Domaradzki 2006; Lutman et al. 1988). Given the complex interplay of variables that collectively influence emergence timing, understanding the best timing of measures for control is difficult. Thus, elucidating the emergence timing of cleavers is key to better managing this weed, even if effective herbicides are available.

The over reliance on ALS and auxin-type herbicides for cleavers control has resulted in the development of resistance (Beckie et al. 2012; Hall et al. 1998). A >6.7 and >14 fold level of resistance to quinclorac and ALS herbicides, respectively, has been identified in *G. spurium*

populations in western Canada (Hall et al. 1998). The resistance to ALS herbicides can be caused by the $Trp_{574}Leu$, $Ser_{653}Asn$, and the $Asp_{376}Glu$ mutation within the ALS gene (Beckie et al. 2012). Characterization of quinclorac resistance in *G. spurium* by Van Eerd et al. (2004) showed that resistance is facilitated by a recessive allele at a single nuclear locus. A recessive trait in a highly self-pollinating species like *G. spurium* can support the accumulation of resistant individuals in a population, but limited spread of the trait occurs due to the lack of outcrossing (Jasieniuk et al. 1996). Additionally, cleavers are considered to be one of the species at risk to develop resistance to glyphosate if growers continue to overuse this herbicide for weed control (Beckie et al. 2013). This is primarily due to the high abundance of cleavers on the Prairies (especially the black soil zone) and the high selection pressure induced by multiple applications of glyphosate each year.

Unfortunately, the focus of successfully controlling cleavers has led to significant oversights in species biology and identification. There are clear misunderstandings of genetic and environmental influences on individual populations, such as *G. aparine* and *G. spurium*, which could have aided in their dramatic increase in abundance over the past 30 years. By determining the presence or absence of particular *Galium* species and the plasticity of their emergence timing and biological characteristics, we can begin to understand their success and hinder further expansions. Thus, the overall objective of this thesis is to provide information of the current *Galium* situation in western Canada that can be used to improve the efficiency of current control practices.

3.0 Evaluation of the ITS Region to Distinguish *Galium* Species and Relatedness of Western Canadian Populations

3.1 Introduction

Galium aparine L. and *Galium spurium* L. are weed species that have proven to be problematic in field crops. For example, *G. spurium* infestations in Canadian rapeseed fields caused yield losses of 18-38% (Malik and Vanden Born 1987a). Winter wheat yields were reduced by 12-57% in the United Kingdom (Wright and Wilson 1987) and 4-32% in Pakistan (Aziz et al. 2009) due to competition from *G. aparine*. Both species have shown significant impacts on crop yield, but the extent of losses due to the *Galium* complex of cleavers populations in western Canada is unknown. In addition, *Galium boreale* L., another member of the *Galium* genus, is often found around field edges and may serve to facilitate gene flow between species, thus potentially serving as a reserve for traits such as herbicide resistance (Moore 1975). Resistance to ALS (Group 2) and auxin-type (Group 4) herbicides has already been identified in *Galium* species in Canada (Hall et al. 1998); furthermore, cleavers species are believed to be one of the next weeds that develop resistance to glyphosate (Beckie et al. 2013).

G. boreale is easy to differentiate from its *Galium* counterparts as it has a short stature, four leaves per whorl, and a perennial lifecycle (Pratt et al. 2015). *G. aparine* and *G. spurium* are more difficult to distinguish visually, as many of their distinguishing characteristics overlap. For example, *G. aparine* has whitish flowers 2 mm in diameter, seeds 2.8-4 mm long, and linear leaves that are oblanceolate and up to 50 mm long and 5 mm wide. *G. spurium*, on the other hand, has similar traits including greenish-yellow flowers 1-1.5 mm in diameter, fruits 1.5-2.8 mm long, and narrow leaves that are linear to lanceolate in shape (Malik and Vanden Born 1988; Moore 1975). Such small differences in morphological traits would suggest that these species are unlikely to be differentiated by producers or agronomists, which can create challenges to

recommending effective and efficient control options. Additionally, field surveys enumerating cleavers (Leeson et al. 2005, Leeson 2012) and research papers assessing cleavers management do not take any measures to distinguish between *Galium* species (Malik and Vanden Born 1978a; Sapsford et al. 2011). Therefore, potential differences in the response of each species to herbicides and other weed control measures are currently undocumented. While both cleavers species may share common vegetative traits, the potential for different species to exhibit dissimilar competitive abilities also necessitates accurate identification.

Currently, the only method of discriminating between *G. aparine* and *G. spurium* is by chromosome counts followed by measurements of ploidy. *G. aparine* is commonly found as a hexaploid (Podlech and Dieterle 1969) or tetraploid with a chromosome count of 2n=66 or 2n=44, while *G. spurium* has chromosome number 2n=20, with no record of polyploidy (Malik and Vanden Born 1988). While these methods can successfully identify species, they are time consuming, logistically challenging, and resource intensive. Additionally, chromosomes of most *Galium* species are very small and are nearly identical between species, which can make the process tedious and methodologically challenging. However, it may be possible to develop a molecular marker to discriminate these species quickly and economically using real-time PCR techniques.

The internal transcribed spacer (ITS) regions are located in the nuclear genome between ribosomal DNA genes. The ITS region has many distinct features that lend itself well to the identification of species. Benefits of choosing the ITS region include: 1) it is small in size and highly conserved within species, 2) it is highly repeated in the nuclear genome, and 3) copies are uniform throughout the genome (Baldwin et al. 1995). Additional features such as biparental inheritance, intragenomic uniformity (within a plant) and intergenomic variability (between

populations or species), and low functional restrictions are useful for distinct species identification over interspecific relationships between species (Álvarez and Wendel 2003). The ITS1 region in one species, *Stylosanthes guianensis*, has been used to detect variation in the *Stylosanthes* species complex. The results showed that variation in the ITS1 region was successful in differentiating *Stylosanthes guianensis* genotypes. Similarly, the ITS region of *Galium* species has previously been sequenced in European populations (Hübner et al. 2003). Significant amounts of variation were found between various populations, and a dendrogram showed two distinct groups of *Galium* population that may be different species. However, a major limitation of this study was that no known *Galium* reference samples were used in the assessment of species variation, and the authors did not distinguish between *Galium* species. Therefore, the primary objective of this study was to identify one single nucleotide polymorphism (SNP) marker using variation within the 18S-5.8S-28S ribosomal DNA region to distinguish *G. aparine* from *G. spurium.* Once a marker had been identified, a second objective was to develop a SNP based marker assay (eg. TaqMan) that could be used to assess species composition and relatedness of *Galium* populations from across western Canada. It is hypothesized that variation detected in the ITS1-5.8S-ITS2 complex could differentiate *G. aparine* from *G. spurium*, and that Canadian populations are a mixture of both species.

3.2 Materials and Methods

3.2.1 Plant material and flow cytometry

Reference populations consisting of known *G. aparine* and *G. spurium* were obtained from the University of Manitoba. These samples were originally purchased from Herbiseed in Twyford, England in 2001. Additional *G. aparine* seed was also purchased from Herbiseed in 2012, but was proven to be contaminated with *G. spurium* and thus, was not used in the study.

Galium boreale plant material was collected in August of 2014 from northern Saskatchewan (53°23'10.1"N 108°06'56.6"W) and stored at 4°C for 3 days until DNA extraction. Canadian cleavers populations were collected from random locations across various regions from seed cleaners or crop research stations (Table 3.1, Figure 3.1).

Table 3.1. Canadian cleavers populations to determine species complex

Name	GPS Coordinates
[a]Lacombe	52°27’17.3”N, 113°44’55.6”W
[a]Vegreville	53°34’17.5”N, 112°00’11.6”W
[a]Carrot River – Clancy	53°16’46.1”N, 103°35’4.6”W
[a]Saskatoon – SPG	52°3’49.3”N, 106°26’38.4”W
Moosomin	49°59’47.1”N, 101°53’39.1”W
[a]Melfort - Heavin	52°52’15.6”N, 104°36’42.1”W
[a]Melfort- Trawin	52°52’8.5”N, 104°31’11.4”W
Yorkton	51°38’13.2”N, 102°25’47.28”W
Ontario	N/A
Manitoba	N/A

[a] Canadian samples that were sequenced with reference populations
N/A – Not Available

Figure 3.1. Map of western Canada marked with locations of collected *Galium* populations for molecular and TaqMan analysis.

To ensure the *G. aparine* and *G. spurium* reference populations were congeners but not conspecific, flow cytometry was conducted in 2015 at Agriculture and Agri-Food Canada. Flow cytometry was not done on Canadian populations as their purpose was to evaluate the success of the chosen marker. It should be noted that chromosome counts and flow cytometry had previously been performed on the reference (2001) samples of *G. aparine* and *G. spurium* in a previous study (Reid 2003). Results from Reid's (2003) study confirmed reference samples were pure for *G. aparine* and *G. spurium.*

Plant tissue, approximately 1 cm in diameter, was collected from the ends of shoots and stored on ice until chopped with a razor blade in 750 µL of Galbraith extraction buffer (Doležel and Bartos 2005). The chopped material was then aspirated in a pipette and filtered through a 30 µm nylon filter into a 5 mL round-bottom tube on ice. Propidium iodide (250 µL of a 100 µg/mL solution) was added to the samples which were then incubated for 30-40 minutes at 4°C before being run on a Gallios flow cytometer. The internal standard was *Raphanus sativus* L., which has a DNA content of 1.1 pg/2C (Doležel et al. 2007). Relative DNA content was determined using a fluorescence area (585/42 nm detector) and fluorescence peak means. DNA content of the nuclei was calculated as:

$$DNA\ content\ (pg) = \frac{Mean\ of\ Sample}{Mean\ of\ Standard}\ x\ DNA\ content\ of\ Standard$$

3.2.2 DNA extraction and PCR

Prior to DNA extraction, five plants of each reference population and ten plants of each Canadian population were grown in a controlled growth chamber at the University of Saskatchewan in 11 cm diameter pots filled with a soil-less mix (Sunshine Mix 3, Sun Gro Horticulture). Seeds were germinated in 24 hr darkness at subsequent temperatures of 10°C for 8

hrs and 15°C for 16 hrs. After germination, plants were established under a 16 hr photoperiod at 18°C/12°C day/night cycle. Plant material was harvested at the two-whorl stage 4-6 weeks after planting, and stored on ice before DNA extraction.

DNA was extracted from the five plant samples of each *Galium* spp. reference population, and the ten plant samples of each Canadian population using a modified version of cetyltrimethyl ammonium bromide (CTAB) extraction from Procunier et al. (1990). Plant tissue was ground using blue micro pestles in a 2 mL microtube. Ground tissue was incubated in (65°C) CTAB extraction buffer and purified via chloroform/isoamyl alcohol (24:1). Ethanol precipitated DNA was re-suspended in distilled water, quantified, and normalized before being stored at -20°C.

The target ITS1-5.8S-ITS2 complex was isolated from the cleavers ribosomal DNA using polymerase chain reaction (PCR) with the ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers as designed by White et al. (1990) (Figure 3.2). PCRs were prepared to a total volume of 25 µL, with a final concentration of 1X Taq Buffer, 2.0 mM $MgCl_2$, 100 µM of each dNTP, 200 nM of each primer, 1 unit of Taq Polymerase, and approximately 50 ng of DNA template. PCR reactions were run on a GeneAmp 9700 thermal-cycler machine under the following conditions: 94°C for 45 s, 65°C for 45 s, and 72°C for 1 min for 35 cycles after the DNA was initially denatured at 94°C for 3 min. PCR products were separated and visualized on 1.2% agarose, 0.5X TBE gels. Five µL of PCR reactions were saved for cloning.

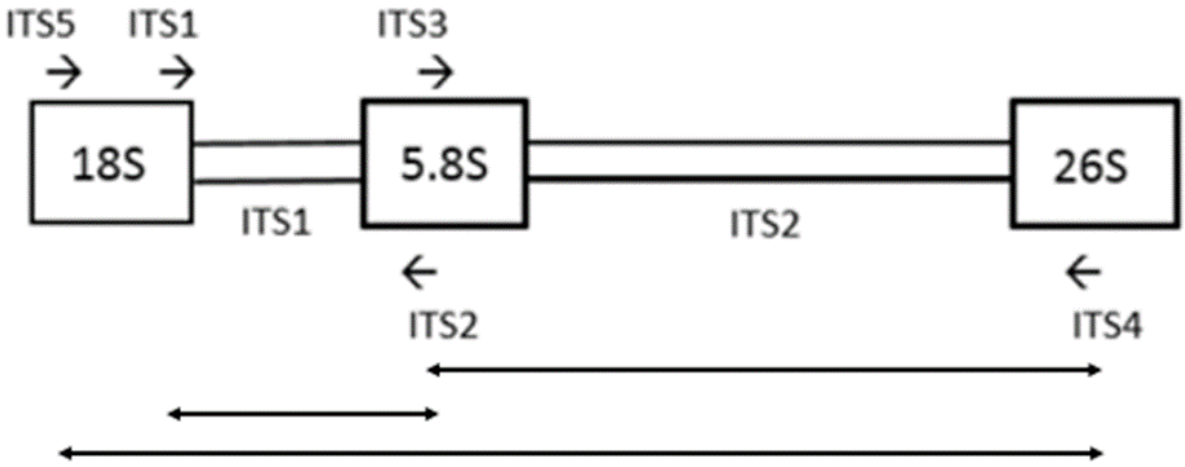

Figure 3.2. The ribosomal DNA region of biological organisms. Long arrows identify fragments that can be amplified using PCR. Short arrows indicate locations of primers use for PCR amplification.

3.2.3 Cloning, sequencing, and marker selection

Cloning and transformation were carried out using a TOPO TA Cloning Kit and One Shot TOP10 *E. coli* Chemically Competent Cells (Invitrogen Canada Inc, Burlington, Ontario). Ligation was achieved with saved PCR product, salt solution, and TOPO vector provided by the kit. Ligation reactions were transformed into competent *E. coli* cells by heat shocking at 42°C for 30 seconds. Cells were incubated at 37°C for 1 hr for recovery and initiation of the ampicillin resistance gene, and spread on individual plates comprised of LB agar and ampicillin (100 μg/mL). Plates were incubated at 37°C overnight. Three colonies from each plate were selected and individually grown in LB-AMP broth in a shaking incubator set at 37°C for 24 hrs. Each culture was checked for the presence of the ITS fragment using PCR and M13 primers. Plasmid preparation for sequencing was done as instructed using QIAprep Spin MiniPrep Kit (Qiagen, Mississauga, Ontario). DNA content of each sample was measured using a spectrometer to ensure there was an appropriate amount for sequencing. Samples were then sent to Eurofins Genomics (Louisville, Kentucky) for DNA sequencing via the Sanger method (Sanger et al. 1977).

Sequences were aligned using software Geneious 6.1.8 (Biomatters Ltd., Auckland, New Zealand). The DNA sequence of each plant's ITS copy was extracted from the bacterial DNA sequence and then aligned with the two other ITS copies to determine a consensus sequence for each plant to eliminate error variation potentially introduced during PCR and sequencing. Consensus sequences for each species were aligned using individual plant sequences and variation within populations was identified. Consistent SNPs were identified between the reference population sequences, and a point of variation in the 5.8S gene was selected for use as

a molecular marker. The sequenced populations were also compared to the reference *G. aparine* and *G. spurium* sequences to discriminate between species of each population.

3.2.4 TaqMan for screening of Canadian populations

A real-time PCR assay (TaqMan) was developed, which was comprised of MasterMix, distilled water, and assay (Applied Biosystems, Burlington, Ontario). DNA was extracted using a modified version of alkali treatment extraction outlined by Klimyuk et al. (1993). A 3 mm piece of plant tissue from leaves of the first whorl was placed in individual wells in a microplate and used to extract DNA. After samples were harvested, 40 μL of 0.25M NaOH was added and mixed with the tissue at 1650 rpm on a shaker. The microplate was placed in a heating block for 45 s and moved to room temperature before adding 60 μL of 0.5 Tris-HCL. The microplate was mixed at 1250 rpm and then placed on the heating block for another 3 minutes. The plate was then removed and placed on ice before being added to the assay. The assay was first tested on the previously sequenced reference samples and then on the DNA of the Canadian populations to confirm it could successfully differentiate *G. aparine* and *G. spurium*, before being tested on new plant tissue.

3.3 Results

3.3.1 Characteristics of the internal transcribed spacer sequences of Galium species

Flow cytometry results on the previously identified *G. aparine* and *G. spurium* populations from Herbiseed indicated two different types of *Galium* species. The higher DNA content in the *G. aparine* is indicative of polyploidy that is common in *G. aparine* populations. The work previously done by Reid (2003) and additional flow cytometry during this study by Dr.

Sara Martin are sufficient for the proper identification of *G. aparine* and *G. spurium* samples to be used as reference populations for identifying a SNP marker in the ITS region (Table 3.2).

Table 3.2. Mean DNA content (pg/2c) within plant cells of reference populations obtained using flow cytometry.

Plant ID	*G. spurium* 2001 (Reid)	*G. aparine* 2001 (Reid)	*G. spurium* 2001 (Martin)	*G. aparine* 2001 (Martin)
1	68	167	73	196
2	72	165	75	193
3	69	171	75	190
4	71	161	75	201
5	67	155	76	N/A
Mean	69	164	75	196
Index	1.00	2.38	1.00	2.61
Designation	Diploid	Tetraploid	Diploid	Tetraploid

The ITS1-5.8S-ITS2 complex of the three *Galium* species was amplified using the ITS1 and ITS4 primers designed by White et al. (1990). The ITS region of all the species were amplified, cloned, and sequenced successfully, producing products between 672-741 bp in length. The conserved nature of the 5.8S gene was used to align the boundaries of the ITS1 and ITS2 spacer regions for comparison. The 5.8S gene was 138 bp for the three species, and the ITS1 region was 210, 201, and 221 bp in *G. spurium*, *G. aparine*, and *G. boreale*, respectively. The ITS2 region was 332-333 bp in *G. boreale* and *G. aparine*, but was much longer, 392 bp, in *G. spurium*. Little to no sequence variation was detected within each species. Since the primary objective was to find consistent variation between species, variation within species was eliminated by constructing a single consensus sequence using the most common nucleotide at variable positions. Alignments showed 82.8% pairwise identity between all three species, which implies there is approximately 17% sequence variation. The similarity between *G. aparine* and *G. spurium* was higher at 87.3%, suggesting that they are more closely related to each other in comparison to *G. boreale*.

Variation between species was found primarily in the ITS2 region. There were 20 polymorphisms between *G. aparine* and *G. spurium* and 49 sites of variation between *G. boreale* and the other species throughout the ITS2 region. *G. aparine* and *G. spurium* had 24 variable nucleotide sites between each other, and *G. aparine* had 70 nucleotide deletions. *G. boreale* had 91 sites of variation from the other species, which also included a substantial number of deletions. Because the majority of the variation between *G. aparine* and *G. spurium* occurred in the ITS2 region, this suggests that it is a practical region for the development of a marker.

In contrast, the ITS1 region exhibited only 3 nucleotide differences between *G. aparine* and *G. spurium* at positions 72, 108 and 171. *G. boreale* exhibited substantial variability in the

ITS1 region compared with the other species, which was similar to that observed for the ITS2 region. There were 41 nucleotide differences between *G. boreale* and the other species in the ITS1 region, although the nucleotides matched those of *G. spurium* at positions 72, 108, and 171.

Unexpectedly, the 5.8S gene also showed variation between the three different species, which is uncommon as this is a highly conserved region that codes for the 5.8S ribosomal units in plant cells. *G. boreale* exhibited two sites of variation at positions 238 and 359 that differentiate it from *G. aparine* and *G. spurium*. A SNP at position 352 in the 5.8S gene distinguished *G. aparine* and *G. spurium* from one another. The highly conserved nature of the 5.8S gene makes it an ideal candidate over the ITS1 and ITS2 regions for a marker, as this variation is often species specific and less predisposed to evolutionary changes.

3.3.2 TaqMan Assay

The [A/G] SNP detected in the 5.8S gene at position 352 was used for the TaqMan assay to discriminate between *G. aparine* and *G. spurium* (Figure 3.3). A SNP within the rDNA was chosen because these genes are highly conserved across taxa and very useful for phylogenetic relationships (Baldwin et al. 1995). To confirm the TaqMan assay would select for individual species, the assay was run on the earlier sequenced DNA samples of the reference populations (5 plants per sepecies). The assay ran successfully and an additional 25 plant samples from the Canadian *Galium* populations were collected for analysis. The results of the TaqMan analysis showed that all plants sampled from numerous populations collected across western Canada were *G. spurium*. None of the plants from any of the populations contained the SNP associated with *G. aparine*.

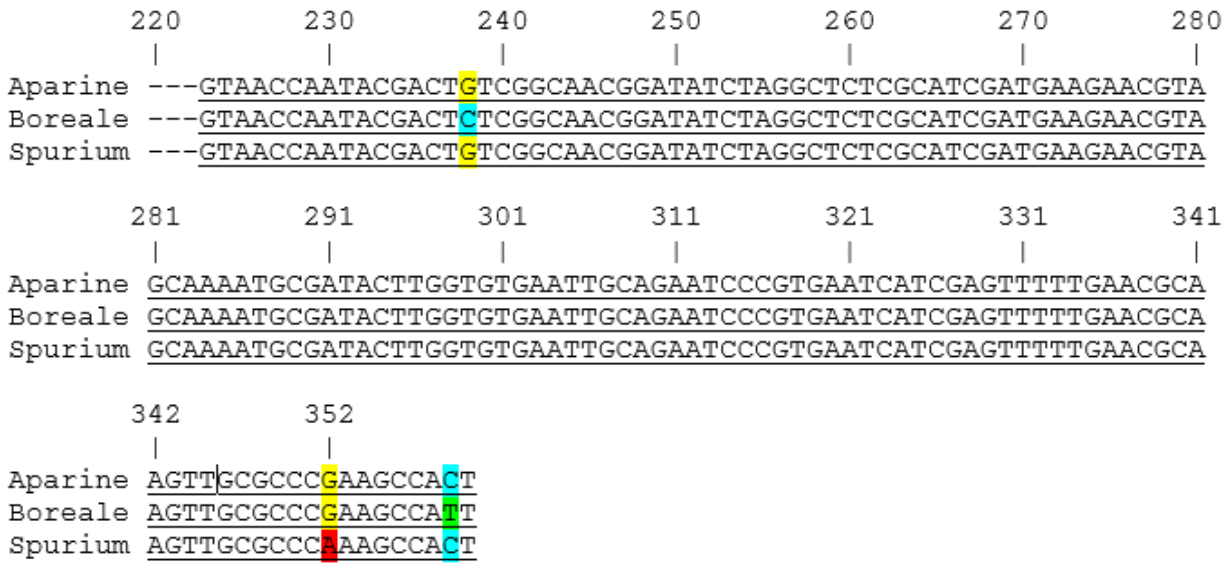

Figure 3.3. 5.8S Gene of the three *Galium* species that shows SNPs used to differentiate the species via TaqMan Assay screening. Colors correspond to individual nucleotides (Red-A, Yellow-G, Blue-C and Green-T)

3.3.3 Sequences of Canadian Galium field populations

Sequencing of samples from the Canadian populations resulted in various ITS lengths from 740 -744 bp long, similar to that of *G. spurium*. Little to no variation was found between individual plants of each population and therefore, sequences were combined to give one consensus sequence for population comparisons. Very little variation occurred within ITS1 and ITS2 between the populations, which suggests that they are highly related. One point of variation at position 189 in the ITS1 region linked the Lacombe and Moosomin populations in similarity to each other. Populations Clancy, Heavin, and Lacombe had similar variation at positions 410, 411, and 441, while Manitoba, Trawin, and Yorkton had the same SNPs at positions 404 and 437 in the ITS2 region. Other areas of variation at positions 428 and 670 linked SPG, Trawin, and Yorkton and Clancy and Moosomin, respectively. No differences were found between any of the populations within the 5.8S gene, and all species exhibited an identity consistent with *G. spurium* (Figure 3.4.). Additionally, the Canadian populations matched *G. spurium* at numerous locations of variation between *G. aparine* and *G. spurium* in the ITS1 and ITS2 regions, including larger insertions, which further supports that cleavers populations on the prairies are likely *G. spurium.*

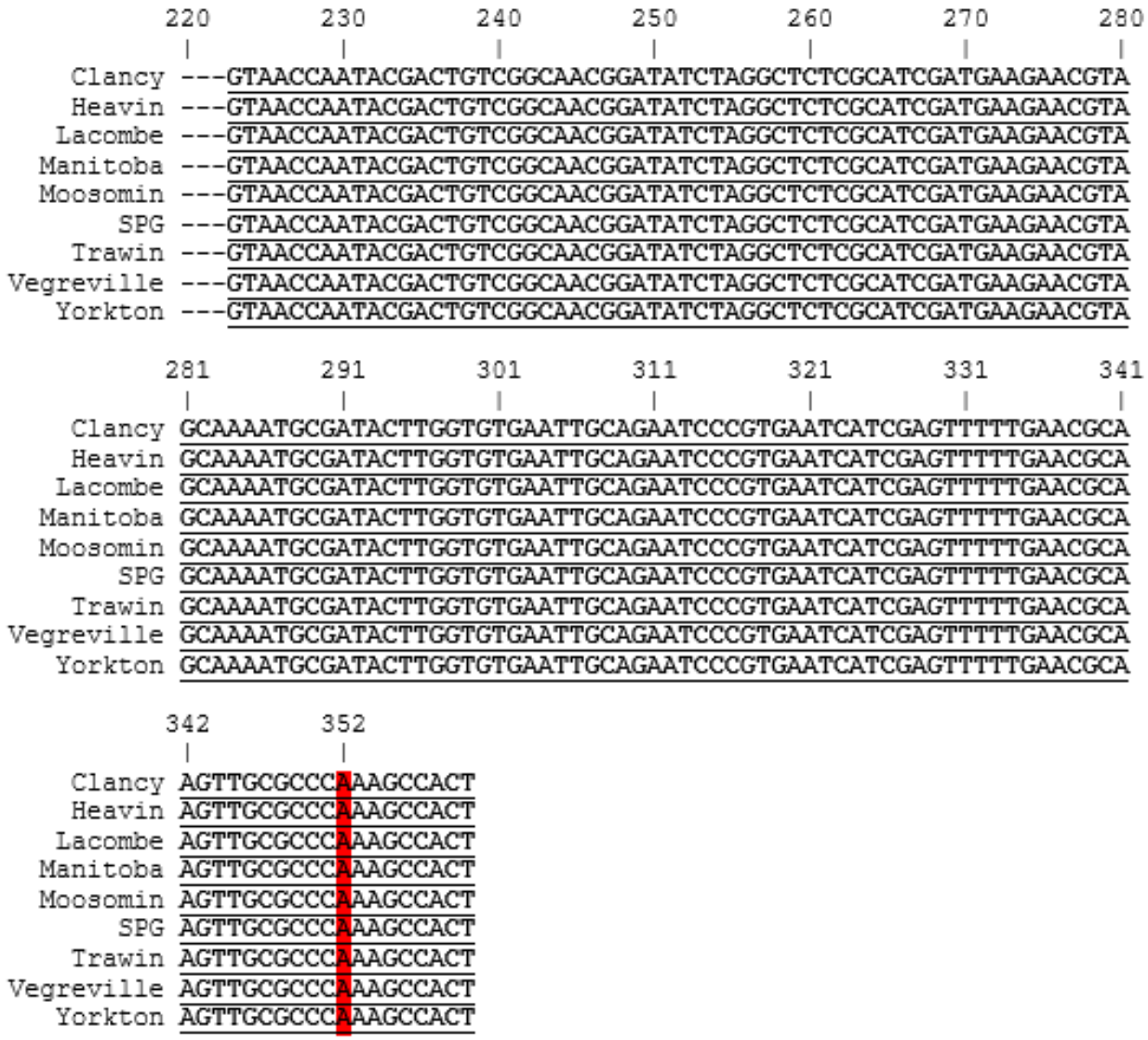

Figure 3.4. 5.8S Gene of the Canadian *Galium* species that were sequenced showing nucleotide at position 352 is consistent with the 'A' SNP in the *G. spurium* species.

The final consensus sequences of the three reference *Galium* species and sampled plants from the western Canadian populations were separated in a dendrogram that depicts the relatedness of populations to each other using genetic variation (Figure 3.5). Twenty additional designated *G. aparine* European population sequences (designated B-: Belgium, G: Germany, N-: Norway, and S: Sweden) obtained from Hübner et al. (2003) were also included in the dendrogram. The degree of variation between the reference populations separated the dendrogram into three groups; *G. aparine, G. spurium,* and *G. boreale.* All of the collected western Canadian populations included in this study grouped with *G. spurium*. Within the *G. spurium* group, none of the western Canadian populations grouped across provincial boundaries. In contrast to the western Canadian populations, half of the European sequences from Hübner et al. (2003) were grouped with *G. aparine* and the other half were grouped with *G. spurium*.

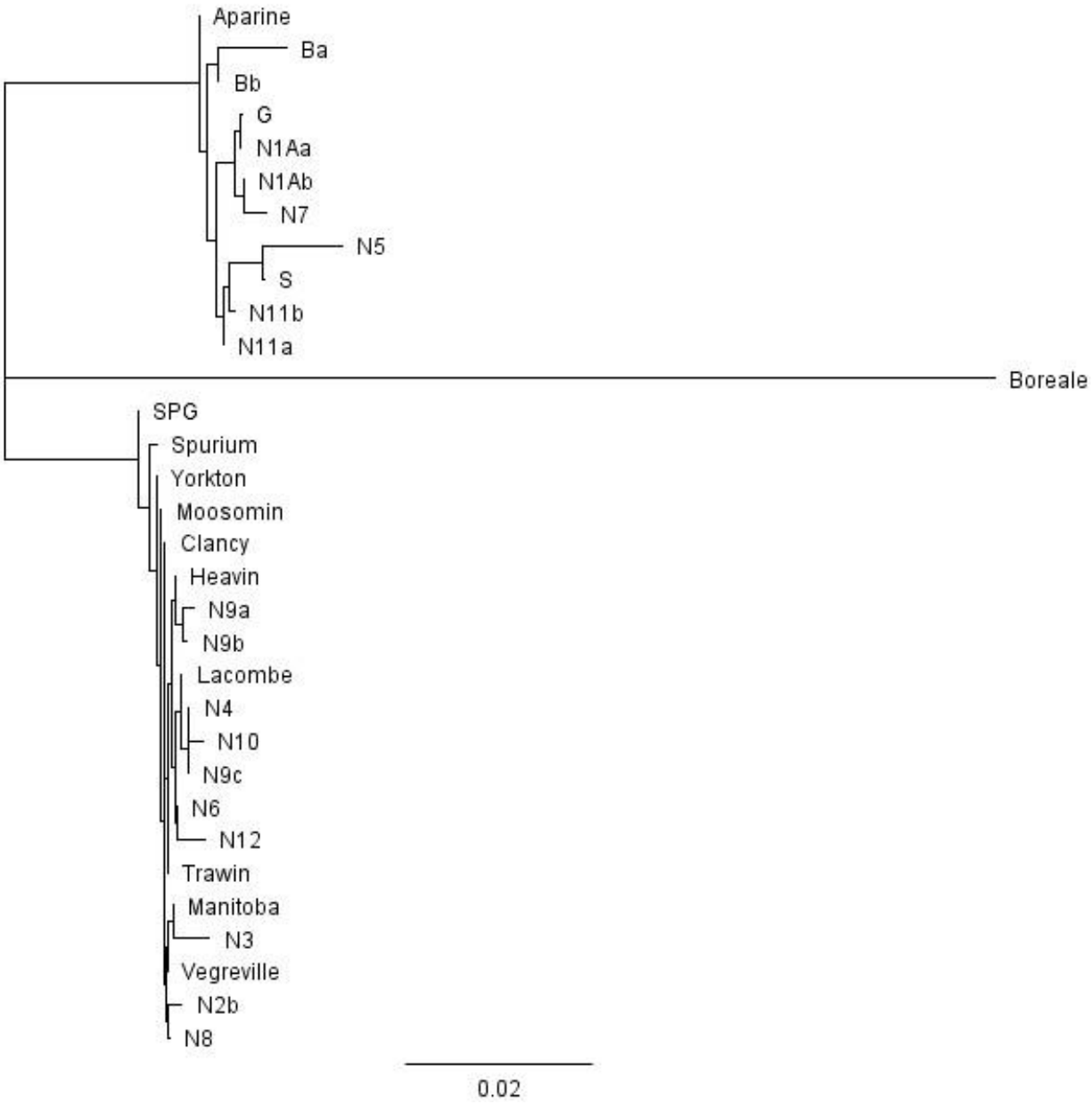

Figure 3.5. Dendrogram created using genetic variability in the ITS1-5.8S-ITS2 complex between the reference populations, Canadian cleavers populations and designated (B-Belgium, S-Sweden, G-Germany, and N-Norway) *G. aparine* populations from Europe (Hübner et al. 2003) detected by Geneious 6.8.1. The three *Galium* spp. of interest separate out from each other, while the Canadian populations all correspond with *G. spurium.* The European populations separated with *G. aparine* and *G. spurium.*

3.4 Discussion

The significant differences in the ITS regions of *G. boreale* from *G. aparine* and *G. spurium* were not unexpected given their visual and behavioural differences. It is relatively simple to differentiate *G. boreale* from other *Galium* species; therefore, the objective was to select a molecular marker to distinguish *G. aparine* and *G. spurium* from each other as the visual similarity between *G. aparine* and *G. spurium* make them hard to differentiate in field environments. Currents methods to separate the species are often inaccurate and tedious to perform, hence the need for quicker, more efficient methods. To find a simpler way of identifying species, the ITS region in 5 plants each of *G. aparine*, *G. spurium*, and *G. boreale* were used for genetic analysis. Previous studies (Hsiao et al. 1995; Hübner et al. 2003) have used fewer plants to investigate genetic variation, but we used five plants to reduce random variation and error in our analysis for a more conclusive marker. Since *G. aparine* and *G. spurium* are difficult to separate, the identity of references samples were confirmed using chromosome counts and flow cytometry in previous work by Reid (2003).

White et al. (1990) initially suggested that the ITS complex could be used to differentiate and characterize closely related species or populations of the same species, and this appears to be true for distinguishing *Galium* species. The variability in length (672-744 bp) of this region initially suggests that the ITS complex could be used to differentiate species, but these small differences in length may not be discriminatory enough to verify species identification via PCR and gel electrophoresis. Extensive research within the ITS region of flowering angiosperms has shown that the ITS1 spacer is between 187 to 298 bp, ITS2 spacer is 187 to 252 bp long, and the 5.8S gene was 163 or 164 bp long (Baldwin et al. 1995). Together, the total length of the ITS1-5.8S-ITS2 complex is normally under 700 bp. *G. aparine* and *G. boreale* sequences from our

study fit the general description of the angiosperm ITS region, with the exception of a shorter 5.8S gene. *G. spurium*'s ITS complex length was 40 bp over the pre-determined angiosperm length, and the insertion appears to be in the ITS2 spacer. This could be specific to *G. spurium*, or characteristics of the ITS complex of angiosperms could have evolved since last evaluated. Aside from the insert in the ITS2 region, the nucleotide variation between *G. aparine* and *G. spurium* was generally in the ITS2 region. The trend of high variation in the ITS2 region was also observed in species of the *Asteraceae* family (Baldwin 1993) and in European *G. aparine* populations (Hübner et al. 2003).

The spacer regions (ITS1 and ITS2) evolve quickly and often differ between species within a genus and even within some populations (Baldwin et al. 1995). The variation found in these regions is ideal for evaluating evolutionary proponents, but the specificity needed to identify species is stronger in rDNA. Nuclear small-subunit rDNA (18S, 5.8S, and 28S) sequences are useful for studying closely related species, as these regions evolve slowly and variation is unique (White et al. 1990). Furthermore, variation in the 5.8S gene is unusual within a single species, which makes it an ideal candidate target site for molecular markers (Hübner et al. 2003). Hence, the SNP between *G. aparine* and *G. spurium* at position 352 within the 5.8S gene was used for the TaqMan assay to determine the species complex of the various Canadian cleavers populations.

Sequences of the Canadian populations had little variation in the ITS1 and ITS2 regions and no variation in the 5.8S gene when compared to the complexity of the reference populations. Additionally, comparing points of distinction in the whole ITS1-5.8S-ITS2 region between *G. aparine* and *G. spurium* to the Canadian sequences showed that all sampled field populations were comprised of *G. spurium*. Previous molecular work on *G. aparine* used the ITS complex to

evaluate population variation (Hübner et al. 2003). European *G. aparine* populations showed a lot of variation between populations, and then could be subdivided into different accessions for analysis. Variation between the Canadian populations in our study was considerably lower than in the European populations as evidenced by tight dendrogram clustering of the *G. spurium* group (Figure 3.6). The European populations in the *G. spurium* group were also tightly clustered, while populations in the *G. aparine* group had greater variation throughout the ITS complex. The base substitution variation in the 5.8S gene used for our marker was also variable in the *G. aparine* species in Europe (Hübner et al. 2003). However, there was no evidence of identification measures or reference samples used in the European study and therefore, it is possible that European populations contain a mixed species complex.

Differentiation between populations in the dendrogram is a result of various polymorphisms between them in the ITS1-5.8S-ITS2 complex. The variation found between the Canadian populations was considerably lower than variation found in European populations (Hübner et al. 2003), likely due to the pooling of multiple plant sequences in our study after little individual plant variation was found. While pooling sequences diluted the variation found on an individual plant level, the variation consistent between field populations could be determined. The ability to separate populations of the same species could be useful for the evaluation of evolutionary traits or the migration of populations across regions. For example, the degree of polymorphisms in garden and field populations, respectively, of common lambsquarters (*Chenopodium album* L.) were correlated to increasing use of triazine herbicides (Mouemar and Gasquez 1983). In another study, significant amounts of intra- and interspecific variation was detected in wild mustard (*Sinapis arvensis* L.) populations collected from 12 locations under herbicide treated and untreated sites (Moodie et al. 1997). Interestingly, results in wild mustard

showed genetic variation was just as high in herbicide regimes as in untreated regimes, and genetic diversity was maintained in conventionally grown field populations. Variation in the ITS1 region of the Lacombe population was like that of Moosomin, but variation in the ITS2 region aligned with the Heavin and Clancy populations, meaning common ancestors or environmental influences could be shared. It is unlikely that pollen flow would influence shared genetic variation as both *G. aparine* and *G. spurium* are known to be predominantly self-fertilizing species (Malik and Vanden Born 1998; Moore 1975). Since little is known about the agronomic practices preceding the collection of population samples, it is unclear if the variation in the western Canadian cleavers ITS regions can be correlated to environmental influences.

3.5 Conclusions

G. aparine and *G. spurium* are two species that are difficult and tedious to differentiate visually or through cytology. Molecular variation in *Galium* species in Europe, together with our research, suggests that the ITS complex is an acceptable region of DNA that can be used successfully to differentiate between *Galium* species. Sequences of *Galium* populations sampled across western Canada suggest they are *G. spurium.* Moreover, the populations are highly related, with little variation in the ITS1-5.8S-ITS2 region. Implications of a *G. spurium* dominant complex are problematic as this species is known to possess resistance to Group 2 and 4 herbicides. Because this study identified variation in the ITS region that can be linked to each species, the marker can be implemented in plant surveys to verify the distribution and speciation of *Galium* species in Canada.

4.0 Emergence Timing and Morphological Characteristics of Cleavers (*Galium* spp.) Populations

4.1 Introduction

Galium aparine is a weedy species that can be found around the world in many different types of ecosystems (Holm et al. 1977). Woodland populations of *G. aparine* are considered native to Canada, while field populations were reportedly introduced from Eurasia along with *Galium spurium* (Moore 1975). North American populations of *G. aparine* require nutrient rich, shaded, moist environments to complete their lifecycle (Holm et al. 1977), which may be why more accessions are found in forests (Malik and Vanden Born 1988). *G. spurium* is more commonly found in open, lighted habitats with high moisture and fertility, such as fields in crop production (Malik and Vanden Born 1984; Moore 1975). Within Canada, both species are collectively called 'cleavers' as they have common growth patterns and are difficult to distinguish visually.

Cleavers have become a dominant weed species on the Canadian Prairies. The latest Prairie weed survey shows cleavers were ranked 9th in abundance in all field crops in the 2000's, increasing from 30th position in the 1970's (Leeson et al. 2005). Similarly, an increasing incidence of cleavers populations was reported in a Saskatchewan canola (*Brassica napus* L.) field survey, where cleavers were 6th in relative abundance, up from 31st in the 1970's (Leeson 2012). While the reasons for this increase in abundance are not known, the similar size and shape of cleavers to canola make separation difficult, resulting in seed lots that are seeded back into the field or spread through purchased seed (Malik and Vanden Born 1988).

Cleavers are considered one of the most competitive species in grain crops around the world (Cussans 2000). Yields are severely reduced when high numbers of cleavers are found in

within cropped fields. For example, *G. aparine* reduced wheat (*Triticum aestivum* L.) yields up to 60%, mainly due to crop lodging and interference with harvesting operations (Rola 1969). These findings were confirmed by Wright and Wilson (1987), who noted yield reductions of up to 57% in winter wheat infested with *G. aparine*. Research in Canada determined rapeseed (*Brassicae napus* L.) yields could be reduced by 13-28% when competing with *G. spurium*, with the resulting rapeseed samples contaminated with 30-70 cleavers seeds per gram of rapeseed (Malik and Vanden Born 1987b). The ability of *Galium* species to compete with crops for resources, to lodge crops, and to contaminate grain lots highlights the importance of finding more effective control options (Malik and Vanden Born 1988).

Measurements of morphological characteristics are a useful tool for evaluating competitiveness of different weed species, communities, and populations. Crop breeders study various traits to increase crop competitive ability with weeds, and these same traits can be measured to study the competitiveness of weeds. Small differences in plant traits have been found between *G. aparine* populations in Europe (Hübner et al. 2003). Leaf size, shoot length, and plant weight varied with environmental conditions, while length-width ratio of cotyledons, the number of leaflets per whorl, length of internodes, and thousand seed weight were generally controlled by genetic factors. Specific competitive differences between closely related species, such as *G. aparine* and *G. spurium*, stems from their genetic code. However, a plant's genetic ability to respond to stresses in the environment can also contribute to their competitive ability.

The emergence timing of weeds greatly influences the success of various control strategies. If control measures are implemented too early, growers can miss later emerging flushes of weeds. Conversely, if weeds are controlled too late, there is a risk of poor control and crop losses from competition (Harker et al. 2001). Little research on cleavers emergence timing

has been conducted in Canada, however. Recent studies in Europe have shown that *G. aparine* exhibits two cohorts of emergence, one in spring and fall (Royo et al. 2010a). The early fall emerging cohort emerges just after seeding the winter crop (commonly wheat) and is often more prolonged than that of other broadleaf weed species. Spring emerging populations exhibited high variability in overall emergence, ranging between 2 and 18% (Cussans and Ingle 1999). The cause behind the variability of these populations is unknown, although the environmental variation among sites and years likely contributed to the unpredictable emergence patterns. Fall emerging cleavers tend to be very competitive with crops, causing significant yield loss, and also contribute large amounts of seed to the seedbank (Cussans 2000). Cleavers emerging later in the spring also produce large quantities of seed that serve to regenerate the population, but they are generally less competitive with the crop.

Understanding the emergence timing and plant characteristics of weeds and the potential variability across populations can aid in developing effective weed management strategies. The increased abundance of cleavers across western Canada, for example, may be due to development of resistance to ALS inhibitors and synthetic auxins (Hall et al. 1998), sub-optimal herbicide timing driven by emergence periodicity of *Galium* species, or differential tolerance of *Galium* species or populations to registered herbicides. Glufosinate must be applied to cleavers before the 2-whorl stage in glufosinate-resistant canola to be effective as the herbicide works best when the plant is adequately covered. Larger plants means greater coverage is needed for control (Saskatchewan Ministry of Agriculture 2015). This narrow window of application means that variable emergence timing may have a large impact on glufosinate efficacy. Non-selective herbicides options in herbicide-resistant crops may be successful if applied at the appropriate growth stage (Brenzil 2012), but poor control in recent years may be indicated that their success

is limited. Therefore, the objective of this study was to characterize the emergence periodicity and morphological traits of cleavers populations from across western Canada. The primary objective of this experiment was to determine the emergence timing of cleavers populations in spring and fall of western Canadian populations. The second objective was to identify if differences existed in the emergence timing of cleavers populations from different geographic locations. The final objective of this experiment was to evaluate the morphological characteristics that influence competitive ability differed among cleavers populations. It was hypothesized that emergence timing and plant characteristics would vary between cleavers populations, but populations in close geographical range would have similar emergence patterns. Knowledge derived from this study could be used to further develop effective and efficient control strategies.

4.2 Materials and Methods

4.2.1 Experimental location and design

Field experiments were conducted in 2013 and 2014 at two locations, the Kernen Crop Research Farm (52°09'10.3"N 106°32'41.5"W) and the Goodale Research Farm (52°03'48.6"N 106°29'59.7"W) near Saskatoon, SK. The Kernen site is located on Black Chernozemic loam soil with a pH of 7.2 and organic matter content of 3.8%. The soil at Goodale is a Dark Brown Chernozemic loam with a pH of 6.4 and 2.6% organic matter. The experimental design was a randomized complete block with four replicates. Each replicate had a factorial treatment design (two factors) with the first factor being spring and fall seeding and the second factor being eight cleavers populations. The plot size was 1 x 2 meters. Treatments consisted of eight different cleavers populations for the plant traits study, and eight populations planted in spring and fall for the emergence timing study.

4.2.2 Experimental populations and seeding

Six cleavers samples were obtained from various seed cleaners across western Canada to observe a broader, random range of the species complex than what could be done with seeds from individual plants. Seed was stored in paper bags at -4°C before seeding to induce dormancy for storage. The original seed source was used to measure emergence and plant traits in both years to avoid introduction of foreign material. The approximate coordinates of the fields from which the cleavers were sampled is given in Table 4.1.

Table 4.1. Approximate GPS coordinates of collection sites of cleavers seed for emergence timing and plant characteristic measurements

Name	Land Location
Lacombe	52°27’17.3”N, 113°44’55.6”W
Vegreville	53°34’17.5”N, 112°00’11.6”W
Carrot River – Clancy	53°16’46.1”N, 103°35’4.6”W
Melfort - Heavin	52°52’15.6”N, 104°36’42.1”W
Melfort- Trawin	52°52’8.5”N, 104°31’11.4”W
Saskatoon – SPG	52°3’49.3”N, 106°26’38.4”W

Germination tests were initially conducted to determine approximate germination percentages for each sample. Each petri dish was lined with two layers of filter paper. Fifty seeds of each sample were placed in their respective petri dish and wetted with deionized water before being placed in the dark. Supplemental water was added when necessary, and germinated seeds were counted and identified when the radicle broke through the seed coat after fourteen days. Seed samples were also sent to Discovery Seed Labs (Saskatoon, Sk) for viability testing using tetrazolium chloride.

Prior to sowing cleavers populations in a common garden, the background cleavers population was quantified at each site using the 'grow out' method described by Forcella (1992). Two 10 inch diameter soil cores taken from each rep were placed in individual containers. A total of 16 containers were placed in a growth chamber set for a 16 hr daylight cycle with temperatures at 18/12°C day/night. Cleavers were counted and removed as they emerged. Once emergence stopped, the containers were dried, stirred, and re-watered. That cycle was repeated 3 times before the trays were chilled at 4°C for 14 days, and then re-exposed to the growth light.

A common garden experiment was conducted using microplots (1 m x 2) established on tilled fallow. The experimental area was soil tested prior to seeding to confirm sufficient nutrient content for plant growth. Tillage was conducted using a harrow prior to seeding to break down plant residue, followed by a glyphosate application of a rate of 900 g ae ha^{-1} to control emerged weeds. Seeds of each population were blended with sand and then broadcasted at a rate of 400 seeds m^{-2} as in Reid and Van Acker (2005). Germination percentages were not taken into account so that total field emergence of each population could be quantified. Plots were raked individually to cover the seeds with a shallow layer of soil. Sowing dates for the spring treatment in 2013 were May 21st and 23rd at the Kernen and Goodale sites, respectively. Fall seeded

treatments were sown on August 21st at both sites in 2013. In 2014, the spring treatment was sown on May 13th at both sites, while the fall treatment was sown on August 14th and 15th at the Kernen and Goodale sites, respectively. To maintain weed-free plots, broadleaf weeds were hand-weeded as required, and grassy weeds were managed by applying clethodim at 45 g ai ha^{-1} in 2013 and quizalofop at 47 g ai ha^{-1} in 2014.

Emergence of cleavers populations was recorded daily in three randomly placed 0.15 m^2 quadrats in each microplot. Emergence was monitored from the time that the first plant emerged until emergence was complete and no further plants emerged over a two-week period. Newly emerged seedlings were recorded and marked with a colored toothpick to ensure they were not counted twice. Cumulative (final) emergence percentages were obtained by dividing the final number if emerged plants by the number of seeds planted.

For morphological traits, leaves were removed from five randomly selected plants collected from each plot to determine the leaf area index. The leaf material of each plot was analyzed with a leaf area meter and then dried and weighed to assess leaf weight. The remaining plant material from the collected leaf tissue was dried for three days at 71°C, weighed, and added to the leaf weight to give the total biomass on a per plot basis. Shoot length was measured on five randomly selected plants per plot when plants entered the reproductive stage (flowering). Branches were also counted at the base of five different plants in each plot. Time to 5%, 50%, and 95% flowering were visually assessed for each plot during the reproductive stage. Once reproduction was complete and the plant tissue had dried down, each plot was hand-harvested and threshed by a small plot combine. Average seed production was calculated by dividing the plot seed weight by the number of plants in each plot. A thousand seeds from each plot were counted and weighed to determine the thousand seed weight.

4.2.3 Data analysis

Emergence curves and plant trait data were analyzed for the Goodale site only as a sizeable native population of cleavers was found at Kernen in 2013. The Kernen site in 2014 had no background cleavers population, but the emergence was too low to properly establish a good model fit for predicting emergence timing and provide enough plants for trait measurements.

Cumulative growing degree days (GDD) were calculated using Equation 4.1 and Equation 4.2

Equation 4.1 Daily growing degree day: $GDD_{daily} = \left(\frac{[T_{max} + T_{min}]}{2}\right) - T_{base}$

Equation 4.2 Cumulative growing degree day: $GDD = \sum_{i=1}^{n} GDD_{daily}$

where T_{max} is the maximum daily air temperature, T_{min} is the minimum daily air temperature, T_{base} is the base temperature (2°C) at which no biological activity occurs, and *n* is the number of elapsed days from seeding to the end of the emergence period. The base temperature of 2°C was used as it is the lowest recorded germination temperature of cleavers (Malik and Vanden Born 1988). Maximum and minimum air temperatures were obtained from an Environment Canada weather station located at the Kernen Research Farm. Air temperatures were used in proxy of soil temperature as the seeding depth of cleavers was very shallow.

Emergence periodicity was presented as a proportion of the total emergence. Cumulative daily values of emergence over each plot were recorded and used for statistical analysis. Non-linear models were fit to the emergence data using the DRC statistical package (Ritz and Streibig 2005) in R (R Core Team 2013). The 3 parameter Weibull model (Equation 4.3) was utilized to

model the emergence of cleavers populations in both spring years and fall of 2014, and a 2 parameter model was used for the fall 2013 emergence data (Equation 4.4)

Equation 4.3 Three parameter Weibull equation for cleaver emergence.

$$Y = d(\exp(-\exp(b(\log(x) - e))))$$

Equation 4.4 Two parameter Weibull equation for cleavers emergence.

$$Y = exp(-\exp(b(\log(x) - e)))$$

where *Y* represents the proportion of cleavers emergence, the *d* parameter represents that upper limit or maximum emergence, the *e* parameter is the ED_{50} or the time to 50% emergence, and the *b* parameters is the slope around the ED_{50}. The lower limit, typically parameter *c*, was fixed at 0 since all samples exhibited a delayed period where germination was zero, and germination percentages cannot be negative. Model selection was based on the extra sum of squares lack-of-fit test using the DRC package in R, as well as on the examination of model residuals and Akaike's Information Criterion (AIC) values. The extra sum of squares lack-of-fit test was also used to determine if years and populations could be combined for analyses.

Final emergence percentages and plant traits were analyzed with ANOVA using a mixed model analysis (PROC MIXED) in SAS (SAS Institute Inc. 2011). The UNIVARIATE procedure and Bartlett's test was used to examine normality and homogeneity of variance, respectively. Residuals for all plant traits and emergence percentages did not violate the assumptions of ANOVA. In the mixed model, population was treated as a fixed effect while year, rep within year, and year by population interactions were considered random effects. Means were separated using Tukey's honestly significant difference (HSD) at $P < 0.05$. Letter groupings of the means were determined using the PDMIX800 macro in SAS (Saxton 1998).

4.3 Results

4.3.1 Emergence timing

In both years, the temperature was similar between years but significantly warmer than the 30-year average (Table 4.2). Precipitation was 18% below and 11% above the long-term average in 2013 and 2014, respectively. The cool, wet environment in 2014 was favorable for cleavers emergence and growth. Cleavers emergence in spring was fairly consistent across years, commencing at 223 growing degree-days (GDD) on June 3rd in 2013 and 214 GDD on June 1st in 2014 (data not shown). Fall emergence, on the other hand, was extremely variable between years. In 2013, cleavers began emerging on September 6th (307 GDD) while emergence in 2014 began on August 26th (201 GDD).

Emergence models could not be combined across years, as years were significantly different from each other within spring and fall emergence. Similarly, a single emergence curve could not be fit for populations originating from the same province based on extra sum of squares lack of fit test (Table 4.3). Moisture and temperature during the emergence period were different between years and sites, and likely had an effect on the periodicity of emergence of cleavers populations. Additionally, germination and seed viability differed between populations, but was not accounted for to simulate natural populations.

Table 4.2. Monthly rainfall (mm) and mean daily temperature (°C) for Saskatoon, Saskatchewan and climate normals (30-year average).

Month	Rainfall			Temperature		
	2013	2014	Normal[a]	2013	2014	Normal[a]
	---------- (mm) ----------			---------- (°C) ----------		
May	19.4	73.0	43.0	19.6	16.3	11.2
June	123.0	103.2	65.8	20.8	19.4	15.8
July	40.2	65.6	60.3	23.3	23.7	18.5
August	13.8	22.2	42.6	25.6	24.0	17.6
September	16.6	12.8	35.4	22.4	19.0	11.4
October	5.6	17.6	18.8	9.7	12.7	4.0
Total	218.6	294.4	265.9	-	-	-

[a]1970-2000 Canadian climate normals for Saskatoon obtained from Environment Canada (2010)

Table 4.3. F and p-values obtained by comparing models with the extra sum of squares lack of fit test using the ANOVA function in R to determine if years, Alberta (AB) populations or Saskatchewan (SK) populations could be combined.

	Combined Years	Combined AB	Combined SK
Spring 13	F- 5.65; P-<0.0001	F- 26.18; P-<0.0001	F- 14.60; P-<0.0001
Spring 14	F- 3.61; P-<0.0001	F- 15.26; P-<0.0001	F- 10.15; P-<0.0001
Fall 13	F- 33.61; P-<0.0001	F- 21.26; P-<0.0001	F- 93.34; P-<0.0001
Fall 14	F- 66.27; P-<0.0001	F- 117.86; P-<0.0001	F- 29.08; P-<0.0001

Spring emergence parameters varied between populations and years (Table 4.4, Table 4.5, Figure 4.1). In 2013, median emergence time (ED_{50}) was significantly different between all populations, with the exception of Heavin and SPG (Table 4.5). Lacombe was the slowest population to emerge, and took 380 GDD to reach 50% emergence compared to 274 GDD for Clancy, which was the most rapidly emerging population (Table 4.4). Median emergence time of populations in 2014 was longer than in 2013, and populations generally were comprised of three groups. Vegreville, SPG, and Clancy were not significantly different from each other, but took about 100 GDD (8-9 days) longer to emerge than Heavin and Trawin, and 50 GDD (4-5 days) less than Lacombe (Table 4.5). Populations did not individually exhibit the same 50% emergence timing and emergence rate from year to year, indicating that optimal control timing may vary from pre-seeding to in-crop, depending on environment (Table 4.4).

With regard to the slope parameter (*b*) the Lacombe population's rate of emergence (slope) in 2013 was 28% lower than SPG, while Trawin and Vegreville were 24-35% slower than Heavin and SPG, respectively (Table 4.4 and Table 4.5). In 2014, the rate of emergence in 2014 was more uniform among populations. The Vegreville populations's emergence was 31% greater than that of Trawin. Rate of emergence (slope) of the individual populations decreased between 2013 and 2014 with the exception of Trawin, which increased.

Table 4.4. Parameter estimates and standard errors of spring emergence timing data of cleavers populations across western Canada in 2013 and 2014. Standard errors are in brackets.

Population	2013			2014		
	Slope	ED50	Upper Limit	Slope	ED50	Upper Limit
Vegreville	4.68 (0.334)	338.29 (5.590)	1.02 (0.020)	3.60 (0.260)	417.54 (11.508)	1.03 (0.038)
Lacombe	5.24 (0.455)	380.07 (6.808)	1.06 (0.040)	4.32 (0.351)	467.58 (11.701)	1.07 (0.047)
SPG	7.25 (0.721)	318.99 (3.700)	0.97 (0.019)	4.04 (0.291)	425.89 (10.063)	1.02 (0.035)
Heavin	6.13 (0.538)	308.58 (4.081)	0.99 (0.020)	4.30 (0.401)	363.80 (8.832)	0.97 (0.027)
Trawin	4.26 (0.364)	290.58 (5.982)	0.95 (0.023)	5.15 (1.013)	328.81 (11.137)	0.88 (0.029)
Clancy	5.75 (0.818)	273.85 (6.137)	0.83 (0.020)	4.10 (0.293)	403.24 (9.102)	1.00 (0.031)

Table 4.5. Approximate *t*-test for comparison of relative difference of ED_{50} and slope values among cleavers populations for spring emergence in 2013 and 2014.

	2013 Estimated Difference of Variance			
Population	ED50	P>F	Slope	P>F
Vegreville:Lacombe	0.8901	<0.001*	0.8924	0.285
Vegreville:Clancy	1.2353	<0.001*	0.8130	0.150
Vegreville:Heavin	1.0963	<0.001*	0.7634	0.007*
Vegreville:Trawin	1.1642	<0.001*	1.0985	0.422
Vegreville:SPG	1.0605	0.005*	0.6456	<0.001*
Lacombe:Clancy	1.3879	<0.001*	0.9110	0.558
Lacombe:Heavin	1.2317	<0.001*	0.8555	0.173
Lacombe:Trawin	1.3080	<0.001*	1.2310	0.125
Lacombe:SPG	1.1915	<0.001*	0.7235	0.004*
Clancy:Heavin	0.8875	<0.001*	0.9391	0.698
Clancy:Trawin	0.9424	0.046*	1.3512	0.119
Clancy:SPG	0.8585	<0.001*	0.7941	0.137
Heavin:Trawin	1.0619	0.018*	1.4389	0.014*
Heavin:SPG	0.9674	0.056	0.8457	0.171
Trawin:SPG	0.9109	<0.001*	0.5877	<0.001*
	2014 Estimated Difference of Variance			
Population	ED50	P>F	Slope	P>F
Vegreville:Lacombe	0.8931	0.002*	0.8323	0.065
Vegreville:Clancy	1.0356	0.336	0.8764	0.167
Vegreville:Heavin	1.1478	0.001*	0.8366	0.099
Vegreville:Trawin	1.2719	<0.001*	0.6909	0.035*
Vegreville:SPG	0.9802	0.580	0.8920	0.237
Lacombe:Clancy	1.1595	<0.001*	1.0530	0.642
Lacombe:Heavin	1.2852	<0.001*	1.0051	0.967
Lacombe:Trawin	1.4241	<0.001*	0.8302	0.340
Lacombe:SPG	1.0975	0.010*	1.0717	0.538
Clancy:Heavin	1.1084	0.003*	0.9546	0.686
Clancy:Trawin	1.2282	<0.001*	0.7884	0.203
Clancy:SPG	0.9466	0.085	1.0178	0.863
Heavin:Trawin	1.1081	0.019*	0.8259	0.336
Heavin:SPG	0.8540	<0.001*	1.0662	0.599
Trawin:SPG	0.7707	<0.001*	1.2910	0.285

* indicated significance difference between populations

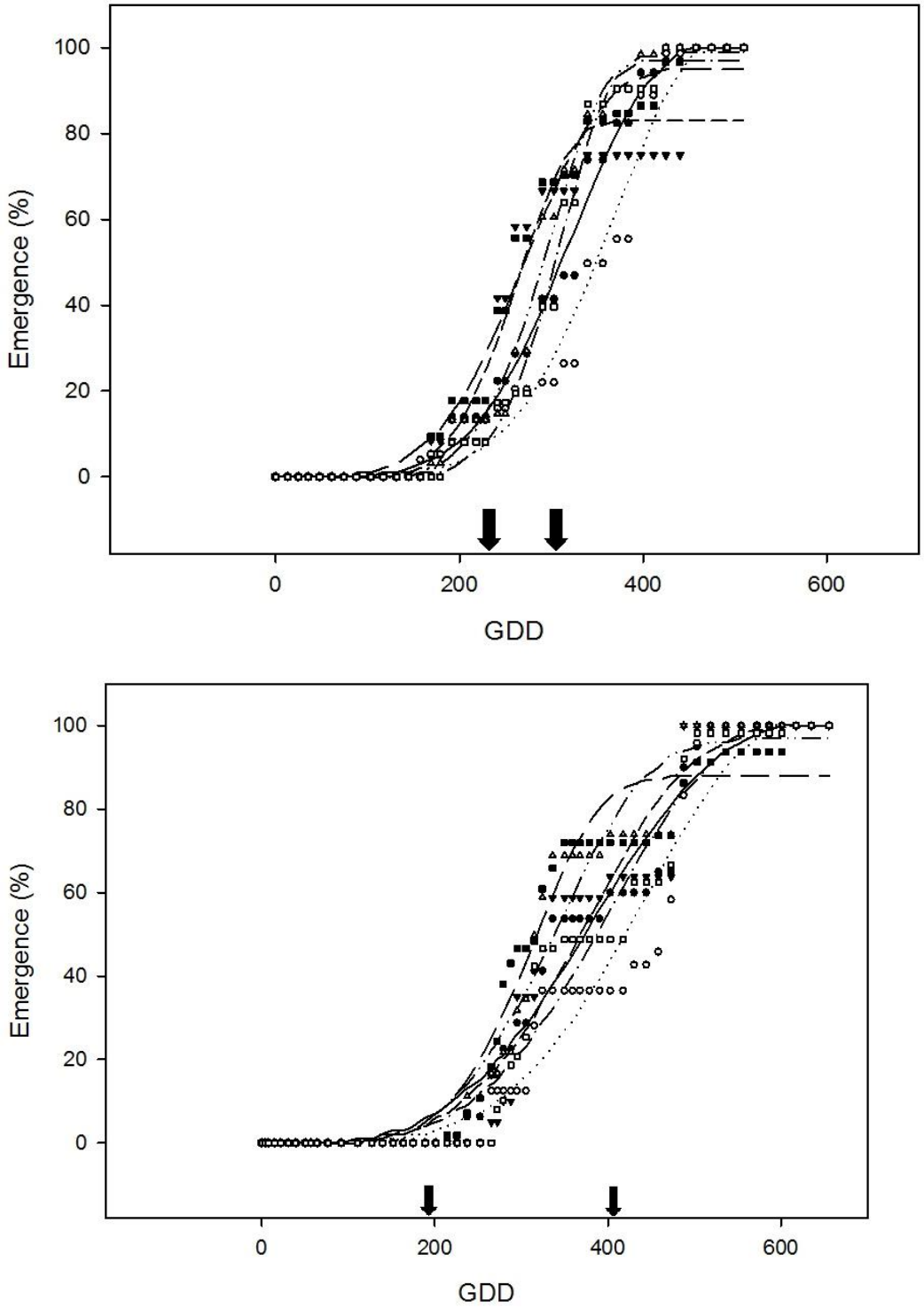

Figure 4.1. Emergence timing of cleavers Vegreville (●, ▬), Lacombe (○, ····), Clancy (▼,– – –), Heavin (△,–··–··), Trawin (■, ▬ ▬ ▬), and SPG (□, –·–·–) populations (observed and predicted values, respectively) at Goodale in the spring of 2013 (top) and 2014 (bottom). Growing degree-days (GDD) were determined with a base temperature of 2°C. Arrows indicate significant rainfall events.

The parameter that sets the upper limit of the curve (*d*) also varied between populations (Table 4.4). In 2013, Clancy exhibited an upper limit that was 20% lower than the other populations, while the *d* parameter for Lacombe was 10% higher than Trawin. In contrast, Trawin was the only population that had a significantly different upper limit from that of the other populations (9-18% lower) in 2014 (Table 4.5). The differences in the *d* parameter suggest that there was no regional similarity among the populations from Saskatchewan or Alberta populations, indicating cleavers populations in close proximity to each other did not necessarily have similar emergence patterns.

Timing of precipitation events appeared to impact cleavers emergence (Figure 4.1). Relatively consistent rain events in 2013 after the start of emergence led to even, gradual emergence of all populations in the spring. While 2014 saw more moisture than 2013, there appeared to be two cohorts of emergence after significant rain events at 200 (51 mm from May 27th to 31st) and 400 (63.6 mm from June 19th to 23rd) GDD. Vegreville, Clancy, SPG, and Heavin had about a week period of no emergence. The Lacombe and Trawin populations had a longer period of no emergence, about 10 days. The rapid growth of early emerging cleavers and the late emergence of a second cohort will undoubtedly lead to some herbicide timing issues with certain herbicides.

Fall of 2013 was particularly dry early in the season. Despite the drought-like conditions, Vegreville, Heavin, and Trawin all started to emerge at approximately 300 GDD. Immediately after the start of emergence, these populations did not continue to emerge and took 21, 31, and 29 days, respectively, to emerge again with the remaining populations. Emergence of all populations started after a short window of rain events on September 26-27 (16.2 mm) and October 2-4 (3.2 mm) around 600 GDD. The fall of 2014 had significantly more moisture than

the fall of 2013. The consistent rainfall resulted in more uniform emergence with the exception of the Lacombe and Clancy populations, which was evident in the 7-9 day break at 250 GDD near the end of emergence.

Fall emergence of cleavers populations in 2013 were fit to a 2-parameter curve where the upper and lower limits are 1 and 0, respectively. Emergence curves in the fall of 2014 fit to a 3-parameter curve where the lower limit is equal to 0. This data showed that the rate of emergence (*b*) for Heavin was 2.6-fold lower than Vegreville and 13-17 fold lower than the other populations in 2013 (Table 4.6, Table 4.7, Figure 4.2). With regard to median emergence timing (ED_{50}) in 2013, the populations separated into three groupings (Table 4.7). Heavin exhibited a significantly higher median emergence time than all other populations, indicating that it had the slowest emergence of all populations (Table 4.6). Although the Lacombe, Clancy, and SPG populations did not differ from each other, these populations exhibited significantly greater median emergence times than Trawin and Vegreville (Table 4.6, Table 4.7). Median emergence time in 2014 differed between all populations, however, and ranged from 249 to 328 GDD. In contrast to 2013, Heavin exhibited the lowest median emergence value, while Lacombe took the greatest amount of time to reach 50% emergence. The upper limit results in 2014 show Trawin was significantly lower than the upper limit of Lacombe and Vegreville. Once again, parameters of the populations did not cluster by region of origin.

Table 4.6. Parameter estimates and standard errors of fall emergence timing data of cleavers populations across western Canada in 2013 and 2014. Standard errors are in brackets.

Population	2013		2014		
	Slope	ED50	Slope	ED50	Upper Limit
Vegreville	8.20 (2.078)	676.20 (4.955)	8.64 (0.798)	256.55 (2.166)	1.00 (0.012)
Lacombe	42.65 (6.925)	690.20 (1.345)	4.55 (0.274)	328.44 (5.358)	1.01 (0.028)
SPG	41.66 (4.369)	691.56 (1.297)	10.23 (1.016)	265.91 (2.198)	0.97 (0.012)
Heavin	3.17 (0.379)	743.32 (17.630)	6.13 (0.462)	248.83 (2.997)	0.98 (0.012)
Trawin	53.63 (7.642)	674.37 (1.373)	8.29 (0.838)	276.66 (2.440)	0.95 (0.012)
Clancy	43.20 (4.298)	690.42 (1.283)	4.27 (0.254)	312.31 (5.775)	0.99 (0.027)

Table 4.7. Approximate *t*-test for comparison of relative difference of ED_{50} and slope values among cleavers populations for fall emergence in 2013 and 2014.

	2013 Estimated Difference of Variance			
Population	ED50	P>F	Slope	P>F
Vegreville:Lacombe	0.9797	0.007*	0.8924	<0.001*
Vegreville:Clancy	0.9794	0.006*	0.8130	<0.001*
Vegreville:Heavin	0.9097	<0.001*	0.7634	0.029*
Vegreville:Trawin	1.0027	0.722	1.0985	<0.001*
Vegreville:SPG	0.9778	0.003*	0.6456	<0.001*
Lacombe:Clancy	0.9997	0.906	0.9110	0.946
Lacombe:Heavin	0.9285	0.001*	0.8555	<0.001*
Lacombe:Trawin	1.0235	<0.001*	1.2310	0.234
Lacombe:SPG	0.9980	0.467	0.7235	0.904
Clancy:Heavin	0.9288	0.001*	0.9391	<0.001*
Clancy:Trawin	1.0238	<0.001*	1.3512	0.166
Clancy:SPG	0.9983	0.532	0.7941	0.805
Heavin:Trawin	1.1022	<0.001*	1.4389	<0.001*
Heavin:SPG	1.0748	0.004*	0.8457	<0.001*
Trawin:SPG	0.9751	<0.001*	0.5877	0.208
	2014 Estimated Difference of Variance			
Population	ED50	P>F	Slope	P>F
Vegreville:Lacombe	0.7811	<0.001*	1.8985	<0.001*
Vegreville:Clancy	0.8214	<0.001*	2.0232	<0.001*
Vegreville:Heavin	1.0310	0.042*	1.4096	0.016*
Vegreville:Trawin	0.9273	<0.001*	1.0421	0.768
Vegreville:SPG	0.9648	0.002*	0.8447	0.176
Lacombe:Clancy	1.0516	0.048*	1.0657	0.467
Lacombe:Heavin	1.3199	<0.001*	0.7425	<0.001*
Lacombe:Trawin	1.1872	<0.001*	0.5489	<0.001*
Lacombe:SPG	1.2352	<0.001*	0.4449	<0.001*
Clancy:Heavin	1.2551	<0.001*	0.6967	<0.001*
Clancy:Trawin	1.1289	<0.001*	0.5151	<0.001*
Clancy:SPG	1.1745	<0.001*	0.4175	<0.001*
Heavin:Trawin	0.8994	<0.001*	0.7393	0.006*
Heavin:SPG	0.9358	<0.001*	0.5993	<0.001*
Trawin:SPG	1.0404	0.002*	0.8106	0.100

* indicated significance difference between populations

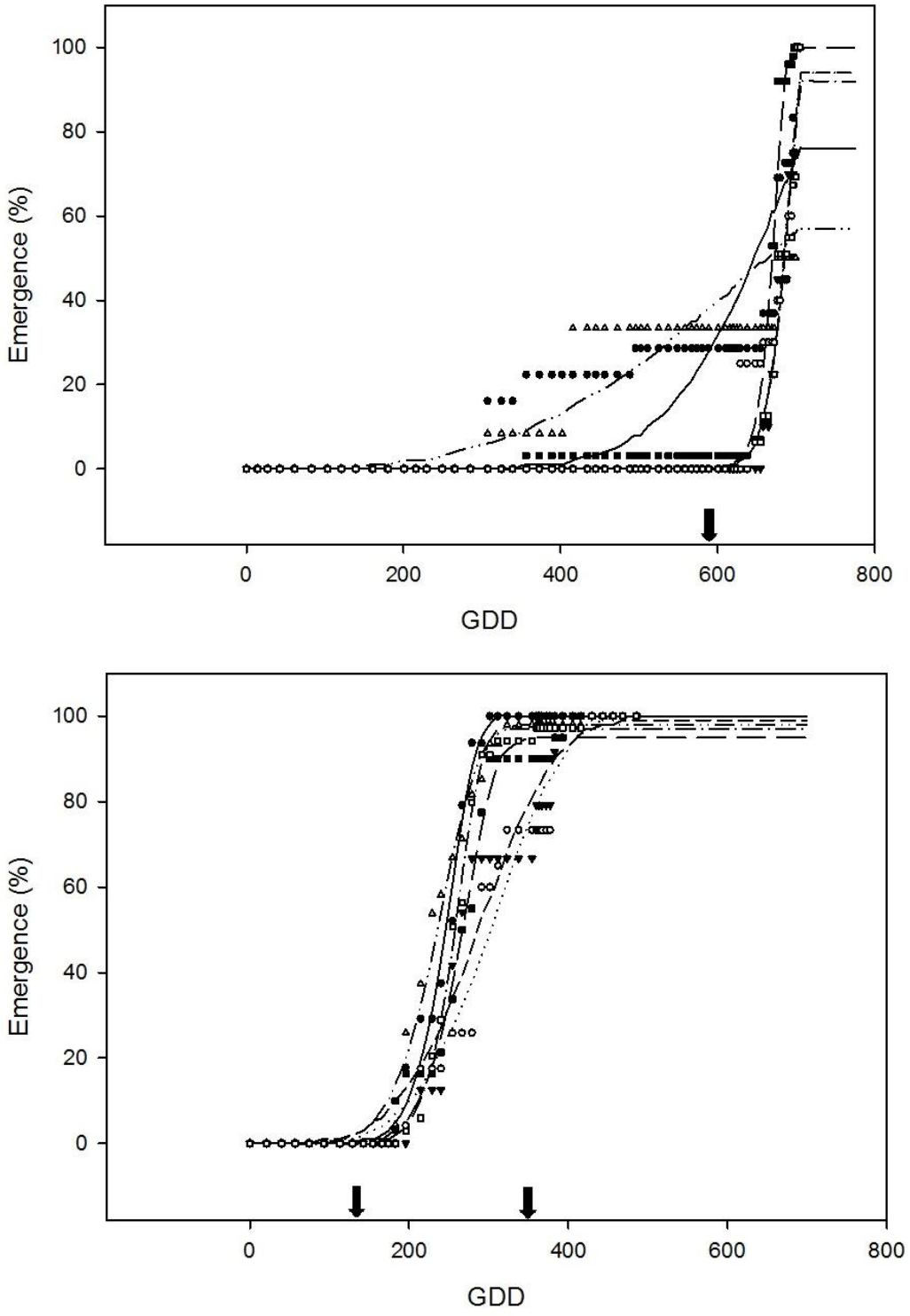

Figure 4.2. Emergence timing of cleavers Vegreville (●, ▬), Lacombe (○, ····), Clancy (▼,– – –), Heavin (△ ,–··–··), Trawin (■, ▬ ▬ ▬), and SPG (□, –·–·–) populations (observed and predicted values, respectively) at Goodale in the fall of 2013 (top) and 2014 (bottom). Growing degree-days (GDD) were determined with a base temperature of 2°C. Arrows indicate significant rainfall events.

4.3.2 Cumulative emergence

Cumulative (final) emergence percentages were analyzed separately for each year. Cumulative spring emergence was significant for populations in 2013 (P=0.022) and 2014 (P=0.001). In 2013, the Clancy population exhibited emergence percentages that were 5-8-fold lower than the other populations (Figure 4.3). In 2014, emergence of the SPG population was 2-4-fold greater than the other populations, with the exception of Trawin. The emergence percentage of Trawin was approximately 3-fold higher than Clancy and Vegreville. Statistically significant differences were detected between populations that were collected in close proximity to each other, showing that overall emergence percentages will likely vary between fields.

The F-values for fall cumulative emergence were P=0.08 and P=0.07 for 2013 and 2014, respectively; however, means separation detected differences between populations in both years (Figure 4.4). In 2013, the Trawin population exhibited emergence that was 3-8.5-fold higher than Lacombe or Clancy, and Heavin, respectively. Trends for differences between populations were similar across years, with the exception of Heavin. Despite having the lowest fall emergence of any population in 2013, Heavin had the highest emergence in 2014. In contrast, the SPG, Clancy, and Trawin populations exhibited decreased fall emergence in 2014, which was unlike their spring emergence patterns.

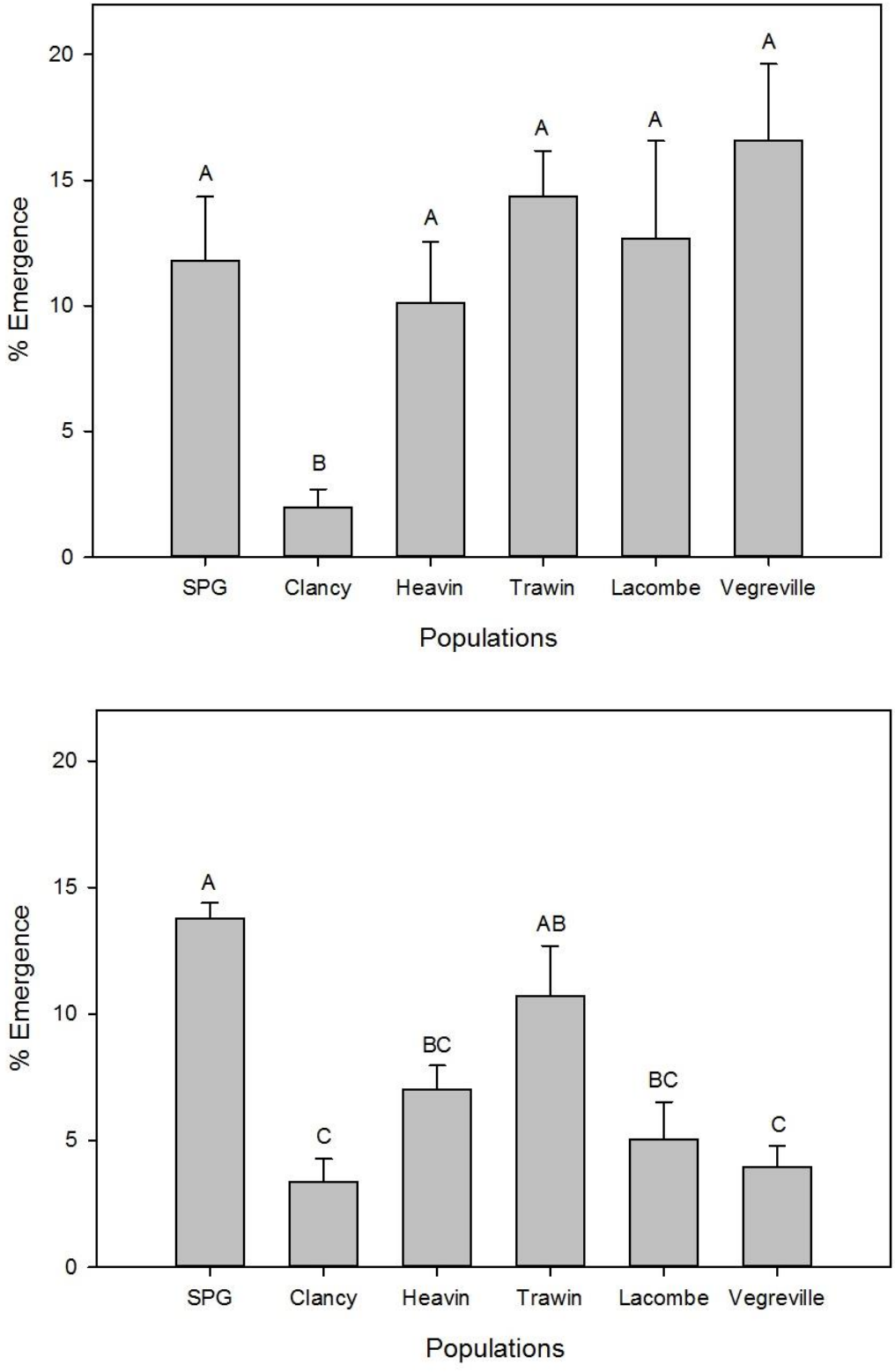

Figure 4.3. Emergence percentage of Canadian populations in spring of 2013 (top) and 2014 (bottom). Error bars represent standard error and letters indicate significant differences at $P<0.05$.

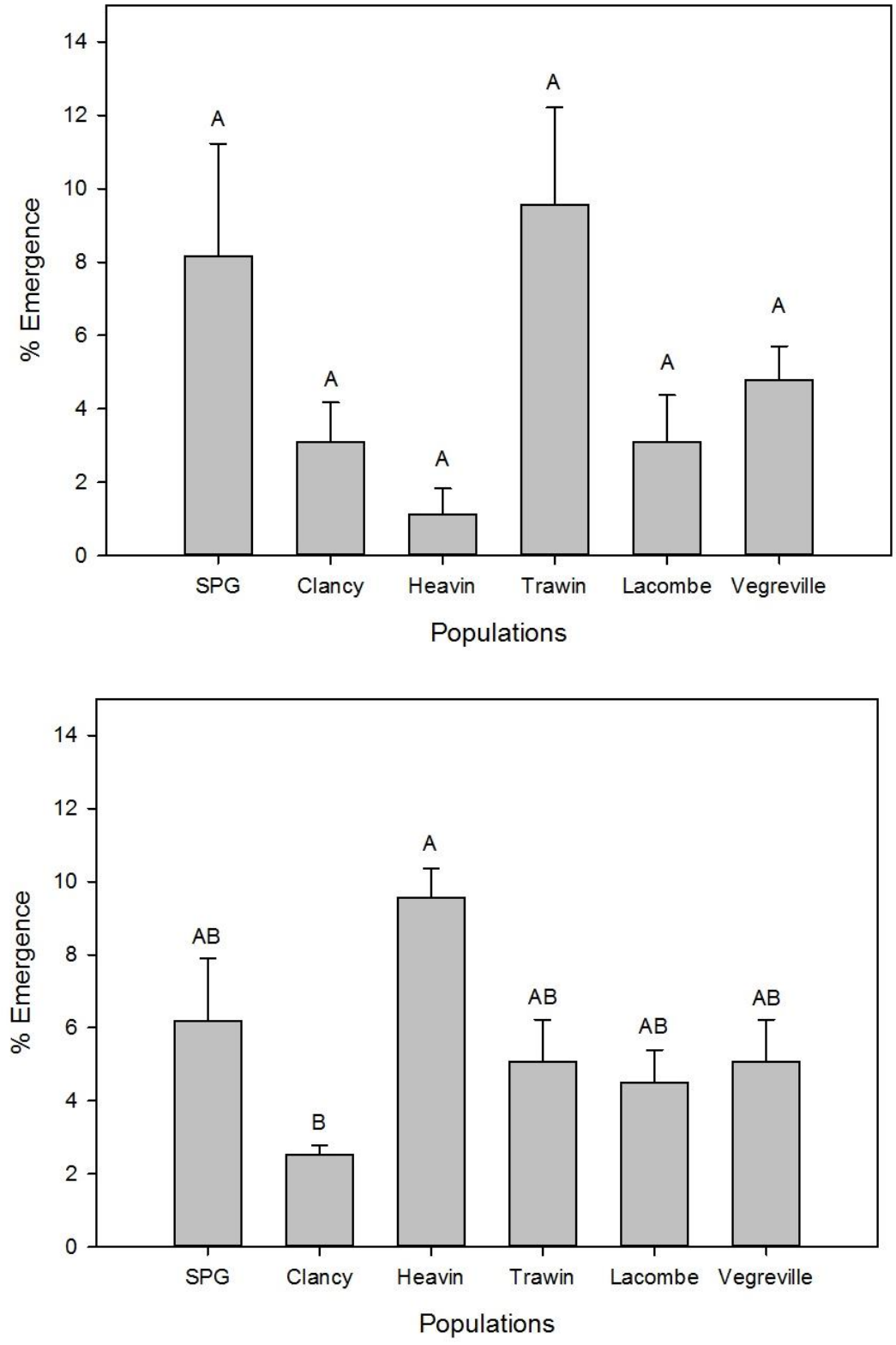

Figure 4.4. Emergence percentage of Canadian populations in fall of 2013 (top) and 2014 (bottom). Error bars represent standard error and letters indicate significant differences at $P<0.05$.

4.3.3 Morphological characteristics

Residual data for all plant characteristics was normally distributed and no heterogeneity was detected between populations. There was a significant year by population interaction for plant height and therefore data were separated by years (Table 4.8). However, when separated by year, plant heights were not significantly different from each other either in 2013 (P=0.463) or 2014 (P=0.255). Days to start of flowering, 5%, (P=0.028) and end of flowering, 95%, (P=0.049) were the only traits found to be significantly different between populations as none of the other traits measure in this study differed between populations (Figure 4.5). Clancy started flowering approximately 4 d before SPG, Trawin, and Vegreville, while Heavin took 5 d longer to finish flowering than Vegreville. Overall, the time each population spent in the flowering period was not significantly different from one another.

Table 4.8. Analysis of variance results (P-values) for leaf area index (LAI), leaf weight (LW), total weight (TW), base branch number (BBN), height, start of flowering (FLW5), mid-flowering (FLW50), end of flowering (FLW95), thousand seed weight (TSW), and fecundity (FED) of cleavers grown in field plots.

Source	LAI	LW	TW	BBN	Height	FLW5	FLW50	FLW95	TSW	FED
Population (Pop)	0.783	0.787	0.689	0.422	N/A	0.028*	0.274	0.049*	0.291	0.078
Year	0.464	0.465	0.460	0.541	0.633	0.480	0.243	0.481	0.278	0.251
Rep (Year)	0.195	0.148	0.148	0.866	0.336	0.142	0.190	0.567	0.461	0.328
Year*Pop	0.152	0.279	0.343	0.987	0.005**	0.870	0.125	0.214	0.276	0.422

*, ** denotes significance at the 0.05 and 0.01 probability levels, respectively.

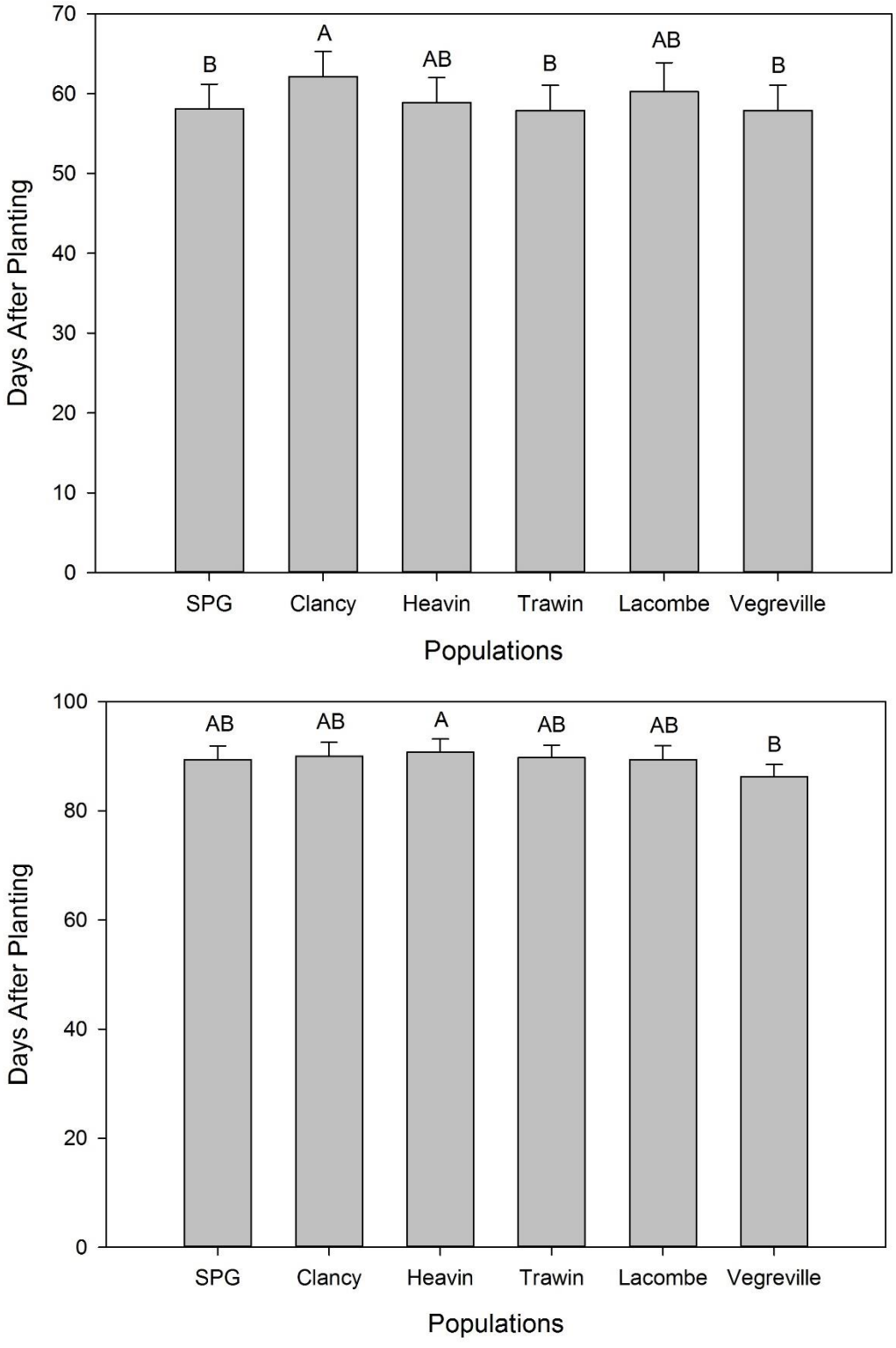

Figure 4.5. Days to the start (5%) and the end (95%) of flowering, (top and bottom, respectively), between cleavers populations. Means were separated using the Tukey test ($p<0.05$). Bars represent the standard error around each mean.

4.4 Discussion

Over the past 30 years, surveys have shown that the abundance of cleavers in western Canada has increased significantly (Leeson et al. 2005; Leeson 2012). Results from this study showed that variation in emergence characteristics between populations may be one explanation for increases in the relative abundance of cleavers. Emergence parameters of Canadian populations varied between populations within each year, but also varied between years. The higher moisture and cooler temperatures in 2014 were favourable for these populations over the others. The lower emergence of the Clancy population in both years means this cleavers population may be potentially less threatening to crop competition. The Alberta populations were more variable from year to year and were less predictable, while the Saskatchewan populations, other than Clancy, had similar cumulative emergence percentages from year to year. Variation in cumulative emergence from year to year and between populations can have an effect on the return of seed to the seedbank; therefore long-term control could be compromised. This concurs with previous studies on *Galium* species emergence in Spain, where variation between years was related to temperature and moisture during the emergence period (Royo et al. 2010b). Excess moisture in their study and in May of 2014 in our study likely promoted cleavers emergence and extended the emergence period. For example, the emergence period of the western Canadian populations in the spring of 2014 was longer and more gradual than in 2013, which was likely the result of greater moisture received in May and June of 2014. It has been shown that *G. spurium* (-1.2 MPa) is more sensitive to soil water potential than *G. aparine* (-2.5 MPa) and thus, has a greater requirement for moisture (Royo et al. 2010b).

Some populations in our study were very responsive to moisture as indicated by an increase in median emergence time in 2013 compared with 2014. The significant increase in

emergence period suggests that Canadian populations, like *G. spurium*, respond to moisture. Seedlings in Spain started to emerge after 250-300 GDD, and took 400-500 GDD to reach 50% emergence (Royo et al. 2010b). Spring emergence of cleavers in our study showed Canadian populations emerged earlier (~150-215 GDD) than European populations and took less time to reach 50% emergence (~275-470 GDD). Malik and Vanden Born (1987b) found that emergence of *G. spurium* in Canada occurred throughout the growing season under ideal, uncompetitive environments. Emergence in their study occurred predominantly from mid-May to early-July, which was similar to that observed in the current study. High temperatures typically experienced in late June to early August are likely not conducive to cleavers germination.

The initiation of emergence and time to median emergence in the fall was varied considerably between years in our study likely due to moisture. In 2013, populations started to emerge from 300 GDD to 600 GDD after planting and took 670-750 GDD to reach 50% emergence, while in 2014 it took 180-200 GDD to start emergence and 250-330 GDD to reach 50% emergence. In both years, the majority of fall emergence occurred after periods of rain. The drought like conditions early in fall of 2013 and rapid emergence after significant rain events in both years shows that moisture is required for emergence of fall cleavers populations. A review of the literature confirms that some fall emerging populations have been reported, but those studies did not investigate emergence timing. Sporadic emergence, likely influenced by moisture availability, can explain why control measures, such as herbicides, have not met expectations. Populations with higher fall emergence are more problematic as these plants have an advanced start on competition with the crop next year and are more tolerant of herbicides. Cleavers plants that escape control measures can make significant contributions to the next generation, meaning

variation in emergence percentages could explain why cleavers have increased in abundance. Overall, all cleavers populations showed higher emergence in the spring than in the fall.

The number of cleavers that germinated and emerged varied between seasons and years. Spring emergence ranged between 2 and 17%, while fall emergence ranged from 1 to 10% in this study. Although emergence may be lower, fall emerging cleavers that overwinter are very competitive. Malik and Vanden Born (1987b) found that *G. spurium* emergence could occur throughout the growing season and that seedlings emerging in July could potentially overwinter. In Spain, *G. aparine* and *G. spurium* plants that emerged late in the season were able to overwinter when conditions were favorable (Royo et al. 2010a). Similar to our study, differing emergence percentages was also observed in different accessions of European populations. Cleavers populations collected from cereal fields had higher overall emergence percentages than populations collected from riverbanks and irrigated fields (Royo et al. 2012). Different *Galium* species can also have different emergence characteristics. Royo et al. (2012) reported that emergence percentage differed significantly between populations of both *G. aparine* and *G. spurium*. Although these differences did influence cleavers plant density, differences in growth stages between plants disappeared during early vegetative growth (Royo et al. 2012). Even though both species have similar lifecycles, their differences in emergence timing, early competitive ability, and contributions to the seedbank all influence efficacy of herbicide control.

The variation we observed in emergence periodicity and percentage between populations is not uncommon, as some populations show greater plasticity in emergence characteristics. A common garden approach allowed us to evaluate if there were genetic differences among the populations for emergence timing by neutralizing the environment. Although specific genetic evaluations on emergence were not utilized to in this study, it can be presumed origins of the

cleavers seed influenced genetic variation between populations. Genetic differences between populations can stem from by characteristics of the maternal environment. The maternal environment has been shown to influence seed characteristics, like dormancy, in offspring through maternal conditioning (Roach and Wulff 1987). For example, shorter photoperiods in the maternal environment in *Arabidopsis thaliana* L. increased the germination response of progeny when secondary dormancy was released with cold stratification (Munir et al. 2001). Additionally, different collections of *A. thaliana* resulted in altered plasticity responses to combinations of variable maternal photoperiod and stratification, further increasing variation. In our study, cleavers were collected from as far as 800 km away from each other east to west and 400 km north to south. This could have influenced the photoperiod exposure of maternal plants and therefore; influence genetic variation between populations.

Geographic variation of germination characteristics has been well documented in various species, and the disparity in germination traits has a significant impact on the emergence timing and life-cycle of the plant. When exposed to various temperatures, populations of *Stellaria media* L. produced seed that had significantly different levels of germination, which then resulted in different life-cycle characteristics (van der Vegte 1978). Another study on three different species, *Thlaspi arvense* L., *Sinapis arvensis* L., and *Spergula arvensis* L., showed that the germination percentage differed significantly between seeds of individual plants and from seeds of different populations in various controlled environments (Andersson and Milberg 1998). The different temperatures in the maternal environments had an influence on progeny's germination. We did not measure dormancy in our emergence study, but maternal effects on these processes can indirectly influence emergence timing. Mechanisms of these physiological processes would need

to be studied in great depth to narrow the error in emergence timing predictions of *Galium* species.

The genetic influences on dormancy and germination ultimately determine timing of emergence. Timing of emergence then influences the successful completion of the plant's lifecycle, as shown in a study on *Helianthus annus* L., where early emerging plants of common sunflower had twice the probability of survival than later emerging plants (Mercer et al. 2011). The competitive advantage of fall emerging and early spring emerging plants in the *Helianthus annus* study warranted the implementation of earlier control measures. Genetic variation influencing emergence characteristics results in unique field populations that need to be managed individually each year.

The lack of significance between morphological traits of cleavers populations, with the exception of the beginning and the end of the flowering period, suggests that they all behave alike, and likely inflict the same competitive ability on field crops. The lack of significance in traits suggests that these populations are very similar and when compared to the literature, could possibly of the same species. For example, the fecundity of *G. aparine* is 300-400 per plant (Hanf 1983), while *G. spurium* can produce up to 3500 seeds per plant (Malik and Vanden Born 1987b). The populations in our study are all likely to be of one species, *G. spurium* (Chapter 3). Not surprisingly, Canadian populations tested in our study produced seed yields between 1800-14034 seeds per plant, consistent with the seed production of *G. spurium*. Seed weight is also different between species. A thousand seeds of *G. aparine* weighs between 3-6 grams (Moore 1975), while Malik and Vanden Born (1988) observed *G. spurium* averaged 2.8 grams per thousand seeds. Like fecundity, thousand seed weight of Canadian populations in this study were more similar to *G. spurium*, between 1.5 and 3.0 grams. *G. aparine* populations in Europe did

not produce significantly different seed yields per plant, but did have significantly higher thousand seed weights, 4.5-9.1 grams (Hübner et al 2003). The days to the start and end of flowering were significantly different between populations in this study, but flowering period was not. Other evaluations of flower timing of cleavers species also showed variation between populations that may be influenced by the original environments of the population (Hübner et al. 2003) or timing of emergence (Malik and Vanden Born 1987b). No comments in the literature are found regarding different flowering periods between species.

Few vegetative growth characteristics can differentiate species, and can be highly variable between populations. Early studies on *G. aparine* showed plants grew to 1.2 m tall in Canada (Moore 1975), but later research in Europe showed *G. aparine* populations varied between 1.2 and 1.9 m in height (Hübner et al. 2003). Observations of *G. spurium* heights up to 2.0 m have been reported (Malik and Vanden Born 1988). The heights of our Canadian populations were shorter and more like *G. aparine*, but height variation is not uncommon within both species. Additionally, growing conditions of cleavers in the literature were under supportive structures unlike the barren environment experienced by the Canadian populations in our study. Malik and Vanden Born's (1987b) *G. spurium* growth experiment also indicated that characteristics like height, number of nodes, and total dry weight are variable due to timing of emergence. Plants collected to measure traits may have had different emergence timing as they were selected at random. The lack of significant differences in reproductive and vegetative traits of Canadian cleavers populations support the earlier molecular conclusion that they are all the same species. Although there were no significant difference in plant traits, the literature on both species suggests that our Canadian populations are characteristically similar to *G. spurium.*

Further research on both species under controlled or competitive environments would need to be done to prove this.

4.5 Conclusions

Emergence timing and final emergence were significantly different between cleavers populations within years and between years in the spring and fall. In each year, some populations were significantly different from each other in regard to rate of emergence and median emergence time. Plant traits did not differ between the Canadian populations, with the exception of the start and end of the flowering period. The differences between populations were not a function of provincial boundaries for emergence or flower timing. Understanding traits that contribute to the aggressiveness of cleavers can result in better control strategies that still control less competitive species.

There is a significant genotype by environment interaction that needs to be further examined to create an accurate model to predict emergence of cleavers in Canada from year to year. Genetic differences causing variation among populations' within any given year means growers will have to take an individualistic approach to controlling cleavers in their fields. More importantly, cleavers populations are now emerging in the fall and can potentially overwinter into spring, making post-harvest weed control even more important. Thus, in years likely to be favorable for emergence, scouting fields for appropriate control timing will aid in greater control efforts. Additionally, more research on various characteristics of cleavers populations is needed to evaluate new parameters of competitive traits.

5.0 General Discussion

The general objective of this research was to find possible explanations as to why cleavers populations have increased in abundance over the last 30 years. More specifically, studies were conducted to determine the species composition of *Galium* populations in the field, and to identify some potential ecological explanations as to why herbicide applications are not as effective as expected. Overall, the original hypothesis that cleavers populations were mixed species was false after evaluation of the ITS region identified no *G. aparine* plants. The emergence timing and morphological trait hypothesis that cleavers populations would vary was true for emergence, but false for traits. The field study showed the rate of emergence and time to 50% emergence could not be combined among populations, while no significant differences were found in morphological traits. It is possible that the variability in emergence timing overshadowed differences in morphological traits. Lastly, our assumption that cleavers in close proximity to each other would exhibit similar emergence patterns was false after statistical analysis showed no cleavers populations were the same. The *G. spurium* conclusions from the ITS study is supported by the lack of significance found in morphological traits. With the results, growers, agronomists, and companies can adjust control strategies to improve efficacy of herbicides on cleavers. Moreover, further research can be conducted to enhance our understanding of the complicated biological processes influencing weed populations.

5.1 Considerations for Further Molecular Analysis

Molecular analysis of the ITS1-5.8S-ITS2 complex in *Galium* species found enough variation to differentiate species quickly and efficiently using TaqMan. The majority of the variation was in the ITS2 region, but an unanticipated SNP was identified in the 5.8S gene, which was used to discriminate between species in western Canada. Small amounts of consistent

variation were found between populations, but populations were highly related to each other and the *G. spurium* reference population.

While additional measures were taken to confirm the species of the reference populations, more populations of *G. aparine* and *G. spurium* are needed to confirm the SNP in other populations. Research evaluating the ITS regions for variation in species do not use extraordinary measures to identify species before sequencing (Hübner et al. 2003; Vander Stappen et al. 1998). Moreover, research looking at species variation focuses solely on the ITS1 or ITS2 regions over the ribosomal genes due to less conserved behaviour and greater variation. The highly conserved nature of the 5.8S gene where the species identification SNP was located in this study lends greater confidence than in the spacers (Baldwin et al. 1995). Although if additional SNP's were necessary, variation detected within the ITS2 region of *G. aparine* and *G. spurium* could be used with the 5.8S marker to confirm species identification. The extra precautions to confirm the reference populations and selection of a SNP in the 5.8S gene suggests confidence in the results until further investigations can be undertaken.

The influence of polyploidy on differences and changes in the ITS1-5.8S-ITS2 region should also be further evaluated in *Galium* species. *G. spurium* has no known polyploidy, but *G. aparine* is most commonly found as a hexaploid (Kliphuis 1962; Podlech and Dieterle 1969; Moore 1975). Previous research also shows that tetraploid plants (Kliphuis 1962) and possibly an octoploid plant (Fagerlind 1934) could contribute to more variation. The ITS region of *Alternanthera philoxeroides* (Mart.) Griseb. was evaluated over three kinds of ploidy in the species (Chen et al. 2015). While biotypes of *A. philoxeroides* with higher ploidy have a stronger invasive ability, there was little variation found in the ITS sequences. If the ITS region in *G. aparine* also shows no variation between ploidy levels, the SNP used in our study is practicable

across all populations. Variable ploidy *G. aparine* populations were not evaluated in this study, but they are not expected to change the efficiency of the 5.8S SNP.

Outside of the ribosomal gene and ITS regions, other locations within the genome could be evaluated for species variation. The most common alternative genome location for species identification is the chloroplast region. Chloroplast genes have been used extensively in the *Echinochloa* species for taxonomic and evolution studies (Ye et al. 2014). The chloroplast genome of two species, *Echinochloa oryzicola* and *Echinochloa crus-galli*, have been sequenced using high-throughput sequencing data to understand the diversification of the genus and evolution of the genome. Research evaluating non-coding spacer regions in chloroplast DNA between *Echinochloa crus-galli*, *Echinochloa oryzoides*, and *Echinochloa oryzicola* found variation that could be used to identify the species from one another (Mennan and Kaya-Altop 2012). Genetic differentiation between species was then used to check if 178 accessions of *E. oryzicola* were properly identified. Eight of the *E. oryzicola* accessions were misidentified and were actually *E. oryzoides*, which could hinder control strategies if species differed in their response to control.

5.2 *Galium spurium* Abundance in Western Canada

Population sequences in the molecular analysis aligned with the *G. spurium* sequence. Small amounts of variation were detected throughout the complex of the populations, but the strong alignment of populations to *G. spurium* and each other suggests they are highly related. The lack of significant differences in traits also suggests that the populations are comprised of one species, but further trait evaluations with *G. aparine* and *G. spurium* would need to be conducted to support the *G. spurium* conclusions.

No *G. aparine* plants were found in cleavers populations collected from western Canada during molecular analysis. While no *G. aparine* was found in the collected samples, it does not mean none is present in fields across the prairies. Earlier surveys reported that *G. aparine* can be found on the coast of B.C. and the Maritimes, across the prairies, and throughout southern Ontario and Quebec (Scoggan 1979). Light to moderate infestations of *G. spurium* can be found across Canada, as well as throughout Europe, with the exception of north and western Asia (Malik and Vanden Born 1988). It is possible the selection of field populations would select against *G. aparine*. *G. aparine* prefers nutrient rich, moist habitats (Holm et al. 1977), while *G. spurium* thrives in dry, sunny habitats that are more characteristic of fields in crop production (Moore 1975; Malik and Vanden Born 1984). Extensive sampling of field and bush cleavers populations would need to be done to confirm the *Galium* spp. complex in western Canada is primarily *G. spurium*. It is likely that the complex in western Canada is a mix of *G. aparine* and *G. spurium*, but the dominant species causing problems in crop production is the more competitive species, *G. spurium*.

No research has been done exclusively on gene flow between *Galium* species that exist in and around cropping systems. Flowers of *G. aparine* and *G. spurium* are self-pollinated and self-compatible, meaning that the probability of gene flow is low (Moore 1975). When many small *Galium* flowers are clustered together, small insects like beetles, ants, flies, and bees will interact with them and move pollen (Batra 1984). The base number chromosomal mismatch between *G. aparine* (n=11) and *G. spurium* (n=10) means offspring are unlikely to survive (Moore 1975; Malik and Vanden Born 1988), but nothing is known about *G. boreale*. A possible hybrid was observed between *G. spurium* and *G. boreale* by Moore (1975), but little research has been done since to determine if hybridity was the cause for an abnormal *G. spurium* type plant with reduced

fertility. If a hybrid plant could exist between *G. boreale* and *G. spurium*, *G. boreale* plants could serve as a gene reserve for aggressive competitive traits and, perhaps herbicide resistance traits.

5.3 Management Implications

Results from the emergence timing and trait evaluations suggest that cleavers abundance has increased due to variable requirements for emergence timing and not through competitive ability with other weeds or crops. Variation in emergence timing occurred within years and between years, depending on environmental conditions during the emergence period. The lack of differences in traits from the molecular studies also suggests that all populations only consist of a single species, *G. spurium*.

Cleavers in canola are particularly problematic and worrisome to growers, and the abundance of cleavers has increased over the last 30 years. The critical period of weed control (CPWC) in canola is the 4-6 leaf stage (Martin et al. 2001). Seeding of canola occurs in late April to late May, and this study showed that cleavers emergence overlaps with the later seeding of canola, which is well into crop emergence. Malik and Vanden Born (1987b) suggest that control measures should attempt to contain *G. spurium* for at least 6 weeks from mid-May to end of June. Understanding emergence timing and the factors that affect weed emergence benefits the effectiveness of all types of control (chemical, mechanical, and cultural) by ensuring the weed is managed through the CPWC. CPWC is influenced by seeding date and emergence of the crop relative to the emergence of the weeds (Knezevic et al. 2002; Martin et al. 2001). Research by Knezevic et al. (1997) showed that the relative time of redroot pigweed (*Amaranthus retroflexus* L.) emergence was more important to competition with sorghum than the actual weed density.

However, the crop, weed, and environmental characteristics can collectively affect the CPWC. Competitive ability of crops and weeds is heavily dependent on environmental conditions (Lindquist et al. 1999). Conditions such as warm winters and spring droughts can reduce or delay cleavers emergence and may render herbicide applications unnecessary, although high moisture after the initial emergence period can induce a secondary flush (Royo et al. 2010a). If adequate moisture is consistent throughout the year, cleavers emergence can be continuous throughout the growing season, making populations more difficult to maintain and control. While weeds need to be controlled in particular period during crop development to reduce yield loss, weeds also need to be of a certain stage for herbicides to be effective. Results obtained using the Weibull model to evaluate emergence showed growers can expect cleavers to start emerging in late May or early June. Multiple flushes of cleavers can occur after significant rainfall events, which requires appropriate evaluation of populations in the field. Contact herbicides like glufosinate have to be applied while cleavers are large enough to come in contact with the herbicide, but small enough that cleavers will not produce new shoots. Emergence models can give significant insight into what growers can expect over the emergence period.

5.4 Final Remarks

The variable emergence of cleavers should result in growers and agronomists taking more rigorous reports when scouting. A long period of emergence in the spring, and additional emergence in the fall, means scouting fields and implementing control strategies will need to be done more often. The lack of similarity between populations shows that field populations need to be treated on an individual basis in regards to scouting and weed control operations.

The cleavers species commonly found in crops is undoubtedly *G. spurium*. While this result has no immediate impact on cleavers control, given the uncertainty of speciation in other

research, the use of the 5.8S gene SNP can be used to further understand this species and its *G. aparine* counterpart. Furthermore, the SNP can be implemented in weed surveys to confirm the absence or presence of *G. aparine* in field populations on a much wider scale.

Overall, the outcome of this thesis can conclude that variable emergence characteristics of *G. spurium* have contributed to the increased abundance of cleavers populations, and a *G. spurium* dominant complex is causing the majority of yield losses in the field. In addition, our results will enable future research to study, evaluate, monitor, and control cleavers populations in western Canada to be done more efficiently and effectively.

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Appendix A

Sequence results of the ITS1-5.8S-ITS2 region of *G. aparine*, *G. boreale*, and *G. spurium*. The 5.8S gene is bolded, and spots of variation are highlighted according to nucleotide (A-red, C-blue, T-green, and G- yellow)

```
         1         10         20         30         40         50         60
         |         |          |          |          |          |          |
Aparine  TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAATCCTGC--AGACGACCGCGAACACG
Boreale  TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAATCCTGCTCGAACGACCGCGAACAAG
Spurium  TCCGTAGGTGAACCTGCGGAAGGATCATTGTCGAATCCTGC--AGACGACCGCGAACACG

Aparine  -TTAACCAAAAGCGCGGGACGC------TGG----------CCGT-CAGGCCGGCTCCCG
Boreale  TTTAACAAAAAAYGCGGGACGCCGGGCGTGGACGGGGAAACCCRTCCCTCCCGGCCCCG
Spurium  -TTAACCAAAAACGCGGGACGC------TGGCCGGACCTAGCCGT-CCGGCCGGCTCCCG

Aparine  CACCAAACCTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGACTGCCCCTTC
Boreale  CACCAAACCTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATYGCCCCCTT
Spurium  CACCAAACCTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTC

Aparine  CTCGCCAAGGCTTCCTCGGCGGGGAGGAC--GCGTCTGAAAC GTAACCAATACGACTGTC
Boreale  C-CCCCGCGGATTCCGTGGTGGGTCGGGCYGGCATCTGAAAC GTAACCAATACGACTCTC
Spurium  CTCGCCAAGGCTTCCTCGGCGGGGAGGAC--GCGTCTGAAAC GTAACCAATACGACTGTC

Aparine  GGCAACGGATATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTG
Boreale  GGCAACGGATATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTG
Spurium  GGCAACGGATATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTG

Aparine  TGAATTGCAGAATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCACT
Boreale  TGAATTGCAGAATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATT
Spurium  TGAATTGCAGAATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACT

Aparine  CGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCATTC--------
Boreale  CGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCTCGTCGCCACCCCCAGC--------
Spurium  CGGCCGAGGGCACGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCGAGCCTT

Aparine  ------------C--TAAGT----------CCTGGACTCG-GACCGGCGCGCGGTGGATG
Boreale  -------------TC---------------CATTG---CGAGCATGGCGTGTGGCGGAAG
Spurium  CCCGTCTCGGCTCATTAAGTTGGCCCGATCCGTGGGCTCG-GGATGGTGCTCGGTGGATG

Aparine  TTGGCCTCCCGTTCCCCTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGAC
Boreale  CTGGCCTCCCGTTCCCCTAGCGGCGCGGATGGCCCAAATGCGAGTCCTCGGATAAGGGAC
Spurium  TTGGCCTCCCGTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGAC

Aparine  GTCACGACTAAAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCC
Boreale  GTCACGACTACAGGTGATTGAACTAGCTTTCATTTT-GCTCTCGTGTCCTGACGGCCCCC
Spurium  GTCACGACTAAAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCC

Aparine  GGCGGAACTGAACAAGCCCTAGAGCTCCC-----------CAAAT-TAGCTTC----AAA
Boreale  GAYGGAACACAWMGA-CCCTAGAGC-CCCT--------CCCGTCCCGTGCT-CT-AC-GA
Spurium  GGCGGAACTGAACAAACCC-AGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAA

Aparine  GCTGGTCCAAG------------GGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTA
Boreale  GCGCGGGCGCG----------GGCGGAGCCTTCGACCGCGACCCCAGGTCAGGCGGGATTA
Spurium  GCCAGGGGAAGAGAGAGAAAGGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTA
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Aparine CCCGCTGAATTTAAGCATATCAATAAGCGGAGGA
Boreale CCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
Spurium CCCGCTGAATTTAAGCATATCAATAAGCGGAGGA
```

Appendix B

Sequence results of the ITS1-5.8S-ITS2 region of nine western Canadian populations. The 5.8S gene is bolded, and spots of variation are highlighted according to nucleotide (A-red, C-blue, T-green, and G- yellow)

```
            1         10         20         30         40         50         60
            |         |          |          |          |          |          |
     Clancy TCCGTAGGTGAACCTGCGG-AAGGATCATTGTCGAATCCTGCAGACGACCGCG-AACACG
      Heavin TCCGTAGGTGAACCTGCGG-AAGGATCATTGTCGAATCCTGCAGACGACCGCG-AACACG
    Lacombe TCCGTAGGTGAACCTGCGG-AAGGATCATTGTCGAATCCTGCAGACGACCGCG-AACACG
   Manitoba TCCGTAGGTGAACCTGCGG-AAGGATCATTGTCGAATCCTGCAGACGACCGCG-AACACG
   Moosomin TCCGTAGGTGAACCTGCGG-AAGGATCATTGTCGAATCCTGCAGACGACCGCG-AACACG
        SPG TCCGTAGGTGAACCTGCGGNAAGGATCATTGTCGAATCCTGCAGACGACCGCGNNACACG
      Trawin TCCGTAGGTGAACCTGCGG-AAGGATCATTGTCGAATCCTGCAGACGACCGCG-AACACG
 Vegreville TCCGTAGGTGAACCTGCGG-AAGGATCATTGTCGAATCCTGCAGACGACCGCG-AACACG
    Yorkton TCCGTAGGTGAACCTGCGG-AAGGATCATTGTCGAATCCTGCAGACGACCGCG-AACACG

     Clancy TTAACCAAAAACGCGGGACGCTGGCCGGACCTAGCCGTCCGGCCGGCTCCCGCACCAAAC
      Heavin TTAACCAAAAACGCGGGACGCTGGCCGGACCTAACCGTCCGGCCGGCTCCCGCACCAAAC
    Lacombe TTAACCAAAAACGCGGGACGCTGGCCGGACCTAGCCGTCCGGCCGGCTCCCGCACCAAAC
   Manitoba TTAACCAAAAACGCGGGACGCTGGCCGGACCTAGCCGTCCGGCCGGCTCCCGCACCAAAC
   Moosomin TTAACCAAAAACGCGGGACGCTGGCCGGACCTAGCCGTCCGGCCGGCTCCCGCACCAAAC
        SPG TTAACCAAAAACGCGGGACGCTGGCCGGACCTAGCCGTCCGGCGGGCTCCCGCACCNAAC
      Trawin TTAACCAAAAACGCGGGACGCTGGCCGGACCTAGCCGTCCGGCCGGCTCCCGCACCAAAC
 Vegreville TTAACCAAAAACGCGGGACGCTGGCCGGACCTAGCCGTCCGGCCGGCTCCCGCACCAAAC
    Yorkton TTAACCAAAAACGCGGGACGCTGGCCGGACCTAGCCGTCCGGCCGGCTCCCGCACCAAAC

     Clancy CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA
      Heavin CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA
    Lacombe CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA
   Manitoba CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA
   Moosomin CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA
        SPG CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA
      Trawin CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA
 Vegreville CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA
    Yorkton CTAACTCTCGGCGCGGAAAGCGCCAAGGACTACTCAAACGGATTGCCCCTTCCTCGCCAA

     Clancy GGCTTCCTCGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
      Heavin GGCTTCCTCGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
    Lacombe GGCTTCCTTGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
   Manitoba GGCTTCCTCGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
   Moosomin GGCTTCCTTGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
        SPG GGCTTCCTCGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
      Trawin GGCTTCCTCGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
 Vegreville GGCTTCCTCGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
    Yorkton GGCTTCCTCGGCGGGGAGGACGCGTCTGAAACGTAACCAATACGACTGTCGGCAACGGAT
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    Clancy ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG
    Heavin ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG
   Lacombe ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG
  Manitoba ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG
  Moosomin ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG
       SPG ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG
    Trawin ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG
Vegreville ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG
   Yorkton ATCTAGGCTCTCGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAG

    Clancy AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGGC
    Heavin AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGG
   Lacombe AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGG
  Manitoba AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGG
  Moosomin AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGG
       SPG AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGG
    Trawin AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGG
Vegreville AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGG
   Yorkton AATCCCGTGAATCATCGAGTTTTTGAACGCAAGTTGCGCCCAAAGCCACTCGGCCGAGGG

    Clancy ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCAAGCCTTCTCCGTCTCG
    Heavin ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCAAGCCTTCTCCGTCTCG
   Lacombe ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCAAGCCTTCTCCGTCTCG
  Manitoba ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCGAGCCT--TCCGTCTCG
  Moosomin ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCAAGCCTT-TCCGTCTCG
       SPG ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCAAGCCT--TCCGTCTCG
    Trawin ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCGAGCCT--TCCGTCTCG
Vegreville ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCAAGCCT--TCCGTCTCG
   Yorkton ACCGTCTGCCTGGGCGTCACGCATCTCGTCACCGCCCCAATCCGAGCCT--TCCGTCTCG

    Clancy GCTCATTAAGTTGGGCTGATGCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC
    Heavin GCTCATTAAGTTGGGCTGATGCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC
   Lacombe GCTCATTAAGTTGGGCTGATGCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC
  Manitoba GCTCATTAAGTTGGCCCGATCCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC
  Moosomin GCTCATTAAGTTGGGCTGATCCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC
       SPG GCTCATTGAGTTGGGCTGATCCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC
    Trawin GCTCATTGAGTTGGGCCGATCCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC
Vegreville GCTCATTAAGTTGGGCTGATCCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC
   Yorkton GCTCATTGAGTTGGGCCGATCCGTGGGCTCGGGATGGTGCTCGGTGGATGTTGGCCTCCC

    Clancy GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
    Heavin GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
   Lacombe GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
  Manitoba GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
  Moosomin GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
       SPG GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
    Trawin GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
Vegreville GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
   Yorkton GTTCCCTTAGCGGCGCGGATGGCCTAAATTCGAGTCCCCGGACAAGGGACGTCACGACTA
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```
    Clancy AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG
    Heavin AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG
   Lacombe AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG
  Manitoba AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG
  Moosomin AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG
       SPG AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG
    Trawin AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG
Vegreville AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG
   Yorkton AAGGTGGTTGAATTCTCATTCATTCTCGCTCTAGCGTCCTGACGGCCCCCGGCGGAACTG

    Clancy AACAAACCCAGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAAGCCAGGGGAAG
    Heavin AACAAACCCAGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAAGCCAGGGGAAG
   Lacombe AACAAACCCAGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAAGCCAGGGGAAG
  Manitoba AACAAACCCAGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAAGCCAGGGGAAG
  Moosomin AACAAACCCAGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAAGCCAGGGGAAG
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    Trawin AACAAACCCAGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAAGCCAGGGGAAG
Vegreville AACAAACCCAGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAAGCCAGGGGAAG
   Yorkton AACAAACCCAGAGCTCCCTCGCCTCGCCTCGCCTCTTGGTTCGAATAAAGCCAGGGGAAG

    Clancy AGAGAGAAAAGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT
    Heavin AGAGAGAAAGGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT
   Lacombe AGAGAGAAATGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT
  Manitoba AGAGAGAAAGGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT
  Moosomin AGAGAGAAAAGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT
       SPG AGAGAGAAAGGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT
    Trawin AGAGAGAAAGGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT
Vegreville AGAGAGAAAGGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT
   Yorkton AGAGAGAAAGGGGGAGCCTTCGAACGCGACCCCAGGTCAGGCGGGATTACCCGCTGAATT

    Clancy TAAGCATATCAATAAGCGGAGGAA
    Heavin TAAGCATATCAATAAGCGGAGGAA
   Lacombe TAAGCATATCAATAAGCGGAGGAA
  Manitoba TAAGCATATCAATAAGCGGAGGAA
  Moosomin TAAGCATATCAATAAGCGGAGGAA
       SPG TAAGCATATCAATAAGCGGAGGAA
    Trawin TAAGCATATCAATAAGCGGAGGAA
Vegreville TAAGCATATCAATAAGCGGAGGAA
   Yorkton TAAGCATATCAATAAGCGGAGGAA
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