POPULATION GENETICS AND SCARIFICATION REQUIREMENTS OF *GYMNOCLADUS DIOICUS*

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Abstract

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The Kentucky coffee tree (*Gymnocladus dioicus*) is an endangered tree species native to the American Midwest and Southwestern Ontario. Significant habitat loss and fragmentation due to agricultural, industrial and urban development has caused gradual decline across its native range. The aims of this study were to investigate: (1) patterns of genetic diversity and, (2) genetic differentiation (3) relative levels of sexual vs. clonal reproduction, and (4) potential for reduced genetic diversity at range edge for wild *G. dioicus* populations. An analysis of variation at nine microsatellite loci from populations in the core of the species distribution in the U.S.A. and 4 regions of Southwestern Ontario indicated that *G. dioicus* has remarkably high genetic similarity across its range (average pairwise F_{ST}= 0.05). Germination trials revealed that the seed coats require highly invasive treatments (e.g. 17.93 mol/L H_2SO_4) to facilitate imbibition, with negligible germination observed in treatments meant to emulate prevailing conditions in natural populations. Low levels of sexual reproduction, high genetic similarity, and habitat degradation are issues that exist across the entire native range of *G. dioicus*.

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Figure 6, Frequency of alleles at 9 microsatellite loci across Canadian populations, pooled into four geographic groupings: Essex, Lambton, Middle Sister Island, and East Sister Island. Low frequency allele ratios denoted in color while high frequency alleles are shown in greyscale. Pie chart values report observed allele frequencies, however, alleles with very low frequencies (<2.5%) were adjusted to 2.5% for visibility (see Appendix, Table 13 for true values). KC8-1, KC9-1, KC10-1 were fixed across all Canadian populations.

Chapter 1: Introduction

The Carolinian zone (Eastern Deciduous Forest Zone) in Southwestern Ontario is considered one of the main biodiversity hot spots in Canada (Oldham 2017). Although it only covers 0.25% of Canada's total land area, it hosts 40% of its plant species, making this area an excellent target for conservation action (Oldham 2017). In the post-industrial era, 78% of the Carolinian zone has been converted into agricultural land while 7% is occupied by urban development, leaving this area mostly devoid of forested cover (Oldham 2017). The remaining forest fragments provide critical refuges for the rare forest species that occupy this area, and the flora and fauna that they provide habitat for.

One tree species found only in this area (in Canada) is the Kentucky coffee tree (*Gymnocladus dioicus*), a woody member of the legume family (Fabaceae) native to the American Midwest and Southwestern Ontario. The evolutionary history of the *Gymnocladus* genus places its origin in Eastern Asia after *Gymnocladus* and its sister genus *Gleditsia* diverged from the rest of Caesalpiniaceae during the mid-Eocene (44.3-53.3 mya)(Schnabel *et al.* 2003). Following this divergence, *G. dioicus* (or an ancestor) was able to successfully traverse the Bering isthmus and establish in North America, forming an Asian-American disjunction recognized in a number of other tree genera (*Gleditsia, Magnolia, Liriodendron, Sassafras, Liquidambar*; Berry 1899) (Schnabel *et al.* 2003). *G. dioicus* has a maximum life span of ~100 years and grows to a maximum height of ~30m with a relatively narrow crown spread at maturity (12-15 meters) (Row & Geyer 2014). The bipinnately compound leaves are the largest of any Canadian tree species by a large margin with the capacity to reach 90cm in length (Environment Canada 2014). The inconspicuous green-white flowers emerge as individually stalked flowers

(pedicillate) arranged in a long terminal raceme (up to 6 inches long) and typically appear in late spring (immediately following leaf emergence) (Bebeau G. D. 2014). Individuals exhibit either staminate or pistillate flowers (i.e. the plant is dioecious)(Barker 1986; Yanan *et al.* 2004), with some sources describing the rare occurrence of bisexual flowers on otherwise male or female individuals (Minnesota seasons 2018; Ohio State University 2018). When pollinated, pistillate flowers produce large green seed pods (3-7 inches long) which ripen into cutinized brown indehiscent seed pods (Horr 1927; Zaya & Howe 2009). The seed pods contain a fragrant glucose-containing pulp and 4-6 large globular seeds with robust seed coats (Horr 1927). The pods ripen in late fall and remain attached to the tree until at least the following spring (Horr 1927). *G. dioicus* is capable of reproducing through seed germination or vegetative root suckering. *G. dioicus* is currently considered a riparian species as natural populations are found almost exclusively in floodplain habitats, although there is evidence to suggest that it is not physiologically dependant on floodplains (McClain & Jackson 1980; Zaya & Howe 2009).

As habitat and forest cover decline and continue to marginalize many rare plant species in Ontario, land trusts and government agencies in Canada have worked to actively protect and maintain the remaining habitat fragments. These protection efforts require accurate and up-to-date data to reveal population trends of endangered species. The current body of literature pertaining to *G. dioicus* is extremely limited, with sizable knowledge gaps in its reproductive biology and biogeographic history. This thesis will attempt to add to this body of information by mapping genetic variation of natural *G. dioicus* in Canada. This will allow me to quantify levels of genetic diversity,

differentiation, levels of sexual versus asexual reproduction, and potential range-edge effects in natural populations, which will fill the aforementioned knowledge gaps and create a solid backdrop for future conservation efforts. This population genetics assessment was done in collaboration with the University of Iowa who contributed samples from across the American range to enable comparison with Canadian samples. A new set of primers were developed yielding 9 microsatellite loci which were used to genotype trees sampled in this investigation.

An additional investigation was conducted to determine the scarification requirements of the extremely hard-coated seeds. It has been proposed in the current body of literature (VanNatta 2009; Environment Canada 2014) that the seeds of *G. dioicus* do not germinate readily under natural conditions, so this investigation will seek to fill these knowledge gaps and provide inferences regarding actual levels of germination in the wild using genetic data.

Chapter 2: Literature review

General background

Habitat fragmentation due to land-use changes such as industrialization, urbanization, or agricultural development is the primary driver of biodiversity loss in North America (Vellend 2003). These anthropogenic activities reduce existing habitat patches while simultaneously increasing the distance and number of obstacles between patches, decreasing overall connectivity (connectivity defined as the distance and number of obstacles between patches; Vellend 2003; Favre-Bac *et al.* 2017). Loss of connectivity can result in highly isolated populations, increasing vulnerability to stochastic demographic events and/or genetic drift and inbreeding depression (Favre-Bac *et al.*

2017; Hens *et al.* 2017). These effects are particularly pertinent to species at risk which are often already restricted to small fragmented populations (Hens *et al.* 2017; Van Rossum *et al.* 2017). Before the advent of genetics in conservation biology, census data was often utilized as a proxy for overall population vigor (Hens *et al.* 2017). This approach can be a cost effective and practical way to assess populations of flora and fauna in conspicuous taxa, but can be misleading in scenarios where the census size is large but the number of genetic lineages present is small (e.g. clonal plant populations) (Hens *et al.* 2017).

The International Union for the Conservation of Nature (IUCN) has made efforts to incorporate biogeographic and demographic principles into their status designation criteria. Specifically, IUCN has incorporated multiple species-specific criteria including evidence of population decline, range extent, density of occupancy, and estimates of the total number of reproductively mature adults (Willoughby *et al.* 2015). In theory, the inclusion of these parameters should increase the accuracy of IUCN designations, however, some have argued that genetics and population-genetic principles (i.e. genetic diversity and effective population size) need to play a more central role in the process (Frankham *et al.* 2014; Willoughby *et al.* 2015). Inclusion of these genetic data (or metrics generated from genetic data) introduces a complementary way to infer aspects of demographic data which, when used in conjunction with spatial data, provides ecologically relevant insight into the genetic structure of wild populations. Specifically, genetic diversity within populations can indicate which populations are at the highest risk for inbreeding depression and rapid loss of alleles via genetic drift, effectively functioning as a proxy for overall population vigor (Van Dyke 2008). This relationship

between neutral genetic diversity and population vigor allows ecologists to gain some level of inference about the future adaptive potential of the population in question (Spielman *et al.* 2004), which is an important attribute to consider when projecting responses to climatic change or pathogens (Jump *et al.* 2009). Genetic data can also be used to reveal levels of connectivity between populations by quantifying levels of genetic differentiation**.** These insights provide a robust backdrop on which we can assess the current state and future projections of wild populations.

In the temperate regions of Eastern North America (East of 95°W, as described in King & Schlossberg, 2014), intense agricultural and urban development has transitioned much of this region from heavily forested habitat to a mosaic of urban areas and agricultural fields with intermittent fragments of natural or semi-natural habitat. This shift, coupled with non-native species introductions, has rapidly altered the community compositions of eastern forests (Martinuzzi *et al.* 2015). Although community composition changes occur through natural processes, the current anthropogenic landscape transition is causing such frequent and dramatic changes that it is difficult to identify if current biologic communities exhibit stable demographic compositions or if they are still in transition (Santo-Silva *et al.* 2016)**.** For tree species, the latter is the most likely scenario as they often have relatively long generation times, causing a lag in demographic responses to new stressors (Vranckx *et al.* 2011; Talluto *et al.* 2017). This lag creates an 'extinction debt' which refers to the mortalities that occur sometime after the initial disturbance event (Vellend *et al.* 2006). Extinction debts are especially relevant in cases of habitat fragmentation as the resulting population decline can occur over several years following the initial event (Vellend *et al.* 2006). Complicating matters further is the introduction of

non-native forest pests that have substantially altered the hardwood communities that characterize Carolinian forests (e.g. the Emerald ash borer - *Fraxinus* sp.; Dutch elm disease - *Ulmas* sp.; Chestnut blight - *Casttanea sp.* (Fisichelli *et al.* 2014). This turnover of eastern hardwood species has dramatically altered tree community compositions, which has significant implications for the relative abundance of fringe species or species otherwise subordinate to previously dominant hardwoods. From a management perspective, the demographic challenges faced by species-at-risk in small disconnected populations might make them ill-equipped to exist in these altered communities.

Isolated populations that reproduce through a combination of sexual and asexual reproduction may be particularly likely to experience reductions in genetic diversity following disturbance. The ability to root sucker following damage is commonplace among deciduous trees of Eastern North America, and can allow individuals to recover from major trunk injuries (Del Tredici 2001). While this life history trait is typically reserved until life-threatening circumstances arise, some species implement root suckering as a regular part of their reproductive cycles (e.g. *Rhus typhina* and *Populus tremuloides*; Del Tredici 2001). From a conservation perspective, trees capable of asexual reproduction should be assessed with caution as even very large populations can exhibit low genetic diversity (e.g. clonal populations of *Populous tremuloides*; DeWoody *et al.* 2008). This is especially relevant in highly fragmented landscapes in which obligate out-crossers have become too sparsely distributed to be pollinated by their conspecifics, and vegetative propagation becomes their primary mode of reproduction (Beatty *et al.* 2008). Excessive vegetative propagation can be detrimental as it drastically reduces genetic diversity, which can severely limits future adaptive potential (Beatty *et al.* 2008).

The Kentucky coffee-tree (*Gymnocladus dioicus*) is a deciduous tree with a number of unusual life history traits, and appears to be slowly disappearing from the landscape (Zaya & Howe 2009). As the only member of its genus in North America, *G. dioicus* grows exclusively in the Midwest region of North America with the northern extent reaching into Southwestern Ontario, Canada. *G. dioicus* is typically found in riparian areas and grows to a maximum height of ~30 meters over its ~100 year life span (Row & Geyer 2014)**.** Upon reaching sexual maturity (20-30 years; Randall 2017), trees produce unisexual flowers (Zaya & Howe 2009). The inconspicuous white flowers are hypothesized to be entomophilous (Zaya & Howe 2009), which may limit gene flow as insect pollination activity occurs mostly within habitat patches, thereby contributing to genetic isolation (Vranckx *et al.* 2011). When pollinated, female flowers produce brown indehiscent seed pods that encapsulate a glucose-containing mesocarp and 4-8 extremely large seeds (~15-20mm in diameter) with extremely hard seed coats (Horr 1927). *G. dioicus* is also capable of asexual reproduction through root suckering via 'rhizome-like' lateral runners (McClain & Jackson 1980).

The ability to reproduce asexually allows *G. dioicus* to persist in circumstances where sexual reproduction is limited. These limitations are thought to be caused by three main factors: (1) infrequent/non-existent fruit production (Environment Canada 2014), (2) limited seed dispersal capabilities (Barlow 2002; Zaya & Howe 2009), (3) and low germination rates (VanNatta 2009; Environment Canada 2014). Low levels of natural fruit production is a common phenomenon in sparsely distributed *G. dioicus* populations as these conditions are not conducive to the limited traveling distance of insect pollinators, making it difficult for trees to mate with conspecifics (Zaya & Howe 2009).

Limited opportunity for outcrossing in fragmented landscapes largely precludes fruit production in single-sex populations, making vegetative propagation the primary mode of reproduction (White & Oldham 2000). These circumstances tend to reinforce the occurrence of single-sex populations, which, barring somatic mutations, often consist of genetically identical trees (Environment Canada 2014). In Canada, only 4 of 25 extant locations have been identified as fruit-producing populations; surveys at the remaining 21 locations failed to identify any fruit-bearing trees (Environment Canada 2014). The proportion of fruiting populations in the USA has not been formally evaluated, so the extent of sexual reproduction across the American portion of the natural range is currently unknown.

Given that *G. dioicus* primarily occupies the American Midwest, it is at high risk for being marginalized by forest fragmentation associated with agricultural practices. Habitat fragmentation has imposed a new suite of selection pressures on the dispersal capabilities of the flora that occupy highly developed areas of North America. In general, species that invest heavily into seed production may increase their chances of reaching isolated habitats while also increasing their local population size; this in turn can further increase future seed production (Favre-Bac *et al.* 2017). Long dispersal distances are also advantageous in fragmented landscapes, generally favouring anemochorous and hydrochorous species because of their potential for relatively large seed shadows (Favre-Bac *et al.* 2017).

The modern dispersal agent of *G. dioicus* seeds remains unknown (Zaya & Howe 2009). Currently, *G. dioicus* is classified as hydrochorous due to natural stands occurring almost exclusively in riparian areas (VanNatta 2009; Zaya & Howe 2009). However, the

physical weight and dimensions of *G. dioicus* seeds appear inconsistent with aquatic dispersal, as the seeds do not possess any morphological features that would enable prolonged buoyancy (Zaya & Howe 2009). Compounding dispersal issues further is the architecture of waterways surrounding land that has been drained for agricultural purposes. Also known as drains, these waterways are a network of unnaturally straight channels and sharp corners, with other barriers including drain pipes or filter grates (Favre-Bac *et al.* 2017). These obstacles severely limit the dispersal potential of hydrochorous species with seeds that aren't small enough or streamlined enough to traverse them, as is certainly the case with the large bulky seed pods of *G. dioicus*. It is also possible that *G. dioicus* is capable of dispersal via vegetative fragments, especially following destructive events such as uprooting or flooding, which may provide infrequent supplementary dispersal, although there are no records of such events.

The seeds of *G. dioicus* exhibit a form of dormancy known as physical dormancy (as described in Baskin & Baskin 2016) which allows the seeds to remain viable for a period of time after detaching from the parent tree (the exact length of time is currently unknown). In order for germination to occur, this barrier must be removed to allow water to penetrate the testa (seed coat) and make contact with the embryo. Once this occurs, the seed will begin to imbibe water and initiate the process of germination. The exact requirements for natural germination are unknown, but the impermeability of the testa is exemplified by the fact that previous investigations have achieved the highest germination rates after exposing seeds to highly corrosive sulphuric acid (Grbic & Stillnovic 1988: but also see Horr 1927). Although natural germination success rates have not been previously evaluated, it has been suggested that *G. dioicus* experiences low

natural germination rates (<5% ; (VanNatta 2009; Environment Canada 2014)) due largely to the robust testa and its resistance to water penetration. Additionally, the seeds contain two noteworthy chemical constituents: an insecticide (β-hydroxy-γ-methylglutamic acid; Rehr *et al.* 1973) and a toxin (currently unknown (Fitch *et al.* 2009)) capable of killing large herbivorous livestock (Beasley 1999). These constituents presumably work to counteract insect seed predation, and dissuade consumption by large modern-day herbivores.

These seed traits have given rise to hypothesis that the fruit of *G. dioicus* may have been dispersed by one or more (now extinct) species of Pleistocene megafauna (Barlow 2008; Zaya & Howe 2009). If this is the case, the distribution capabilities and germination rates of wild *G. dioicus* should be severely compromised. Although the merit of this hypothesis is debatable, the repercussions of limited dispersal capabilities appear to have manifested themselves in the spatial aggregation patterns of wild *G. dioicus*. Specifically, natural stands of *G. dioicus* are often small and isolated with extremely localized seed shadows, remaining rare throughout the entirety of its historic range, often persisting as small clonal populations on the edges of agricultural drains and forest fragments (McClain & Jackson 1980; VanNatta 2009). This sparse distribution pattern is typical of wild *G. dioicus* populations, indicating dispersal and colonization are likely infrequent events.

Canadian populations of *G. dioicus* are at their northern range limit, meaning they could contain genotypes which are conducive to survival in northern climatic conditions. Although range-edge populations sometimes have low levels of genetic diversity compared to core populations (Eckert *et al.* 2008), these populations could harbour

adaptive potential important for future northward range expansion, hence their potential contribution to the future conservation and persistence of *G. dioicus* must not be undervalued. Canadian populations suffer from two syndromes typical of range-edge existence, the first of which is limited fruit production. Limited fruit production is often a feature of small, isolated populations, and can result from increased self-pollination and/or inbreeding (Beatty *et al.* 2008), the former of which is not possible in *G. dioicus* due to its dioecious nature. Inbreeding is a tangible concern for *G. dioicus*, but limited fruit production in wild populations indicate that most populations in Canada are likely unisexual, with no sexual reproduction at all. The second range edge effect is the scarcity of habitat suitable for *G. dioicus* as agricultural practices continue to clear and drain riparian areas (Oldham 2017). This reduction in suitable habitat is a common phenomenon seen in other range-edge populations, however, the primary cause is typically unsuitable climatic and/or soil conditions (Beatty *et al.* 2008). In the case of *G. dioicus*, clonal propagation and isolated populations are predominant issues across its entire range (McClain & Jackson 1980; VanNatta 2009), indicating that these phenomena might be accentuated by anthropogenic fragmentation. These fragmentation events are the primary driver of suitable habitat loss, which compounds the poor dispersal capabilities, decreasing the already small likelihood of successful dispersal and establishment. Currently, *G. dioicus* is designated as a species-at-risk in Canada ('Threatened' (Brodribb & Oldham 2000; White & Oldham 2000)) and endangered in New York state, but clonal propagation and isolated populations are predominant issues across its entire range (McClain & Jackson 1980).

Objectives

Broadly, the objectives of this thesis were to investigate four aspects of *G. dioicus* ecology that might be influencing current patterns of distribution and diversity: (1) seed germination, (2) sexual vs clonal reproduction, (3) genetic isolation of natural populations, and (4) potential range-edge effects. Germination rates were investigated through *ex situ* germination trials, and the remaining three objectives were investigated through an assessment of the population-genetic diversity of natural *G. dioicus* populations in Southwestern Ontario. Specifically, I examined the genetic diversity within and differentiation among natural *G. dioicus* stands in Southwestern Ontario, Canada. Investigating the genetic variation of wild *G. dioicus* populations offered insight into genetic diversity and gene flow, which allowed me predict the vulnerability of *G. dioicus* populations. Comparisons were also made between Ontario trees and *G. dioicus* sampled from a number of locations in the USA in order to compare genotypes and overall genetic variation between the core of the distribution and the range edge. I evaluated the following hypotheses: (1) the seeds of *G. dioicus* experience low natural germination rates as they require damage that enables water to penetrate to the testa. Given the robust nature of the testa, sources of damage that can provide sufficient scarification are unlikely to be found in natural settings. If this is the case, scarification typical of riparian environments should yield minimal or no successful germinations. (2) Sexual reproduction is relatively infrequent compared to asexual reproduction. If this is true, the patterns of genetic variation within populations should reveal that most trees are products of asexual reproduction. (3) Little to no gene flow occurs between natural populations resulting in high levels of genetic differentiation. This differentiation should be reflected in relatively High Fst values (>0.25; based on Fst thresholds described in

Hartl & Clark 1997, but also see: Balloux & Lugon-Moulin 2002). (4) Genetic diversity is lower in range edge populations in Canada than in the American Midwest. If this is holds true, expected heterozygosity levels should be lower in Canada than in the U.S.

Methods

Germination

Seed collection

Seeds were collected from urban trees in York region of the Greater Toronto Area during the fall of 2016 with the assistance of Shawn Petille (Humber Nurseries Ltd.) (Table 1). Seedpods were collected with 8' Bartlett square pole pruners with a PR-PH3 Marvin pruner head. Seedpods were detached as close to the proximal end as possible to minimize structural damage to the parent tree. Only seedpods still attached to the tree were collected, any seedpods that had fallen were not included in the collection. A total of 468 seedpods (~1500-2000 seeds) were collected.

Table 1, Location and number of seedpods collected from 5 urban fruit bearing *G. dioicus* in the greater Toronto area. UTM Waypoints refer to zone T17.

INDIVIDUAL	EASTING	NORTHING	# OF PODS
DB01	617348	4853940	241
DB02	617357	4853942	126
DB03	617400	4853953	11
DB04	617521	4853990	32
DC01	620851	4844331	58

Seed viability

Prior to the germination trials, 10 seeds from each tree were put through a single germination trial to determine whether seeds from all trees were viable. Seeds were soaked in concentrated (17.93 mol/L; 95.6%) sulphuric acid for 170 minutes following a

protocol that has previously been used to germinate *G. dioicus* seeds (Grbic & Stillnovic 1988). Immediately following this treatment, seeds were submerged in room temperature tap water for 24 hours to facilitate imbibition. Each 10-seed cohort was wrapped in two 30 cm x 30 cm sheets of paper towel, saturated with 80 ml of tap water, and placed in 18 cm x 9 cm x 9 cm glass Mason jars. Seeds were checked daily for radicle protrusion (commencement of germination). After 30 days the experiment was terminated, with seeds from each tree collectively exhibiting germination rates between 80 and 100%. From this, it was concluded that all trees have similar viability (Table 6), allowing for seeds from all trees to be combined into a large cohort (known herein as the test bank), from which all individuals were selected.

1. Mechanical and Osmotic scarification trial

The germination response of *G. dioicus* seeds to a series of abrasion treatments was quantified. It has been proposed that prolonged immersion in stagnant water (Environment Canada 2014) or fire (Horr 1927; Environment Canada 2014) may compromise the seedcoat and lead to germination. Additionally, given its designation as a hydrochoric disperser, abrasion consistent with tumbling along the substrate of a riverbed may be a means of effective scarification for *G. dioicus*. Soaking in water, exposure to flame, and tumbling with sand were therefore three of the treatments used in this experiment, each designed to mimic natural conditions. Two additional treatments that are more invasive, but less likely to occur naturally were also included: blunt force and piercing combined with compression. Three levels were implemented within each treatment (Table 2).

Table 2, Treatments and their respective levels (and descriptions) applied to *G. dioicus* seeds. n=30 seeds for each level within each treatment; n=90 across all levels within each treatment; n=540 across all treatments and levels (n=90 for both positive and negative control).

Treatment	Treatment category	Treatment level	Treatment description
Hammer blow	Invasive condition (*Blunt force*)	Hammer: 1, 3, 5	Values indicate number of hammer blows sustained
Nut cracker	Invasive condition (*Piercing and compression force*)	Nutcracker: High, Medium, Low	See treatment description for exact compression amounts
Open flame	Natural condition (*Forest fire*)	Fire: 15, 45, 75	Values after fire indicates exposure time in seconds
Rock tumbler	Natural condition (*Lotic water system*)	Tumble: 12, 24, 48	Values indicate time spent inside rock tumbler in hours
Water soaking	Natural condition (*Lentic water system*)	Water: 20, 30, 40	Values indicate submersion time in days

Controls

This experimental series incorporated both a positive and negative control. Seeds in the negative control group did not under go any abrasion or soaking, but did undergo the post treatment procedure (described below). The sulphuric acid treatment is the positive control for this experiment, as it is a known effective method for promoting germination of *G. dioicus* seeds (Ball & Kisor 1985; Grbic & Stillnovic 1988). For the positive control, seeds were submerged in (95.6%: 18.44 mol/L) sulphuric acid (BAKER ANALYZED'® A.C.S Reagent), inside a 1.4L Purex® glass baking dish (13.0cm x 22.7cm x 7.6cm) for 170 minutes. Seeds were rinsed with DI water to remove any lingering acid prior to commencing the post treatment procedure. This soaking time was based on the protocol in Ball & Kisor (1985).

Open flame

Seeds were burned over 150g of wood inside a Lixada® stainless steel camping stove (8 x 10.8 x 9 cm). Two steel cross pins were used to suspend the seeds at a height of ~10cm above the center of the stove for the duration of the trial. The camp stove is equipped with a number of lateral holes, which, for the purposes of this experiment, were used as a rest for the cross pins during the trials in order to standardize the roasting height. Preliminary trials on 10 seeds were conducted to determine approximately how long it took to burn through the seed coat, as germination would be unsuccessful if embryos were burned. This took between 90-120 seconds, which is why the trial times were established as 15, 45, and 75 seconds.

Rock tumbler

Seeds were placed inside an Elenco® Discovery Planet rock tumbler. The seeds were contained within the central drum of the apparatus which contained 200ml of DI water and 4 ounces of Premier® play sand. The drum rotated at 60 RPM for the duration of each trial.

Hammer blows

A 16 oz Benchmark® fibreglass handle hammer rotated around a vertically elevated (3.0 cm) fulcrum (a wooden dowel 10cm x 1cm) threaded through a lateral hole in the base of the hammer handle. The hammer was orientated parallel to the counter top on which the apparatus sat, in one of two distinct positions: the starting position (middle of hammer face raised vertically 10.0cm) and a finish position (middle of hammer face resting on counter top). The vertical height of 10cm was determined from pilot studies that investigated at which height the hammer would destroy the embryo. A hammer blow

consisted of the hammer head being manually lifted into the starting position and released to fall to the finish position.

Water soaking

Seeds were soaked in 400 ml of deionized water for their designated treatment length prior to commencing the post treatment procedure. Environment Canada (2014) proposed that immersion for 30 days was a sufficient to enable penetration of the seed coats.

Nut cracker

Seeds were compressed with a Kuraidori® chrome-plated zinc alloy nut/lobster cracker. Seeds were placed between grooves in the primary jaw (closest to the fulcrum). The orientation was such that the outer, widest 'edges' of the seed were the primary point of contact during the treatment. When seeds were initially placed in the instrument, the gap between the terminal ends of the arms was measured. In order to standardize this treatment, all the compressions were relative to the size of each seed based on the distance between the arms of the nutcracker. This was done to ensure that the testa of each seed was compressed by the same amount, regardless of seed size. This measurement was used to calculate the appropriate compression distance for each respective seed/trial, which were as follows:

Low: compressed by ~10% of total diameter

Medium: compressed by ~15% of total diameter

High: compressed by ~20% of total diameter

Compression distances are based on the average maximum physical tolerance of the seeds (i.e. the maximum compression amount without critically damaging or destroying the seed embryo). Measurements were approximate as the trial aimed to investigate the

outcome of a range of compression thresholds with potential to crack open the seed coat without damaging the embryo.

Preliminary trials on 10 seeds were conducted to determine the maximum tolerable compression distance without destroying the embryo. This break point was at approximately 30% of the seeds diameter, which is why the trial values were established as 10%, 15%, and 25%. All measurements were conducted with a Lufkin® 25XL measuring tape.

Post-treatment procedure

I used n=30 seeds for each level within each treatment; yielding n=90 across for each treatment; and n= 540 across all treatments (including n=90 for each of the positive and negative controls). Immediately following treatment, seeds were submerged in room-temperature deionized water for 24 hours to facilitate imbibition. Each 10-seed cohort (the ten seeds from each treatment level) was wrapped in two 30cm x 30cm sheets of paper towel, saturated with 80ml of deionized water, and placed in 18cm x 9cm x 9cm glass Mason jars. These were placed in a greenhouse at the Trent University Environmental Science Building from February 22nd 2017 to March 24th 2017 at 22.1°C (±3.34 °C) (collected with a HOBO MX2300 External Temperature/RH Sensor Data Logger) under a 16:8 lighting regime. Seeds were checked daily for radicle protrusion which signals the start of germination, and thus the endpoint of this experiment (Finch-Savage *et al.* 2006; Koch *et al.* 2010; Smýkal *et al.* 2014). The experiment was terminated after 30 days, which was considered a conservative length of time as the expected time window for radicle protrusion is 2-10 days post-treatment (Fordham 1965).

2. Freeze and Thaw trials

The goal of these trials was to emulate the freezing and thawing regimes associated with a temperate North American winter, as this may serve as a scarification agent for northern populations of *G. dioicu*s. The experiment included a comparison of seeds that were retained within seed pods and seeds that had been removed from seed pods, as the potential effect of seed pods on the outcome of this experiment was unknown. The trial was run for 60 days which was meant to emulate the average permafrost period of a temperate North American winter (or at least the average amount *G. dioicus* would experience given its current range). The 60 day period took place from (18/09/2017 – 18/11/2017), and was conducted a walk-in freezer maintained at -20ºC.

Given that a number of Eastern North American tree species require cold stratification for successful germination (e.g. *Prunus serotina* (Esen *et al.* 2007), *Carya cordiformis* (Vandevender 2014), *Juglans nigra* (Vandevender 2014)), it seemed appropriate to test the reaction of *G. dioicus* seeds to a series of freezing trials. Four distinct treatment groups were used in order to evaluate any potential effects of the seed pod and water submersion. Freeze-thaw germination trials of post-dormancy *G. dioicus* seeds were done with the following treatment groups:

Pod-Dry freeze: Seeds were left in pods, not immersed in water. n=50.

Bare-Dry freeze: Seeds were removed from pods, not immersed in water. n=50.

Pod-Wet freeze: Seeds were left in pods and immersed in 3L of DI water. n=50.

Bare-Wet freeze: Seeds were removed from pods and immersed in 3L of DI water. n=50.

Positive and negative controls were conducted in the same way as the mechanical and osmotic scarification trials (n=50 for each). The seeds used for these trails were taken from the test bank (collected during the fall of 2016) and were maintained at room temperature in a laboratory setting during the interim (~11months).

All freezing trials included a three-day thaw period and had their seed pods removed (where applicable) prior to initiating the post-treatment procedure. All seeds underwent the same post-treatment procedure as the mechanical and osmatic trials with a single modification: the 30-day germination period occurred at room temperature (~22°C) in a laboratory setting as opposed to the greenhouse utilized previously.

Population Genetics

Field collection protocol

Coordinates for extant Canadian stands of Kentucky coffee trees were obtained from the databases of the Essex Region Conservation Authority, the St. Clair Region Conservation Authority, Parks Canada, Ontario Parks, and the Natural Heritage Information Centre (Ontario Ministry of Natural Resources and Forestry). The tissue samples were collected from 31 stands, 16 of which were natural and 15 were planted (as per the documentation provided by the relevant authorities) (Table 3). All Canadian tissue samples (natural and planted) were obtained from individuals in the field.

Field collections began with a preliminary search for a stand based on coordinates provided from each agency. Once located, a visual search was conducted; if trees were not immediately located, a search was conducted within a 30 m radius. For field collection purposes, a “stand” was defined as an area within which trees grew within ~30 meters of a conspecific(s) (NOTE: this rule was not applied to East Sister Island and

Middle Island as these were treated as single stands, regardless of distance between conspecifics).

The clonal nature of *G. dioicus* means trees tended to grow in highly dense 'clumps' as opposed to being evenly distributed across the spatial extent of each site. Due to this, sites were sampled such that all 'outer' trees (i.e. those trees that marked the periphery of the stand), regardless of size class, were sampled. In order to avoid repeated sampling of ramets from the same genet (clone), subsequent sampling within this perimeter targeted only mature trees (>10cm DBH).

In total, leaf tissue samples from 394 trees from 31 sites (combination of planted and natural) were collected between the dates of June 6th 2016 and July 14th 2016. Sites were distributed throughout Essex and Lambton County in Southwestern Ontario (Table 4; Figure 1). Trees were identified as planted based on documentation provided by the Essex region conservation authority and the St. Clair region conservation authority. During the summer of 2017 a full inventory of all stems present at all natural sites was taken in order to generate size class profiles for these stands. In some cases, more trees were located during the 2017 inventory than the 2016 sampling effort.

Trees were placed into bins based on their diameter at breast height (1.3 meters). The bins were as follows: 1-9cm, 10-19cm, 20-29cm, 30-39cm, 40-49cm, 50-59cm, 60-69cm, 70-79cm, 80-89cm. Trees with height <2m were placed into one of two categories: <16mm or 16mm-2000mm. This was done to quantify the size class composition of each stand.

Table 3. Estimated stand size and number of trees sampled across all Southwestern Ontario collection sites. Sites are designated as planted or natural as per information provided by the Essex Region Conservation Authority (Dan Lebedyk, ERCA pers. Comm., 2016), the St. Clair Region Conservation Authority (Tim Payne, SCRCA pers. comm., 2016), and Environment Canada (Environment Canada, 2014). Tree sampled refers to the number of trees sampled in each stand. Number of trees surveyed refers to the total number of stems counted during the 2017 survey. Roadside refers to any stands which were found in roadside ditches or otherwise marginalized into a linear spatial pattern. Forest stand refers to sites which are not restricted by roads or other anthropogenic disturbance.

DATA GROUPING	*LETTER DESIGNATION*	*NUMBER OF TREES (2017 SURVEY)*	*TREES SAMPLED (2016)*	*SITE TYPE*	*NATURAL/ PLANTED*
ESSEX	***AD***	160**	5	Roadside	Natural
ESSEX	***AE***	76	6	Roadside	Natural
ESSEX	***AF***	6	3	Roadside	Natural
ESSEX	***AG***	14	18	Forest stand	Natural
ESSEX	***AH***	83	6	Forest stand	Natural
LAMBTON	***AV***	94	5	Forest stand	Natural
LAMBTON	***AW***	392	18	Forest stand	Natural
MIDDLE ISLAND	***AY***	Not accessible	66	Forest stand	Natural
ESSEX	***BB***	294	3	Roadside	Natural
ESSEX	***BC***	120	27	Roadside	Natural
ESSEX	***BD***	235	33	Roadside	Natural
LAMBTON	***BK***	1	1	Roadside	Natural
LAMBTON	***BO***	97	12	Forest stand	Natural
LAMBTON	***BQ***	1	1	Roadside	Natural
EAST SISTER ISLAND	***ES***	Not Accessible	79	Forest stand	Natural
LAMBTON	***BP***	18	3	Forest stand	Natural
N/A	***AA***	-	3	-	Planted
N/A	***AB***	-	5	-	Planted
N/A	***AC***	-	2	-	Planted
N/A	***AS***	-	1	-	Planted
N/A	***BR***	-	72	-	Planted
N/A	***BS***	-	4	-	Planted
N/A	***BT***	-	29	-	Planted
N/A	***BU***	-	6	-	Planted
N/A	***BV***	-	2	-	Planted
N/A	***BW***	-	2	-	Planted
N/A	***BX***	-	1	-	Planted

N/A	***CA***	-	6	-	Planted
N/A	***CB***	-	8	-	Planted
N/A	***CC***	-	6	-	Planted
N/A	***DA***	-	1	-	Planted

Note: all mature trees at site "AD" were cut down between the summer of 2016 and 2017, the number obtained during the 2017 survey reflect the number of root suckers found at the site.

Table 4, Size class profiles for natural Canadian *G. dioicus* stands. Size classes are based on diameter at breast height (for trees >200cm tall).

	Height		***Diameter at breast height***									
SITE CODE	**<16mm**	**16mm-2000mm**	**1cm-9cm**	**10cm-19cm**	**20cm-29cm**	**30cm-39cm**	**40cm-49cm**	**50cm-59cm**	**60cm-69cm**	**70cm-79cm**	**80cm-89cm**	***TOTAL***
BC	19	26	31	23	16	5						120
BD	19	29	115	51	18	3						235
AD	72	85	3									160
AE	4	38	23	10	1							76
AF	0	2	3	0	1							6
BB	93	175	26									294
AH	5	59	7	6	2	1	2	1				83
AG			3	1	5	2		2		1		14
AV		9	34	31	16	4						94
BP		4	9	4	1							18
AW	17	111	169	40	20	11	12	10	1		1	392
BO	10	31	16	15	20	5	0					97
BK					1							1
TOTAL	239	569	439	181	101	31	14	13	1	1	1	1590

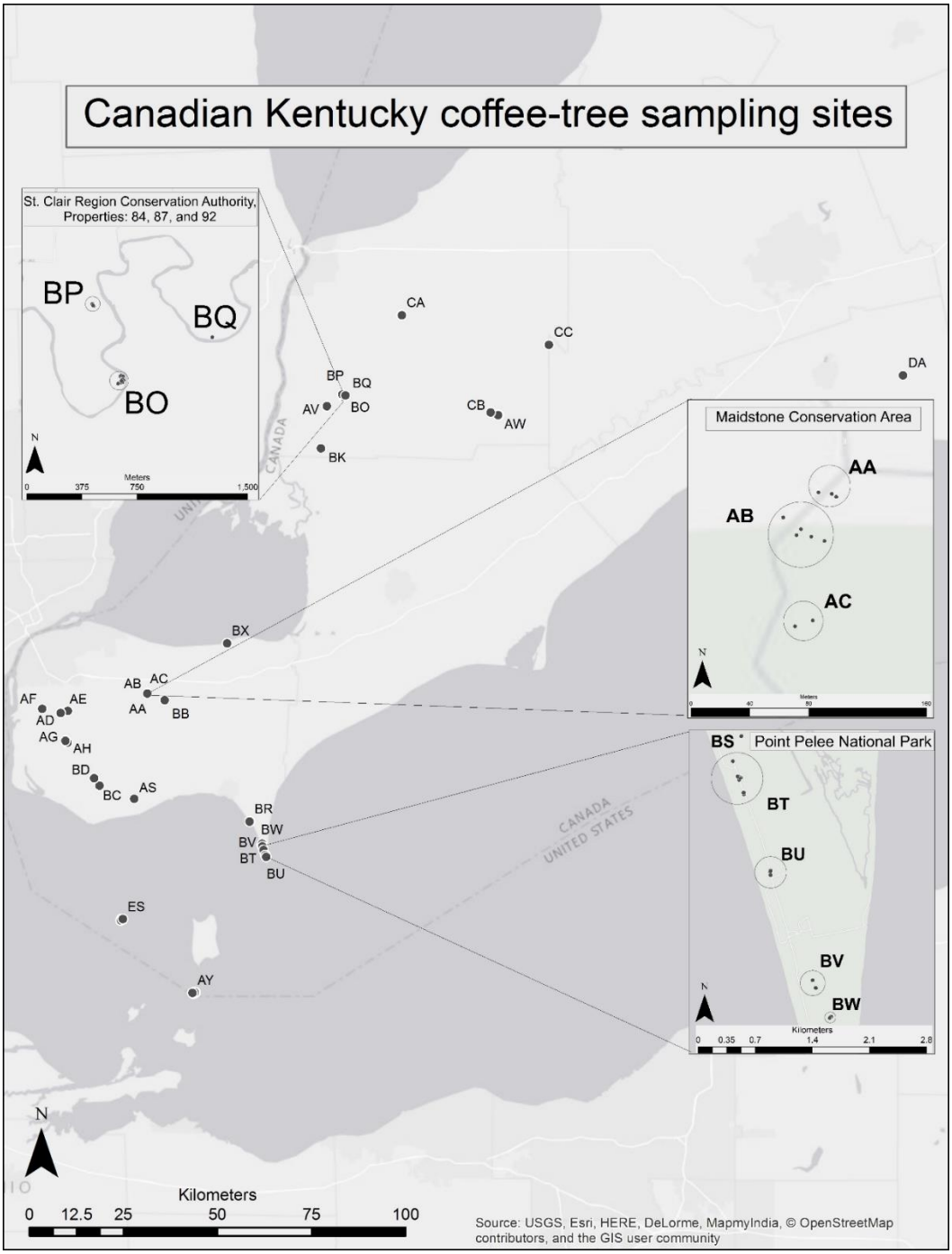

Figure 1, Map of *G. dioicus* sampling sites in Southwestern Ontario. For full site names refer to Table 4.

A UTM waypoint was collected for each sampled tree using a Garmin GPS 64 handheld GPS unit. Diameter at breast height (DBH; 130 cm from base of trunk) was measured for all sampled trees whose trunk did not fork below 130cm (~98% of sampled

trees) Five leaflets were collected from each sampled tree and immediately placed into separate Staples® Kraft coin #1 envelope for temporary storage. After no longer than six hours, leaflets were transferred from envelopes to Ziploc® Brand Snack Bags (16.5cm x 8.2cm) with approximately 100 ml of eCompressedAir® orange to Colorless silica gel beads. The leaflets remained in the bags for 24h to ensure complete desiccation, at which point they were transferred to Ziploc® freezer bags and stored at -20°C.

American tissue samples

Collaborator Jeffery Carstens at Iowa State University germinated and planted seeds collected from natural stands in the USA (Figure 1). In January 2017 (9 weeks post planting), leaflets from 140 saplings were sampled by clipping 1 or 2 leaves at the base of the rachis. Approximately seven leaflets from each sapling were removed and placed in (9 cm x 16.5 cm) coin envelops. Within 30 minutes of harvest, samples were placed into a VirTis^{R} "Freezemobile 25 EL" for freeze drying. After 72 hours in the freeze dryer, samples were shipped to Trent University where they were stored at -20°C in the Freeland laboratory until DNA extraction. The seeds were grouped by the Water Resource Region from which they were collected (Figure 2). The American Water Resource Regions are the broadest of the 6 hydrological units delineated by the United States Geological Survey. The hydrological units are sorted according to a hierarchal system consisting of: Region, Subregion, Basin, Subbasin, Watershed, and Subwatershed (United States Geological Survey 2017). The American samples were collected such that no two samples were taken from any single stand, which precludes any population-level analysis. Despite these restrictions, the American samples were delineated by drainage region, as the hydrochorous nature of *G. dioicus* may have caused some level of

differentiation between these areas. The two-digit Hydrological Unit Codes (HUC) for the regions used in this investigation were as follows: 11 (Arkansas Red-White), 08 (Lower Mississippi), 10 (Missouri), 04 (Great Lakes), 05 (Ohio), 07 (Upper Mississippi).

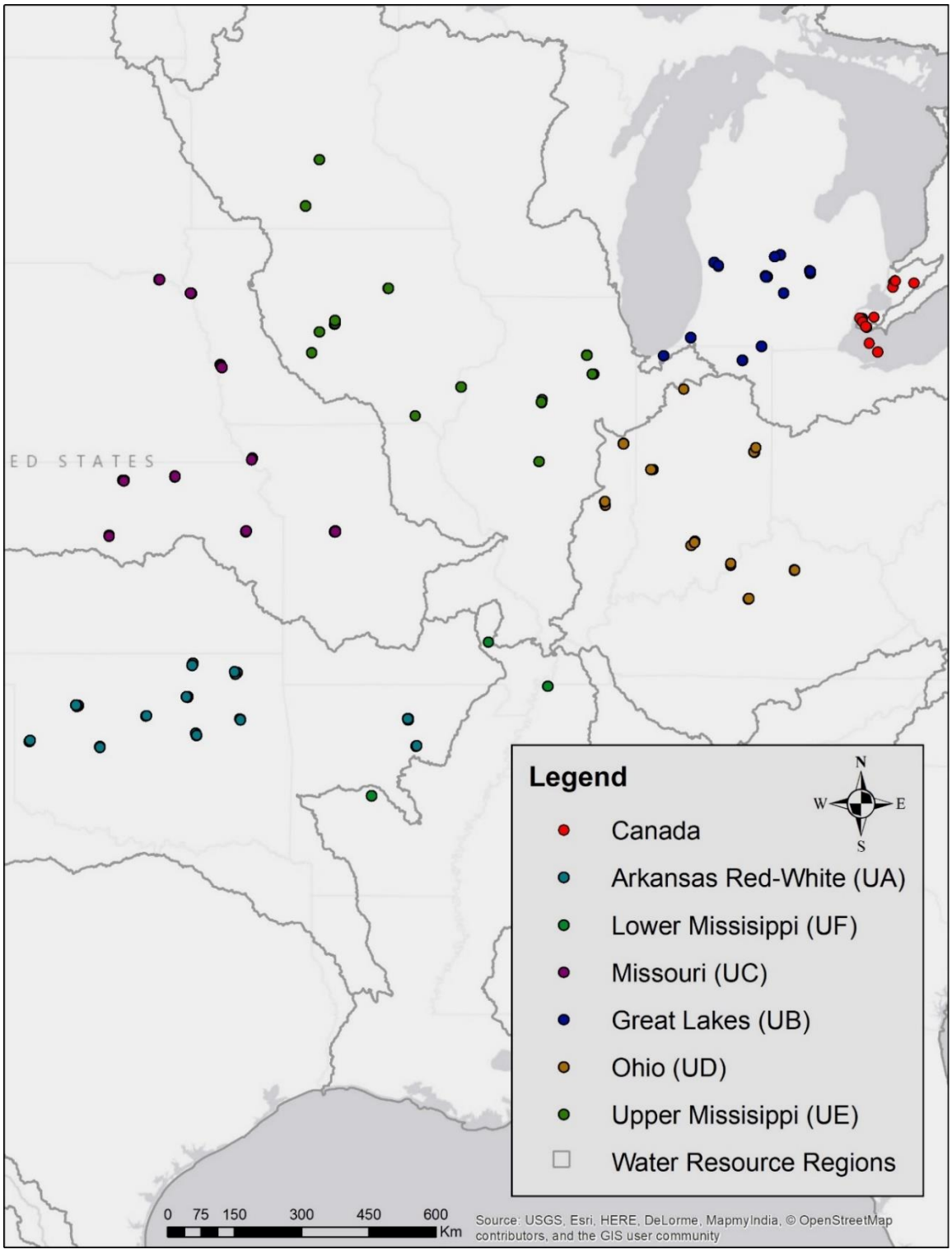

Figure 2, Locations of all American tissue sample acquisitions delineated according to HUC 2 Water resource regions.

Extraction/Genotyping protocol

Approximately 0.2 g of dried leaf from each sample was ground into a semi-fine powder using a MM300 Retsch mixer mill (Haan, Germany). DNA from leaf samples were extracted using E.Z.N.A Plant DNA Kit (OMEGA Bio-tek) following manufacturer's protocol D3485-02. Extractions were visualized on an agrose gel.

Novel microsatellite markers were developed and optimized for characterization via PCR and genotyping on the ABI. Genomic DNA from leaf samples obtained from a single tree in Peterborough, Ontario, was sequenced in a single shot-gun pyrosequencing reaction on a Roche 454 GS Junior (Hoffman La-Roche Ltd., Basel, Switzerland), using a full plate PicoTiterPlate Kit. Sequence data were compiled into contigs in GS Assembler v. 2.5p1/2.5.3 following the manufacturer's protocol. In order to maximize the reliability of our sequence data, only the multiple consensus contigs from the GS assembly were explored for microsatellite repeats. MSATCOMMANDER 0.8.2 (Faircloth 2007) was used with default settings to identify di-, tri-, and tetranucleotide loci with a minimum of six, four, and four tandem repeats, respectively, within the consensus contigs assembly. The output file was exported to Microsoft Excel and the resulting SSR repeats were manually filtered according to their category. Primer pairs were designed using the online version of Primer3Plus Version: 2.3.0 (Utergasser et al. 2007). Primers were designed to amplify products that were 200 – 450 bp in length, using an optimal annealing temperature of between 57 °C and 64 °C. Primer pairs were designed for a total of 22 microsatellite loci; of these, alleles from nine microsatellite loci could be reliably and repeatedly genotyped (Table 5).

Each primer pair was tested using gradient polymerase chain reactions (PCR) with varying annealing temperatures (Table 5). Each PCR included 1x Dream Taq Buffer (Thermo Scientific), 0.5 U of DreamTaq (Thermo Scientific), 0.2mM dNTP (Thermo Scientific), 0.2mm of each primer, and 15.3µL of ddH_20 with 20-30ng/µL of DNA for a total volume of 20µL. PCR negatives were based on the same cocktail but with water instead of DNA. The gradient PCR cycling conditions began with 28 cycles of an initial denaturation at 95°C for 30 seconds, the gradient annealing temperature range shown in Table 5 for 1 minute, and extension at 68°C for 1 minute. The final step was extension of 68°C for 5 minutes and a hold at 4°C.

The PCR product was then run on a 1% agarose gel made using TBE buffer. Bromophenol blue (Thermo Scientific) was used as a loading dye, and a 100bp ladder (FroggaBio) was used to estimate the sizes of the amplicons. Gels were stained with SYBR-Safe (Thermo Scientific), and run for thirty-five minutes at 90V and 0.83 amps.

Once optimal PCR conditions had been identified, fluorescently labelled primers (Table 5) were then used to amplify DNA using the same PCR reagents as mentioned above. The cycling conditions began with a 5 minute initial step at 95°C, followed by 28 cycles of an initial denaturation at 95°C for 30 seconds, the optimized annealing temperature (Table 5) for 90 seconds, an extension step at 72°C for 30 seconds. The final step was extension of 72°C for 30 minutes and a hold at 4°C. 5 µL of the PCR product was then run on a test gel to ensure that the PCR was successful.

Samples were genotyped with an ABI 3730 (Thermo Scientific), using internal lane Rox 500 size standards (Thermo Scientific). Before submission to ABI, samples underwent ethanol precipitation to remove salts and by-products. Optimal dilution ratios

were determined using different dilutions (1 in 16, 1 in 32, 1 in 64) on the pooled combinations using a subset of samples. A qualitative comparison was used to determine which dilution yielded the clearest electropherograms, i.e. high enough to score, but not so high as to have indistinctive peaks. GeneMarker V1.91 software was used to determine the sizes of the amplified alleles.

Table 5, Primer names, sequences, expected product size, PCR gradient temperature ranges and optimized annealing temperature for all loci.

Primer Name	*Primer sequence*	*Fluorescent label*	*Expected Product Size*	*Gradient PCR Temperature Ranges (°C)*	*Optimized Annealing Temperature (°C)*
KC2-2F	TATGGCGGTTTATAGGGCTGAA	Hex	239	57-58	57
KC2-2R	CACGCCAACTTGATCCCTTCT				
KC3-2F	CTTGGTACGTGCATCGCAAAC	Hex	374	59.5-63	60
KC3-2R	GGACGCCTATGAAATGGGTT				
KC4-1F	ACCCTTCATCGGTTGCCATAA	Fam	434	58-59.5	60
KC4-1R	TTTGGAGTGCACACGACAGC				
KC7-2F	AGGGTGGTGATCTTGCCTAC	Fam	148	61-63	60
KC7-2R	ACTCCCTCAAGAGCATTTCGG				
KC8-1F	AGCACGGTTTAAAAATGGGACT	Hex	313	58-60	60
KC8-1R	GATGATGCAGGGAACCACGA				
KC9-1F	CAAGCCCTTAACTTCAGCAAATCA	Hex	376	57-62.5	60
KC9-1R	GAATGGGCCTCATGTAGCAAGG				
KC10-1F	CCGAGAGAACTCGCGATGAG	Hex	390	59-62.5	60
KC10-1R	TCCGGTAATGCAGTCAAGCA				
KC11-1F	AAGCATGATGGAGTAGCAGTT	Fam	243	58-61	60
KC11-1R	AGTGTTGTACTCACCCCTCA				
KC12-1F	AAGACACTTTGGCGTCGTTT	Fam	356	60-62.5	60
KC12-1R	ACCAGAATCTAAAAACAAACCTGGC				

Population genetics data analysis

Due to high levels of genetic similarity and low sample sizes, Canadian stands were grouped such that all trees in Lambton county and Essex county were pooled together, despite there being 6 and 8 distinct stands in these counties, respectively. Middle Island and East Sister Island were treated as separate data groupings due to their large sample sizes and spatial isolation from each other and mainland samples. The planted and U.S. samples were also pooled into their own groups. This was done for the purposes of comparing overall differentiation between these data groupings and the range-edge Canadian populations. Although USA samples were taken from a large geographic region, these data were pooled because of low samples sizes at several loci (Appendix, Table 15). Pairwise Fst values were calculated between each of the data groupings using GenAlex version 6.5. This was calculated between data groupings (Essex, Lambton, Middle Island, East Sister Island, USA, and Planted).

Chapter 3: Results and Discussion

Results

Germination results

Pilot study

Table 6, Number of successful germinations during pilot study among donor trees. All seeds underwent the sulphuric acid treatment (methods). From the pilot study, it was determined that all of the donor trees produced seeds that were approximately equal in viability. These results were utilized to justify amalgamating all of the seeds into the test bank

Individual	# of successful germinations
DB01	9/10
DB02	10/10
DB03	8/10
DB04	8/10
DC01	9/10

Mechanical and Osmotic scarification trial results

The negative and positive control (sulphuric acid) showed 0% and 100% germination respectively. The Fire trials showed a noticeable increase in germination rate from 15 second exposure (3.33%) to 75 second exposure (20%). The nut cracker trials exhibited a similar trend, with a noticeable increase in germination between the 10% compression trial (3.33%) and the 20 percent compression trial (43.33%). None of the seeds in the remaining treatments (Hammer blows, Water soaking, Rock tumbler) germinated.

Although the flame exposure trials yielded germinations in all treatment levels, there were a number of imbibed seeds that did not display radicle protrusion and

therefore were not included in the germination totals. A number of fire-treated seeds showed signs of fire damage to their hilum. After 2-3 days of stunted development, the seeds developed a rancid aroma and were presumed to be unable to continue the germination process. These seeds were counted as 'hilum burns" and did not contribute to the total germination numbers displayed in Figure 3. In total, 6 hilum burns occurred in fire 45, while fire 75 had 11.

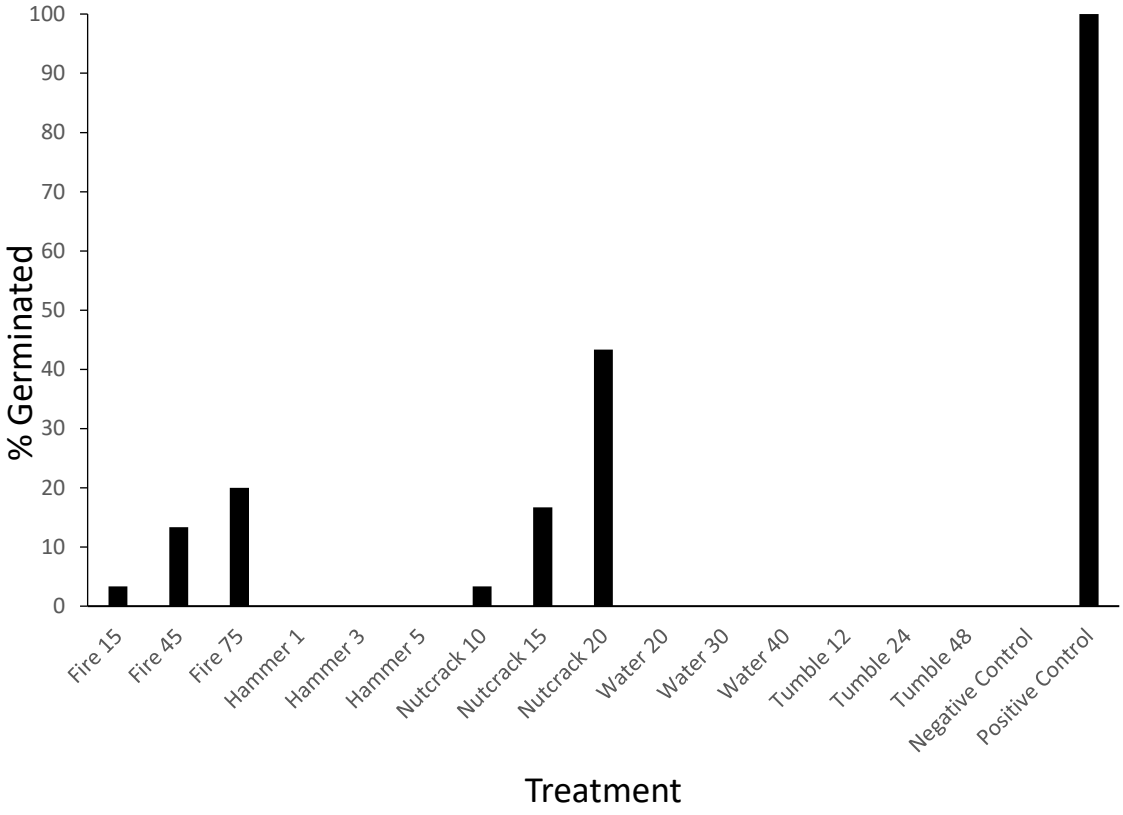

Figure 3, Percent germination of *G. dioicus* seeds across all treatment levels. n=30 for each treatment level. n=90 for the negative and positive (sulphuric acid) control group.

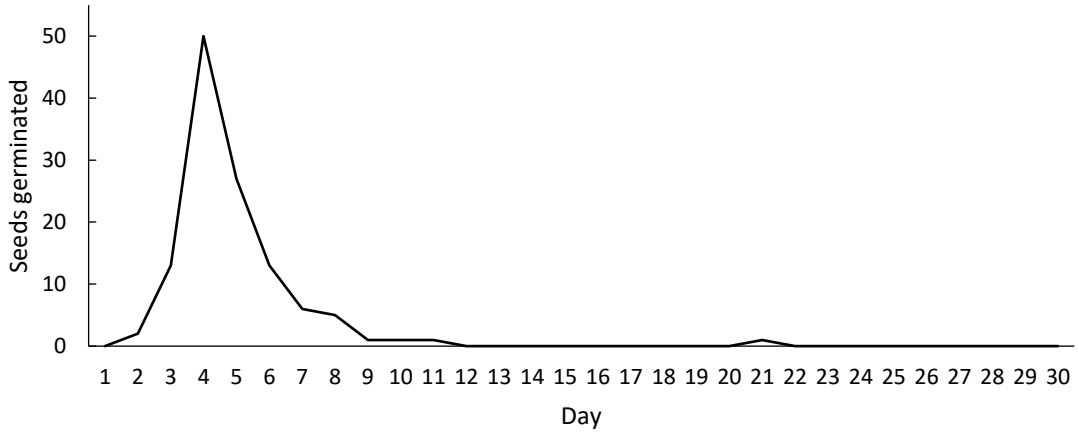

Figure 4, Number of seeds germinated across all trials over a 30 day testing period.

A single germination occurred on the 19th day in Nutcrack 20; all other germinations (119/120) occurred within 11 days following post treatment (Figure 4). A large majority (85.83%) of the germinations occurred between Day 3 and Day 6, with the most occurring on the 4th day (41.67%).

Freeze and thaw trial results

The positive control group showed a 98% germination success rate (49/50 successful germinations) while the negative control and the treatment groups did not lead to any successful germinations.

Population Genetics Results

All loci ranged from 1-3 alleles per locus, which is considerably lower than that of other eastern hardwood species (Table 7).The average number of alleles per locus was similar for trees sampled in Canada and the USA (Table 8). Levels of observed heterozygosity were greater in Canada (0.0784) than the USA (0.0449) except for loci KC4-1 and KC12-

1 (Table 9). Most loci showed observed heterozygosity values of 0. Non-zero values for observed heterozygosity were <0.176, except for Canadian samples at locus KC2-1, which was 0.386. The number of unique genotypes among data groupings (data groupings described in Table 9) were between 3-7, except for Essex and UF which had 11 and one genotype(s), respectively. 22 unique genotypes were observed across all naturally occurring trees ($n_{natural\ USA}$ = 141, $n_{natural\ Canada}$ = 314) (Table 3). Range-wide F_{ST} across all data groupings was very low (Average pairwise F_{ST}= 0.05, SE= 0.00802) (Table 11). USA Data groupings (UA, UB, UC, UD, UE, UF) were grouped due to low sample sizes at several loci (Appendix, Table 15). Two relatively high F_{ST} values occurred between East Sister Island and Essex (0.105), and East sister island and Middle Island (0.121).

There were 3 private alleles between Canada and the USA. [KC2-1] 247, was found exclusively on East Sister Island with no representation in the USA. [KC12-1] 356 occurred once in a single individual in "UC" with no representation in Canada. [KC7-2] 142 occurs at site "BD" in Canada with no representation in the USA (results, Figure 2).

Among planted samples genotyped in this study the allele frequencies largely mirrored those of natural trees. There is a noteworthy appearance of a new allele [KC7-2] 148, which has no representation in either Canada or the United States samples.

Table 7, Number of individuals, populations, loci, and alleles per locus of Microsatellites developed for 5 other eastern hardwood species.

	# of individuals	*# of populations*	*# of Loci*	*Alleles per locus*	*Citation*
G. dioicus	314	16	9	1-3	N/A
Quercus rubra	10	1	14	4-13	Aldrich *et al.* 2002)
Gleditsia triacanthos	36	28	14	3-20	Owusu *et al.* 2013
Acer saccharum	47	1	20	1-10	Harmon *et al.* 2017
Juglans nigra	1250	43	12	9-46	Victory *et al.* 2006
Robinia pseudoacacia	39	1	11	4-12	Mishima *et al.* 2009

Table 8, Proportion of heterozygotes and number of alleles across 9 microsatellite loci for natural Canadian and American populations of *G. dioicus* (n = 314 and n = 141, respectively). Ho = observed heterozygosity, He = expected heterozygosity.

	Canada			***USA***		
Locus	**# of alleles**	**Ho**	**He**	**# of alleles**	**Ho**	**He**
KC2-1	3	0.386	0.546	2	0.16	0.492
KC3-2	3	0.155	0.223	3	0.0482	0.0703
KC4-1	3	0.158	0.178	3	0.175	0.272
KC7-2	2	0.00654	0.0194	1	0	0
KC8-1	1	0	0	1	0	0
KC9-1	1	0	0	1	0	0
KC10-1	1	0	0	1	0	0
KC11-1	2	0	0.207	3	0	0.248
KC12-1	1	0	0	2	0.0208	0.0206
Average	1.888	0.0783	0.130	1.888	0.0448	0.122

Table 9, Number of unique genotypes across all data groupings of Canadian and American *G. dioicus* (Planted samples were not included in this comparison).

Country	Data Grouping	# of Unique Genotypes
Canada + USA	-	22
Canada	-	14
	Essex	11
	Lambton	5
	Middle Island	3
	East sister Island	5
USA	-	16
	UA	6
	UB	4
	UC	7
	UD	4
	UE	3
	UF	1

Table 10, Allele frequency and observed heterozygosity of 9 microsatellite loci among planted *G. dioicus*. Samples were obtained from various planted accessions in SW Ontario with the addition of 2 samples taken from Europe. n = 148.

Loci	***Ho***	***Allele***	***Allele frequency***
KC2-1	0.6	247	0.0188
	-	250	0.625
	-	253	0.356
KC3-2	0.106	372	0.0529
	-	374	0.947
KC4-1	0.271	430	0.159
	-	432	0.836
	-	434	0.00467
KC7-2	0.0122	144	0.994
	-	148	0.00609
KC8-1	0	313	1
KC9-1	0	377	1
KC10-1	0	386	1
KC11-1	0	247	0.96
	-	249	0.04
KC12-1	0.00962	356	0.00481
	-	358	0.996

Table 11, Pairwise Population Fst values for all data groupings. NOTE: American data groupings (UA, UB, UC, UD, UE, and UF) were pooled because of low sample sizes. All planted trees were included as a single data group.

	EAST SISTER ISLAND	ESSEX	LAMBTON	MIDDLE ISLAND	PLANTED	USA
EAST SISTER ISLAND	0.000					
ESSEX	0.105	0.000				
LAMBTON	0.079	0.063	0.000			
MIDDLE ISLAND	0.121	0.042	0.054	0.000		
PLANTED	0.037	0.028	0.031	0.031	0.000	
USA	0.054	0.021	0.044	0.038	0.006	0.000

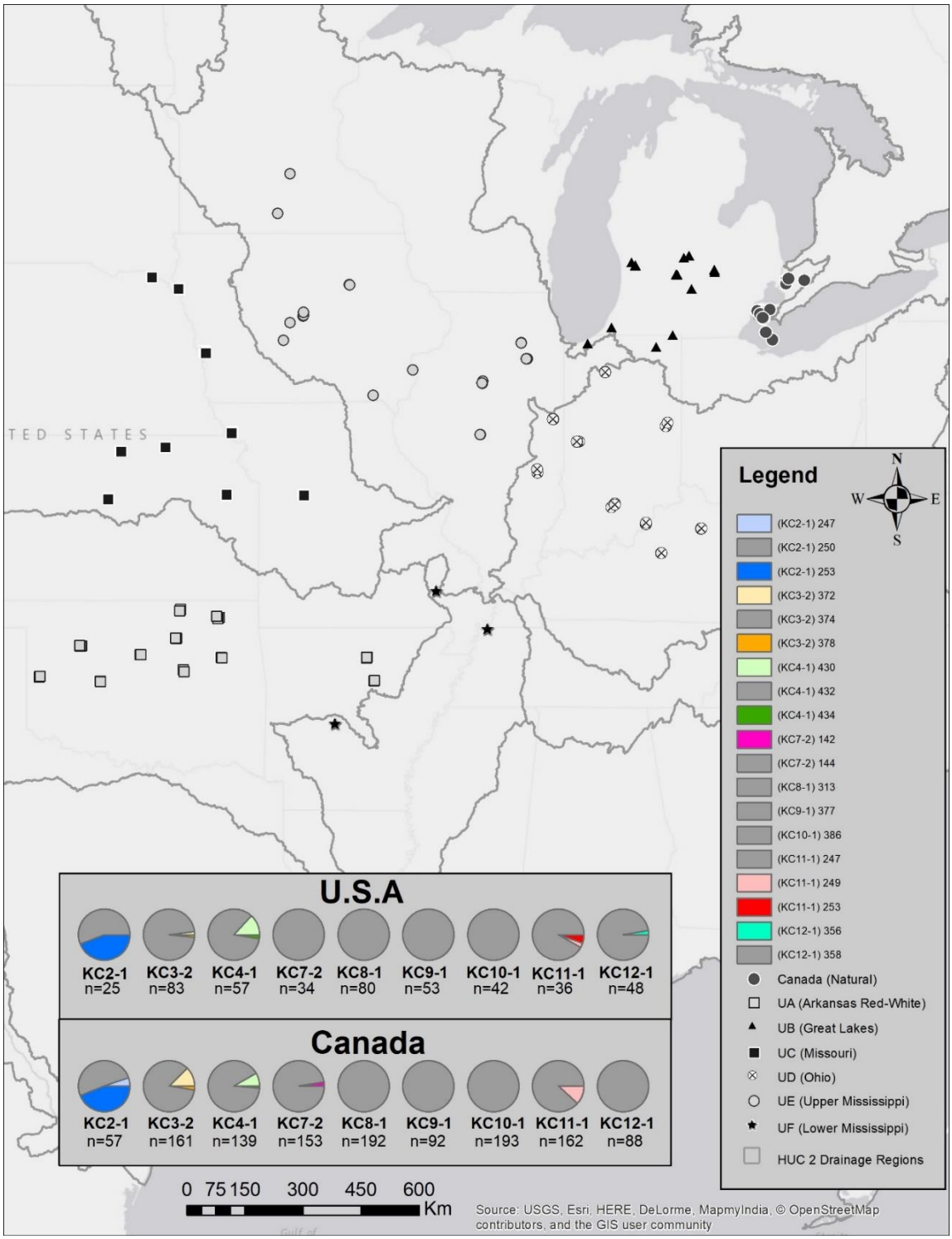

Figure 5, Allele frequencies at 9 microsatellite loci across all Canadian and U.S. *G. dioicus* samples. Low frequency alleles denoted in color while high frequency alleles are shown in greyscale. For the most part, pie charts reflect allele frequencies; the exception to this is very low allele frequencies (<2.5%) which were adjusted to 2.5% for visibility (see Appendix, Table 13 for true values). KC8-1, KC9-1, KC10-1 are fixed across Canadian and U.S. populations.

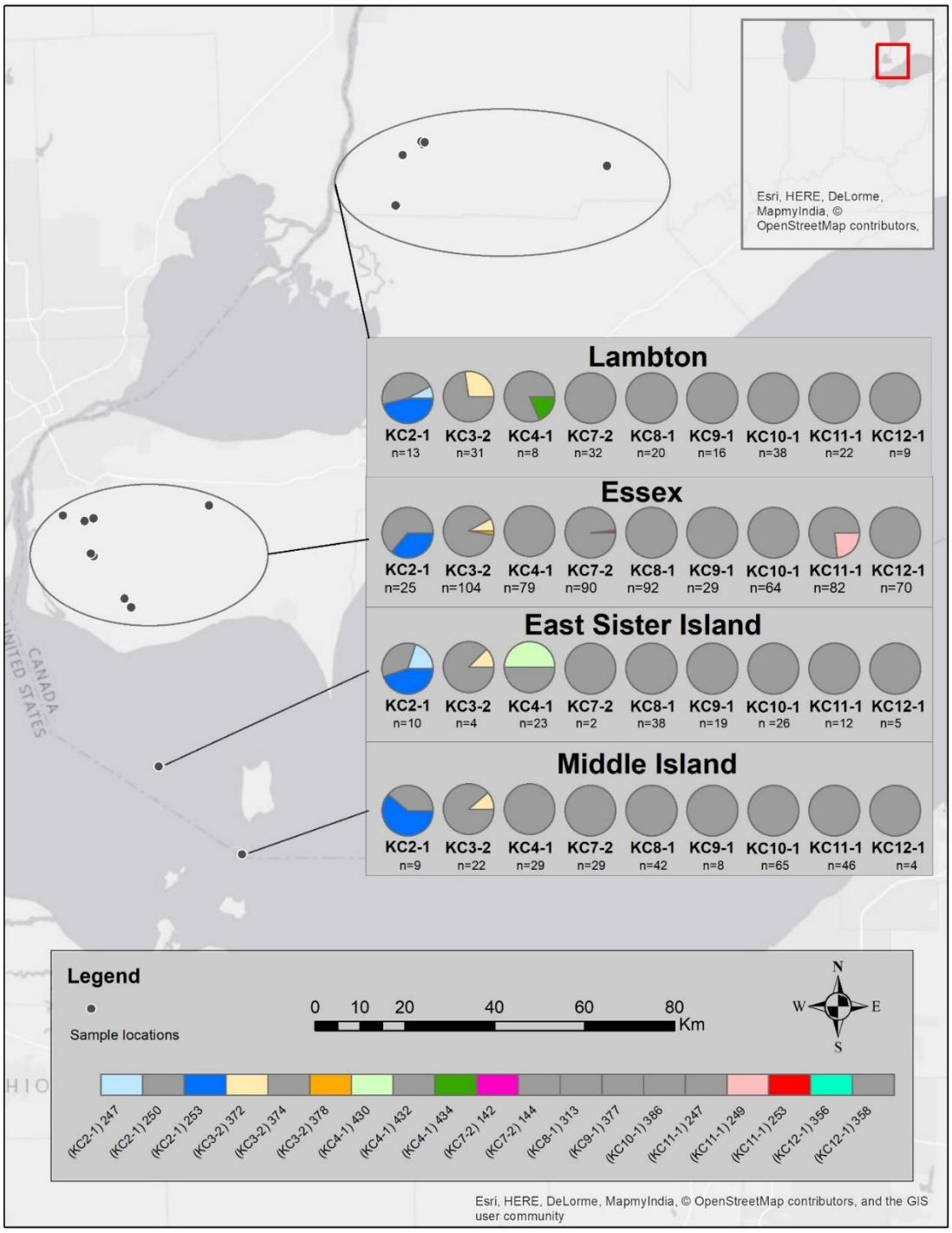

Figure 6, Frequency of alleles at 9 microsatellite loci across Canadian populations, pooled into four geographic groupings: Essex, Lambton, Middle Sister Island, and East Sister Island. Low frequency allele ratios denoted in color while high frequency alleles are shown in greyscale. Pie chart values report observed allele frequencies, however, alleles with very low frequencies (<2.5%) were adjusted to 2.5% for visibility (see Appendix, Table 13 for true values). KC8-1, KC9-1, KC10-1 were fixed across all Canadian populations.

Discussion

This study sought to evaluate the following four aspects of *G. dioicus* ecology: (1) seed germination, (2) sexual vs clonal reproduction, (3) genetic isolation of natural populations, and (4) potential range-edge effects. Of these objectives, genetic isolation of natural populations, and range-edge effects were investigated directly, while the low resolution of the genetic markers precluded quantification of the relative rates of sexual and asexual reproduction. Scarification requirements were addressed through a series of germination experiments, from which inferences regarding wild germination rates were made. Having investigated the above four objectives, the following discussion will put each of these in the context of the contemporary ecology of *G. dioicus*.

Germination

The results of both germination trials indicated that the seeds of *G. dioicus* require scarification beyond that which freezing, tumbling, and soaking can provide. Based on its description as a riparian species, the soaking, tumbling, and freezing trials were intended to emulate scarification regimes that could be commonly encountered in nature. However, these "riparian" trials did not lead to any successful germinations, indicating that these scarification types may not make large contributions to natural recruitment, or that longer exposure times may be required. The more invasive treatments (fire, nut cracker, hammer blows) yielded varied results. The flame treatments provided sufficient scarification if the seed coat was exposed to direct flame for 15 seconds, but insufficient exposure did not lead to germination, and there were several cases in which the embryo was destroyed by flame exposure. These results indicate that fire could be helpful in scarifying the seed coat, but detrimental if the embryo is directly exposed to flame. The

nut cracker trials yielded a number of successful germinations indicating that this type of crushing force is sufficient to make the seed coat permeable to water, as opposed to the blunt force of the hammer blows which did not yield any successful germinations. Overall, the trials meant to emulate natural conditions did not yield any successful germinations, except for flame exposure. These results are consistent with the hypothesis that wild *G. dioicus* seeds are unlikely to germinate naturally, as the scarification sources it would typically be exposed to in a riparian environment do not sufficiently compromise the testa.

The seeds of *G. dioicus* experience low natural germination rates as they require damage that enables water to penetrate to the testa. Given the robust nature of the testa, sources of damage that can provide sufficient scarification are unlikely to be found in natural settings. If this is the case, scarification typical of riparian environments should yield minimal or no successful germinations.

There were several limitations to both germination trials that are worth mentioning. The open flame trial was meant to emulate a forest-fire scenario, but falls short in a couple of respects. Firstly, the duration did not exceed 75 seconds as the seed coat could not endure burning times longer than this (based on preliminary pilot study), but a real forest fire may result in exposure times longer than this. Secondly, because seeds were in all cases removed from the seed pod prior to the experiment, these trials did not account for potential protection provided by the seed pod or being covered in soil and/or detritus, as would be the case in a natural forest setting. The nut cracker trial yielded successful germinations and appeared to show a positive relationship between compression distance and germination success. However, the pressure of compression

was not constant across compression distances, as seeds varied in size (e.g. Pressure applied in the 10% compression treatment group was not the same for all seeds). The discrepancy in compression pressure within treatment groups prevented comparison of germination success among treatment groups, which was inconsequential as the goal of this trial was to investigate whether or not successful germination could be achieved by compression scarification, regardless of magnitude. The main limitation of the remaining trials (hammer blows, rock tumbler, water soaking, and freeze-thaw) was their durations, which is to say that more or less exposure to each treatment could have yielded successful germinations. Nevertheless, the wide range of treatments (and levels within each treatment) applied in this investigation suggest that germination of *G. dioicus* seeds requires fairly intrusive scarification and such events might occur infrequently in the wild.

Natural germination

It has been proposed that seasonal weathering alone can sometimes sufficiently scarify the hard seed coat of *G. dioicus* (~5% (VanNatta 2009; Environment Canada 2014)), but contemporary scarification agent(s) remain largely unknown. The results of this study are consistent with this assertion; treatments designed to mimic natural conditions did not induce germination, suggesting that natural germination might make negligible contributions to overall recruitment. In previous studies, these putative limitations to natural seed germination have sparked discussions of whether *G. dioicus* is a botanical "anachronism" (Zaya & Howe 2009). Specifically, it has been hypothesized and generally accepted that the seeds of *G. dioicus* were dispersed by now-extinct

frugivorous Pleistocene megafauna (Janzen & Martin 1982; Barlow 2002; VanNatta 2009; Zaya & Howe 2009).

Pollen limitations

Like most Eastern North American trees, *G. dioicus* flowers annually, producing inconspicuous unisexual flowers. It has been suggested that they are entomophilous, although the pollinators are unknown (Zaya & Howe 2009). Given the dioecious nature of *G. dioicus*, both male and female individuals are required for fruit production. In highly fragmented landscapes like the North American Midwest and Southwestern Ontario, this often requires that males and females be in close proximity, or at least found within the same habitat patch, as insects tend to remain within patches while pollinating (Vranckx *et al.* 2011). Situations where males and females do not co-occur could lead to pollen limitation of sexual reproduction, making vegetative propagation the only mechanism that can maintain populations in isolated, unisexual populations. These limitations imply that single-clone stands are unlikely to produce fruits. In Canada, at least 4 populations are known to contain both male and female trees, all of which are assumed to be capable of sexual reproduction (Environment Canada 2014). Of those 4 populations, 2 were sampled in this study (East Sister Island – "ES" and Canard River Floodplain – "AH"+"AG"). Additionally, site "BD" was identified as a fruit producing stand which was not previously recognized. The remaining 21 populations are assumed to be unisexual (based on their absence of fruit), which could be due to permanent removal of trees by past stochastic events and/or infrequent colonization. This high incidence of unisexual populations compounds the aforementioned pollen limitations, leaving little opportunity for fruit production.

The Pawpaw (*Asimina triloba*) is another eastern tree species which shares the pollen limitations brought on by habitat fragmentation. Unlike *G. dioicus*, the flowers of *A. triloba* are bisexual (protogynous) with dichogamous flowering patterns (Losada *et al.* 2017), meaning *A. triloba* is capable of self-fertilization, while *G. dioicus* is not, however the protogynous nature of *A. triloba* flowers limits the opportunity for self-fertilization (Willson *et al.* 1980; Peterson 1991). This limited opportunity for self-fertilization almost necessitates outcrossing for fruit production as fruit production appears infrequently in scenarios where conspecific pollen is not available (Willson *et al.* 1980; Lagrange & Tramer 1985). Furthermore, it has been demonstrated that progeny produced by selfing do not compete well with outcrossed progeny or vegetatively-produced ramets (Huang *et al.* 1998)**.** As a shade-tolerant understory species, *A. triloba* occupies a much different niche than the shade-intolerant *G. dioicus*, but the predominately clonal nature of both species indicates that the pollen limitations experienced by *A. triloba* might be similar to that of *G. dioicus*. A population genetics assessment of wild *A. triloba* populations conducted by Wang *et al.* (2005) using AFLP markers indicated below average levels of genetic diversity within populations and high levels of differentiation among populations, and attributed reduced within-population diversity to high levels of clonal reproduction.

Dispersal

In addition to pollen limitation, fragmented landscapes impose a strong restriction on seed dispersal and seedling establishment. The current methods of seed dispersal available to *G. dioicus* are limited as its unpalatable fruit are too heavy to be anemochorous, insufficiently buoyant to be hydrochorous (Zaya & Howe 2009), and too toxic to be dispersed by modern fauna (Beasley 1999; Troxel & Poppenga 2005; Fitch *et*

al. 2009; Environment Canada 2014; Row & Geyer 2014). Additionally, *G. dioicus* is capable of propagating from vegetative fragments from its lateral root runners, which can potentially become dislodged from the main root network during uprooting, flooding, or other large disturbance events. Given the aforementioned dispersal and germination limitations, it is likely that dispersal via vegetative fragments makes up a large proportion of contemporary natural dispersal. Anthropogenic cultivation and planting of *G. dioicus* as an ornamental and urban tree has become a popular practice due to its exceptional tolerance of urban conditions and lack of pathogen and defoliation issues (Barker 1986; Yanan *et al.* 2004). Although there is some resistance to its use in urban scenarios due to its toxicity, landscape plantings of *G. dioicus* have contributed to its present-day distribution.

Population genetics

Levels of genetic diversity and differentiation were low overall. Expected heterozygosity was not lower in Canadian stands, allowing me to reject the hypothesis that range edge populations have lower genetic diversity. Additionally, low genetic differentiation does not support the hypothesis that gene flow between stands is restricted. Three loci were monomorphic across all samples, while the remaining 6 loci showed a single high frequency allele accompanied by one (KC12-1 and KC7-2) or two (KC3-2, KC4-1, KC11) low frequency alleles. KC2-1 was an exception to this trend, as it showed relatively high frequencies of both allele 253 and 250. It is possible that the invariability of the loci used in this investigation could be due to the primers being newly developed, thus there was no *a priori* knowledge of their variability. However, the number of alleles per locus across all 9 loci were considerably lower than that of

microsatellites developed for other eastern trees (e.g. *Acer saccharum* (Harmon *et al.* 2017); *Juglans nigra* (Victory *et al.* 2006); *Robinia pseudoacacia* (Mishima *et al.* 2009), *Gleditsia triacanthos* (Owusu *et al.* 2013); *Quercus* rubra (Aldrich *et al.* 2002)). It therefore seems unlikely that all 9 markers would have such low variability by chance alone. Future investigations should consider higher resolution markers (e.g. SNPs) as they can provide a more accurate representation of genetic variation. Nevertheless, given the unusually low levels of genetic variation across loci, it is reasonable to conclude that genetic diversity is indeed relatively low across the natural range, indicating that *G. dioicus* likely encountered a historical genetic bottleneck(s), which will be discussed below.

Evidence of natural sexual reproduction

As noted above, sexual reproduction in many stands appears limited by the high frequency of unisexual (and likely monoclonal) stands. Although this investigation was not focused on fine-scale genetic structure, direct evidence of sexual reproduction was inferred at site “BD” (not previously documented as sexually reproductive). Specifically, all trees sampled at that site were homozygous for (KC7-2) 144, except for a single female tree (34.6cm in diameter) which was homozygous for (KC7-2) 142. There was also a single female tree (26.1cm in diameter) which was heterozygous at (KC7-2) 142/144 indicating that this tree was likely the product of a past sexual reproduction event between the 142 homozygous female and one of the surrounding homozygous males. Unfortunately, the genetic diversity of the nine microsatellite loci used in this study was too low to allow inference about relative rates of sexual versus clonal reproduction. Future investigations may consider analysing the fine scale spatial genetic

structure of this and other fruiting stands based on a much larger suite of molecular markers. Such an analysis could provide insight into actual levels of wild sexual reproduction and patterns of within-population seed dispersal (Vekemans & Hardy 2004)

A number of attributes indicate that clonal propagation is the primary reproductive mode for *G. dioicus* in Southwestern Ontario. First, spatial clustering of ramets observed during field observations is consistent with clonal expansion. Second, the high-frequency of single-sex stands and low levels of fruit-set by females preclude the production of fruit in natural stands. Third, the germination results indicate that seed propagation is unlikely in the absence of fire or high-force damage to the seed coat.

The low levels of genetic differentiation found in this study were not expected – in general, isolated populations typically contain different mixtures of alleles (Aegisdóttir *et al.* 2009). Although there were populations with different mixtures of alleles (e.g. all trees at site "AW" were fixed for [KC4-1] 372/374 while most other sites were fixed for 374/374), differential fixation of alleles does not appear to be a widespread phenomenon among natural Canadian populations of *G. dioicus*. Moreover, samples from the USA were also genetically similar to Canadian populations, however the U.S. samples were collected such that no two samples came from the same population, precluding direct comparisons between data groupings. It is worth pointing out that U.S. samples were grown from seed, meaning each U.S. sample was the product of a sexual event. Despite this, the allelic composition of U.S. and Canadian samples were similar (Results: Figure 5 and Table 8), and per-sample levels of heterozygosity were lower at most loci in U.S. samples than in Canada (Table 8).

One possible explanation for the low levels of range-wide genetic diversity found in this study is historic genetic bottleneck(s). Such events would be expected to result in drastic reductions in the number of alleles per locus and therefore the genotypic richness in my sample. In North America, historic glacial-interglacial cycles have the potential to cause such bottlenecks, especially when species were restricted to single refugia, and given the current range of *G. dioicus*, it was undoubtedly affected by these cycles. A meta-analysis of 22 Western North America tree species conducted by Roberts & Hamann (2015) found that tree species restricted to a single refugia during the last glacial maximum exhibit negligible differentiation and low genetic diversity, as opposed to species restricted to multiple large refugia which show high levels of differentiation. This finding is consistent with the findings of this study, implying that *G. dioicus* was likely restricted to a single (or very small number of) glacial refugia during the last glacial maximum (LGM).

Biogeographic history

Although fossil evidence is lacking for *G. dioicus*, the macrofossil evidence of its most common site associates (*Juglans nigra* and *Ulmus americana*; McClain & Jackson 1980) suggests that *G. dioicus* was restricted to the lower Mississippi valley during the LGM, occupying a range with a northern extent of 35°N (Jackson *et al.* 2000). If the Mississippi valley migration occurred in *G. dioicus*, it likely caused a sizable reduction in census population size. Given the aforementioned dispersal and germination short comings of *G. dioicus*, it is likely that clonal reproduction was the predominant reproductive mode at the time of this reduction. With infrequent natural sexual reproductive events, opportunity for recombination of alleles would have been rare,

leaving alleles highly vulnerable to loss via genetic drift. Furthermore, mutation rates in trees are relatively low with long generation times (Smith & Donoghue 2008), limiting the possibility of novel allele combinations even further. A combination of the initial Mississippi bottleneck, lack of sexual reproduction, low mutation rates, and genetic drift likely caused dramatic loss of alleles, which is reflected in the genetic homogeneity observed among contemporary wild trees. It is important to note that inferences regarding the glacial range of *U. americana* and *J. nigra* have been inferred from macro fossil evidence of only a few well dated LGM study sites (Jackson *et al.* 2000; Victory *et al.* 2006), with a notable absence of sites in the mid latitudes (30°N and 33°N and east of 91°W). As future sites are discovered, more accurate LGM ranges for temperate hardwoods will emerge, but until then, we can only conclude that cold tolerant hardwoods (likely including *G. dioicus*) were confined to the lower Mississippi valley.

The population genetic data for *G. dioicus* do not show the 'typical' pattern of post glacial expansion: southern populations do not harbour novel genotypes or more genetic diversity than northern populations, as is the case with a number of European tree species (Petit *et al.* 2003). However, a recent meta-analysis conducted by Lumibao *et al.* (2017) suggests that post glacial genetic signatures in North America tree species are vastly different than those observed in the temperate regions of Europe. Lumibao *et al.* (2017) examined the phylogeography of 14 Eastern North American tree species (based on cpDNA) and 21 similar (focus on intra-genus comparisons) European tree species to test for common impacts of quaternary interglacial periods on genetic diversity in the temperate regions of both continents. Their results indicate that Eastern North American tree species do not show north-south clines in genetic diversity, nor do southern

populations harbour private alleles, as northern populations were shown to be just as genetically diverse (if not more so) than their southern counterparts. They attributed this discrepancy to the low number of migration barriers in Eastern North America relative to Europe, while also suggesting the possibility that source populations were simply closer to their northern range limits than those in Europe (i.e. shorter post glacial migration routes). The results of this study agree with these findings as *G. dioicus* shows similar patterns of genetic diversity across its range.

Dispersal by Indigenous Peoples

Assuming that *G. dioicus* was maintained in southerly refugia during the LGM, it managed to disperse broadly over the last ~10,000 years despite limitations to seed dispersal and germination. Following the extinction of its putative historic dispersers at the end of the Pleistocene, it is possible that dispersal of *G. dioicus* occurred via North American indigenous people, who used the seeds to make 'coffee', dice, and instruments (rattles/shakers) (Barlow 2002). The latter options were likely the most common uses, as dice games and music were of widespread cultural significance to Indigenous peoples during pre-colonial times (VanNatta 2009). Given the cultural importance of these seeds, Indigenous people may have contributed to their dispersal and scarification, leading to the creation of satellite populations that coincide with known transportation routes and settlements (VanNatta 2009). The mechanical scarification associated with being repeatedly shaken in a rattle, roasted for 'coffee', or having a design carved into the sides to be used as a dice could act as a form of scarification suitable for germination as these practices could be sufficient to penetrate the hard seed coat. Additionally, the habitat requirements of *G. dioicus* as a riparian species appear congruent with the river-centric

traveling patterns of indigenous people. In addition to inadvertent scarification, it is likely that the cultural importance of *G. dioicus* fueled intentional plantings of this species in areas adjacent to indigenous settlements.

A combination of inadvertent scarification and intentional planting could have contributed to the current distribution of *G. dioicus*, particularly in isolated populations like the Erie islands of Ontario or the shores of Seneca and Cayuga lakes in New York State (VanNatta 2009). Unfortunately, teasing apart natural post glacial expansion and anthropogenic dispersal appears difficult if not impossible given the low levels of genetic-marker resolution available. Indigenous people also utilized the root of *G. dioicus* for medicinal purposes (VanNatta 2009), which could also have contributed to its dispersal as *G. dioicus* is capable of propagating from root cuttings of its lateral runners.

Conservation threats

Although I found comparable genetic diversity between the range edge populations in Canada and the core range in the American Midwest, the threat of non-genetic range edge effects like: ecological marginalization, scarcity of suitable habitat, and isolated populations (Beatty *et al.* 2008) are potential issues for Canadian populations. the Spatial distributions of Canadian populations suggests that these concerns have merit, but previous observations made by McClain & Jackson (1980) suggests that isolated populations and scarcity of suitable habitat are predominant issues across the entirety of *G. dioicus* natural range, indicating that these phenomena are likely due to anthropogenic land use changes rather than range edge marginalization.

As a species which appears to have minimal opportunities for natural seed dispersal and germination, it is not surprising that contemporary populations of *G. dioicus*

are vanishing from the landscape (Zaya & Howe 2009). The disappearance of floodplain habitats in the American Midwest and Southwestern Ontario in favour of drained agricultural fields and irrigation waterways further compounds these issues. It's worth noting that *G. dioicus* is not physiologically dependant on floodplains, as it is capable of persisting in upland habitats, but it flourishes in alkaline (7.5-8pH; McClain & Jackson 1980) well drained floodplains. This is crucial, as upland habitats are often dominated by mesic taxa such as *Acer saccharum, Quercus rubra*, and *Carya spp*., all of which easily outcompete and displace *G. dioicus* due to its shade intolerance. On the other end of the spectrum, perpetually wet sites do not appear to be conducive habitat for *G. dioicus* as it can tolerate only brief, infrequent flooding events, effectively restricting it to well-drained floodplain habitats (McClain & Jackson 1980). Limited seed-mediated dispersal (as discussed above) combined with increasingly scarce suitable habitat should largely preclude the establishment of new sites or the reestablishment of extirpated sites. Unfortunately, due to the low levels of genetic variation available in this study it is difficult to infer past dispersal events, making inferences about contemporary levels of gene flow challenging. For example, allele [KC2-1] 247 is only found on East Sister Island and Lambton County, implying a long distance dispersal event may have taken place between these two areas. Alternatively, the mutation patterns of microsatellite regions make them prone to homoplasy, so it is possible that this similarity is the result of two independent mutation events.

The loss of suitable habitat is the main threat to *G. dioicus* throughout its range, but it may face an additional threat that has only been discussed briefly in the current body of literature. Concerns about altered fire regimes are discussed in Environment

Canada (2014) as a potential detriment to *G. dioicus* as they are thought to play a critical role in removing understory vegetation and opening the forest canopy. This could provide two benefits to *G. dioicus*: low intensity ground fires could provide sufficient scarification to facilitation germination, and clonal stands adjacent to burned over areas would be able to utilize vegetative propagation to quickly establish a presence in the newly available habitat. In view of these potential benefits, controlled burns may act as an effective management strategy which facilitates the proliferation of extant *G. dioicus* populations.

Environment Canada (2014) also suggested that introduction of non-local *G. dioicus* could result in outbreeding depression in natural populations. Although the genotypes of commercial suppliers are unknown, the high level of genetic similarity between wild and cultivated trees genotyped in this study indicates that risk of outbreeding depression is low. However, low levels of genetic diversity may still indicate tangible threats to *G. dioicus*. Assuming these markers provide insights into variation in non-neutral loci, the lack of genetic variation across its range implies low adaptive potential for this species (Strasburg *et al.* 2011; Gossmann *et al.* 2012). Definitive conclusions regarding outbreeding depression and adaptive potential will require future investigations of genetic diversity in coding regions of the genome.

Although *G. dioicus* is facing direct threats from loss of suitable habitat, extant populations are generally not threatened by disease or defoliators, and are able to survive major main trunk injuries (human induced or natural) through vigorous root suckering. These attributes may facilitate the survival of *G. dioicus* despite its low germination and dispersal rates.

Chapter 4: Conclusions

A major finding of this study is that wild populations of *G. dioicus* appear to have very low levels of genetic diversity. Genetic similarity between trees was so high that levels of differentiation among populations in Canada were essentially non-existent. Fruit-producing populations are infrequent in Canada, which is largely attributed to pollination limitation and the tendency for clonal reproduction in fragmented landscapes. *G. dioicus* exhibits negligible germination success without substantial damage to the seed coat. Clonal reproduction is assumed to be the primary reproductive mode of natural *G. dioicus*, although the results of this study indicate that sexual reproduction is occurring in some natural populations. However, in the rare event that sexual reproduction and natural germination do occur, high levels of genetic similarity among wild populations mean that novel allele combinations are unlikely to occur, at least for the loci considered here. Low germination and restricted dispersal are likely issues that will continue to affect the long-term viability of *G. dioicus* populations in Canada.

A study conducted by McClain & Jackson (1980) provided important insights into the current habitat requirements of *G. dioicus*, although they were unable to explain why wild populations seemed to be universally marginalized in contemporary forest stands. Zaya and Howe (2009) proposed that the shade intolerant nature of *G. dioicus* may indicate that it is a pioneer species, meaning it may require large-scale disturbance events in order to maintain a presence on the landscape. Zaya and Howe (2009) go on to propose the idea that Pleistocene herbivores could have been the drivers of such disturbance events, acting as migrating defoliators of eastern forests prior to their extinction at the end of the Pleistocene. They drew comparisons to analogous populations of African

forest elephants which generate sufficient forest destruction to effectively supress (or revert) the establishment of slow-growing shade-tolerant trees in favour of pioneer or gap-colonizing species (Owen-smith 1987). These destructive behaviors of modern African elephants are likely similar to those that would have characterized extinct North American megafauna (eg. Mastodons (*Mammut Americana*), Gomphotheres (elephant-like members of proboscidea), and Giant Ground Sloths (*Nothrotheriops shastense*). Zaya and Howe (2009) used the term "Megafaunal disclimaxes" to describe these phenomena, and argue not only that these likely occurred in North America during the Pleistocene, but proposed that these events acted as a cradle for the proliferation and evolution of *G. dioicus.* Given the pioneer-like life history traits of *G. dioicus,* I agree with this proposition as the putative ecological disturbances would have left parts of the landscape of North America in constant successional flux, oscillating between mature old growth forests and barren shrub lands or savannahs. This constant oscillation would result in the frequent ephemeral appearances of disturbed habitats across the landscape, which one would assume would be beneficial for *G. dioicus* as a shade intolerant species capable of rapid colonization, given the appropriate conditions for seed germination. Additionally, these browsers might also have acted as the dispersal vector for *G. dioicus*, deposition of seeds in recently destroyed woodlands or decimated shrub lands would be ideal conditions for subsequent establishment.

Conservation going forward

Three critical concepts must be considered in the conservation of *G. dioicus* moving forward. First, *G. dioicus* is effectively incapable of establishing new populations or re-establishing historical populations, meaning that any removal of *G. dioicus* stands

brings us one step closer to regional extirpation. Second, natural populations established in optimal habitat (alkaline floodplains) or areas devoid of competition from other over story hardwoods are incredibly resilient to removal due to their ability to root sucker. Thirdly, increasing the number of mixed-sex populations (either by transplanting or cultivar reintroduction efforts) will likely have a negligible impact on potential adaptive variation as the opportunity for novel genotypes to arise via sexual reproduction and seed germination are incredibly limited.

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Appendix

Table 12, American *G. dioicus* sample locations and the water resource regions they occupy.

Tree ID	*Water Resource Region*	*Latitude*	*Longitude*
UA01	Arkansas Red-White	35.93705	-95.4711
UA02	Arkansas Red-White	35.94442	-95.4811
UA03	Arkansas Red-White	35.93482	-95.47
UA04	Arkansas Red-White	36.69669	-95.5326
UA05	Arkansas Red-White	36.67191	-95.5676
UA06	Arkansas Red-White	36.71292	-95.5873
UA07	Arkansas Red-White	36.30185	-96.5305
UA08	Arkansas Red-White	36.30788	-96.5187
UA09	Arkansas Red-White	36.30804	-96.5499
UA10	Arkansas Red-White	35.67582	-96.3525
UA11	Arkansas Red-White	35.71347	-96.3721
UA12	Arkansas Red-White	35.67911	-96.3478
UA13	Arkansas Red-White	35.99337	-97.3709
UA14	Arkansas Red-White	35.99316	-97.3646
UA15	Arkansas Red-White	35.99735	-97.3546
UA16	Arkansas Red-White	35.49279	-98.2971
UA17	Arkansas Red-White	35.49689	-98.2841
UA18	Arkansas Red-White	35.48725	-98.2887
UA19	Arkansas Red-White	36.16222	-98.7261
UA20	Arkansas Red-White	36.15486	-98.7607
UA21	Arkansas Red-White	36.17157	-98.7713
UA22	Arkansas Red-White	35.57676	-99.7059
UA23	Arkansas Red-White	35.58686	-99.6986
UA24	Arkansas Red-White	35.59341	-99.6938
UA25	Arkansas Red-White	36.83372	-96.4316
UA26	Arkansas Red-White	36.84656	-96.4289
UA27	Arkansas Red-White	36.81139	-96.4449
UA28	Arkansas Red-White	35.50438	-91.9439
UB01	Great Lakes	42.95167	-85.8741
UB02	Great Lakes	43.0133	-85.9652
UB03	Great Lakes	42.96438	-85.8775
UB04	Great Lakes	42.80089	-84.9047
UB05	Great Lakes	42.81169	-84.9316
UB06	Great Lakes	42.80063	-84.925
UB07	Great Lakes	43.12132	-84.6349

UB08	Great Lakes	43.12175	-84.6311
UB09	Great Lakes	43.09046	-84.7482
UB10	Great Lakes	42.88888	-84.0457
UB11	Great Lakes	42.84665	-84.0337
UB12	Great Lakes	42.88195	-84.0361
UB13	Great Lakes	42.56043	-84.57
UB14	Great Lakes	42.56122	-84.5697
UB15	Great Lakes	41.55944	-85.3942
UB16	Great Lakes	41.55897	-85.3936
UB17	Great Lakes	41.55875	-85.394
UB18	Great Lakes	42.80062	-84.9053
UB19	Great Lakes	42.80015	-84.9051
UB20	Great Lakes	42.80042	-84.9048
UB21	Great Lakes	42.8004	-84.9243
UB22	Great Lakes	42.8004	-84.9245
UB23	Great Lakes	41.62045	-86.9811
UB24	Great Lakes	41.89922	-86.4324
UB25	Great Lakes	41.76662	-85.0126
UC01	Missouri	38.87434	-98.1016
UC02	Missouri	38.86602	-98.1026
UC03	Missouri	38.8595	-98.1063
UC04	Missouri	39.72587	-97.83
UC05	Missouri	39.72499	-97.8003
UC06	Missouri	39.7161	-97.8097
UC07	Missouri	39.7983	-96.7879
UC08	Missouri	39.78594	-96.784
UC09	Missouri	39.78936	-96.7806
UC10	Missouri	38.93134	-95.3665
UC11	Missouri	38.93849	-95.3653
UC12	Missouri	38.94085	-95.3564
UC13	Missouri	40.068	-95.2254
UC14	Missouri	40.0589	-95.2326
UC15	Missouri	40.04846	-95.2465
UC16	Missouri	41.49047	-95.8729
UC17	Missouri	41.47528	-95.865
UC18	Missouri	41.45182	-95.8426
UC19	Missouri	38.94039	-93.5693
UC20	Missouri	38.93303	-93.5794
UC21	Missouri	38.92946	-93.5675
UC22	Missouri	42.76042	-97.0948

UC23	Missouri	42.76205	-97.1011
UC24	Missouri	42.75929	-97.0912
UC25	Missouri	42.5541	-96.4613
UC26	Missouri	42.56075	-96.4651
UC27	Missouri	42.55849	-96.4732
UD01	Ohio	41.11745	-86.5741
UD02	Ohio	41.12225	-86.5728
UD03	Ohio	41.11882	-86.5731
UD04	Ohio	39.33896	-88.1466
UD05	Ohio	39.3964	-88.1576
UD06	Ohio	39.39867	-88.1542
UD07	Ohio	39.89502	-87.1834
UD08	Ohio	39.8897	-87.2289
UD09	Ohio	39.89592	-87.2323
UD10	Ohio	40.29421	-87.7831
UD11	Ohio	40.29512	-87.7837
UD12	Ohio	40.29256	-87.786
UD13	Ohio	40.16533	-85.1591
UD14	Ohio	40.17088	-85.1677
UD15	Ohio	40.22702	-85.1285
UD16	Ohio	38.71125	-86.4318
UD17	Ohio	38.78409	-86.3487
UD18	Ohio	38.76122	-86.3524
UD19	Ohio	37.8761	-85.2818
UD20	Ohio	37.87761	-85.2689
UD21	Ohio	37.87775	-85.2731
UD22	Ohio	38.41651	-85.6322
UD23	Ohio	38.4051	-85.6311
UD24	Ohio	38.43522	-85.6286
UD25	Ohio	38.33023	-84.3513
UD26	Ohio	38.3205	-84.3526
UD27	Ohio	38.32576	-84.3443
UE01	Upper Mississippi	42.09385	-93.5847
UE02	Upper Mississippi	42.1058	-93.5715
UE03	Upper Mississippi	42.15445	-93.5766
UE04	Upper Mississippi	40.96186	-89.4126
UE05	Upper Mississippi	40.92323	-89.4256
UE06	Upper Mississippi	40.92783	-89.4269
UE07	Upper Mississippi	41.63317	-88.5216
UE08	Upper Mississippi	41.63168	-88.52

UE09	Upper Mississippi	41.62776	-88.5176
UE10	Upper Mississippi	42.63042	-92.5076
UE11	Upper Mississippi	42.63092	-92.5068
UE12	Upper Mississippi	42.62881	-92.4998
UE13	Upper Mississippi	40.01536	-89.4716
UE14	Upper Mississippi	40.01482	-89.47
UE15	Upper Mississippi	40.01793	-89.478
UE16	Upper Mississippi	43.83261	-94.169
UE17	Upper Mississippi	44.49833	-93.8867
UE18	Upper Mississippi	41.15417	-91.0415
UE19	Upper Mississippi	40.715	-91.9592
UE20	Upper Mississippi	41.67298	-94.0378
UE21	Upper Mississippi	41.98091	-93.8902
UE22	Upper Mississippi	41.98049	-93.8904
UE23	Upper Mississippi	41.98172	-93.89
UE24	Upper Mississippi	41.3485	-88.3733
UE25	Upper Mississippi	41.35	-88.4059
UF01	Lower Mississippi	35.9319	-92.1067
UF02	Lower Mississippi	35.95139	-92.1039
UF03	Lower Mississippi	35.95258	-92.1098
UF04	Lower Mississippi	35.9483	-92.1125
UF05	Lower Mississippi	35.94783	-92.1125
UF06	Lower Mississippi	35.5135	-91.929
UF07	Lower Mississippi	36.47498	-89.3024
UF08	Lower Mississippi	34.68933	-92.844
UF09	Lower Mississippi	37.1822	-90.4937

Table 13, allele frequencies at 9 microsatellites loci across all data groupings in Canada and the USA.

Country	*Data grouping*	*Locus*	*Allele*	*Allele frequency*	*#of trees genotyped*
Canada	-	KC2-1	247	0.0526	57
Canada	-	KC2-1	250	0.50877193	57
Canada	-	KC2-1	253	0.438596491	57
Canada	-	KC3-2	372	0.124223602	161
Canada	-	KC3-2	374	0.872670807	161
Canada	-	KC3-2	378	0.00310559	161
Canada	-	KC4-1	430	0.082733813	139
Canada	-	KC4-1	432	0.902877698	139
Canada	-	KC4-1	434	0.014388489	139
Canada	-	KC7-2	142	0.009803922	153
Canada	-	KC7-2	144	0.990196078	153
Canada	-	KC8-1	313	1	192
Canada	-	KC9-1	377	1	72
Canada	-	KC10-1	386	1	193
Canada	-	KC11-1	247	0.882716049	162
Canada	-	KC11-1	249	0.117283951	162
Canada	-	KC12-1	358	1	88
	Essex	KC2-1	250	0.64	25
	Essex	KC2-1	253	0.36	25
	Essex	KC3-2	372	0.081730769	104
	Essex	KC3-2	374	0.913461538	104
	Essex	KC3-2	378	0.004807692	104
	Essex	KC4-1	432	0.993670886	79
	Essex	KC4-1	434	0.006329114	79
	Essex	KC7-2	142	0.016666667	90
	Essex	KC7-2	144	0.983333333	90
	Essex	KC8-1	313	1	92
	Essex	KC9-1	377	1	29
	Essex	KC10-1	386	1	64
	Essex	KC11-1	247	0.768292683	82
	Essex	KC11-1	249	0.231707317	82
	Essex	KC12-1	358	1	70
	Lambton	KC2-1	250	0.461538462	13
	Lambton	KC2-1	253	0.461538462	13
	Lambton	KC3-2	372	0.274193548	31
	Lambton	KC3-2	374	0.725806452	31
	Lambton	KC4-1	432	0.8125	8
	Lambton	KC4-2	434	0.1875	8

	Lambton	KC7-2	144	1	32
	Lambton	KC8-1	313	2	20
	Lambton	KC9-1	377	3	16
	Lambton	KC10-1	386	4	38
	Lambton	KC11-1	247	5	22
	Lambton	KC12-1	358	6	9
	Middle Island	KC2-1	250	0.388888889	9
	Middle Island	KC2-1	253	0.611111111	9
	Middle Island	KC3-2	372	0.113636364	22
	Middle Island	KC3-2	374	0.886363636	22
	Middle Island	KC4-1	432	1	29
	Middle Island	KC7-2	144	1	29
	Middle Island	KC8-1	313	1	42
	Middle Island	KC9-1	377	1	8
	Middle Island	KC10-1	386	1	65
	Middle Island	KC11-1	247	1	46
	Middle Island	KC12-1	358	1	4
	East Sister Island	KC2-1	247	0.2	10
	East Sister Island	KC2-1	250	0.35	10
	East Sister Island	KC2-1	253	0.45	10
	East Sister Island	KC3-2	372	0.125	4
	East Sister Island	KC3-2	374	0.875	4
	East Sister Island	KC4-1	430	0.5	23
	East Sister Island	KC4-1	432	0.5	23
	East Sister Island	KC7-2	144	1	2
	East Sister Island	KC8-1	313	1	38
	East Sister Island	KC9-1	377	1	19
	East Sister Island	KC10-1	386	1	26
	East Sister Island	KC11-1	247	1	12
	East Sister Island	KC12-1	358	1	5
USA	-	KC10-1	386	1	42
USA	-	KC11-1	247	0.916666667	36
USA	-	KC11-1	249	0.027777778	36
USA	-	KC11-1	253	0.055555556	36
USA	-	KC12-1	356	0.010416667	48
USA	-	KC12-1	358	0.989583333	48
USA	-	KC2-1	250	0.56	25
USA	-	KC2-1	253	0.44	25
USA	-	KC3-2	372	0.018072289	83
USA	-	KC3-2	374	0.963855422	83
USA	-	KC3-2	378	0.018072289	83
USA	-	KC4-1	430	0.131578947	57
USA	-	KC4-1	432	0.842105263	57

USA	-	KC4-1	434	0.026315789	57
USA	-	KC7-2	144	1	34
USA	-	KC8-1	313	1	80
USA	-	KC9-1	377	1	53
	UA	KC10-1	386	1	13
	UA	KC11-1	247	1	14
	UA	KC12-1	358	1	11
	UA	KC2-1	250	0.523809524	21
	UA	KC2-1	253	0.476190476	21
	UA	KC3-2	374	1	22
	UA	KC4-1	430	0.108695652	23
	UA	KC4-1	432	0.891304348	23
	UA	KC7-2	144	1	12
	UA	KC8-1	313	1	80
	UA	KC8-1	313	1	29
	UA	KC9-1	377	1	53
	UA	KC9-1	377	1	26
	UB	KC10-1	386	1	1
	UB	KC11-1	247	1	3
	UB	KC12-1	358	1	8
	UB	KC2-1	250	1	2
	UB	KC3-2	372	0.035714286	14
	UB	KC3-2	374	0.964285714	14
	UB	KC4-1	430	0.0625	16
	UB	KC4-1	432	0.875	16
	UB	KC4-1	434	0.0625	16
	UB	KC7-2	144	1	2
	UB	KC8-1	313	1	20
	UB	KC9-1	377	1	10
	UC	KC10-1	386	1	8
	UC	KC11-1	247	1	5
	UC	KC12-1	356	0.055555556	9
	UC	KC12-1	358	0.944444444	9
	UC	KC2-1	250	0.5	2
	UC	KC2-1	253	0.5	2
	UC	KC3-2	372	0.035714286	14
	UC	KC3-2	374	0.857142857	14
	UC	KC3-2	378	0.107142857	14
	UC	KC4-1	430	0.1	5
	UC	KC4-1	432	0.8	5
	UC	KC4-1	434	0.1	5
	UC	KC7-2	144	1	7
	UC	KC8-1	313	1	9
	UC	KC9-1	377	1	6

UD	KC10-1	386	1	13
UD	KC11-1	247	1	6
UD	KC12-1	358	1	10
UD	KC2-1	N/A	N/A	0
UD	KC3-2	372	0.029411765	17
UD	KC3-2	374	0.970588235	17
UD	KC4-1	430	0.25	8
UD	KC4-1	432	0.75	8
UD	KC7-2	144	1	8
UD	KC8-1	313	1	9
UD	KC9-1	377	1	7
UE	KC10-1	386	1	7
UE	KC11-1	247	0.833333333	6
UE	KC11-1	249	0.166666667	6
UE	KC12-1	358	1	7
UE	KC2-1	N/A	#DIV/0!	0
UE	KC3-2	374	1	14
UE	KC4-1	430	0.5	3
UE	KC4-1	432	0.5	3
UE	KC7-2	144	1	3
UE	KC8-1	313	1	10
UE	KC9-1	377	1	2
UF	KC10-1	N/A	#DIV/0!	0
UF	KC11-1	253	1	2
UF	KC12-1	358	1	3
UF	KC2-1	N/A	#DIV/0!	0
UF	KC3-2	374	1	2
UF	KC4-1	432	1	2
UF	KC7-2	144	1	2
UF	KC8-1	313	1	3
UF	KC9-1	377	1	2

Table 14, Locations of mother *G. dioicus* trees and number of seeds collected.

Tree ID	*DBH*	*Easting*	*Northing*	*County*	*# of seed pods collected*
DB01	13.4	617348	4853940	Vaughn	241
DB02	12.4	617357	4853942	Vaughn	126
DB03	12	617400	4853953	Vaughn	11
DB04	10.2	617521	4853990	Vaughn	32
DC01	16.8	620851	4844331	Vaughn	58
DA01	64.2	500789	4735631	Elgin	102
				TOTAL	570

Table 15, Samples sizes across all loci in all data groupings.

Locus	*East Sister Island*	*Essex*	*Lambton*	*Middle Island*	*Planted*	*UA*	*UB*	*UC*	*UD*	*UE*	*UF*
KC2-1	10	25	13	9	80	21	2	2	0	0	0
KC3-2	4	104	31	22	104	22	14	14	17	14	2
KC4-1	23	79	8	29	107	23	16	5	8	3	2
KC7-2	2	90	32	29	82	12	2	7	8	3	2
KC8-1	38	92	20	42	104	29	20	9	9	10	3
KC9-1	19	29	16	8	116	26	10	6	7	2	2
KC10-1	26	64	38	65	96	13	1	8	13	7	0
KC11-1	12	82	22	46	50	14	3	5	6	6	2
KC12-1	5	70	9	4	104	11	8	9	10	7	3

Table 16, Identification codes, diameters (or height for trees <200cm), County, and UTM waypoints (Zone 17T) for All sampled *G. dioicus* in Canada.

Tree ID	*DBH(cm)*	*Height(cm)*	*Easting*	*Northing*	*County*
AA01	13.6	-	351962	4675144	Essex
AA02	15.3	-	351971	4675142	Essex
AA03	6.5	-	351969	4675143	Essex
AB01	18.6	-	351944	4675132	Essex
AB02	23.4	-	351951	4675123	Essex
AB03	9.4	-	351965	4675120	Essex
AB04	12.6	-	351953	4675126	Essex
AB05	16	-	351958	4675122	Essex
AC01	10.9	-	351958	4675080	Essex
AC02	6.1	-	351949	4675077	Essex
AD01	4.4	-	336200	4672052	Essex
AD02	11.2	-	336210	4672051	Essex
AD03	15.1	-	336220	4672048	Essex
AD04	15.7	-	336227	4672048	Essex
AD05	7.1	-	336230	4672049	Essex
AE01	3.2	-	334771	4671646	Essex
AE02	16.7	-	334777	4671663	Essex
AE03	15.5	-	334768	4671664	Essex
AE04	11.6	-	334770	4671675	Essex
AE05	14.4	-	334769	4671684	Essex
AE06	19.4	-	334771	4671692	Essex
AF01	33.8	-	331163	4672577	Essex
AF02	4	-	331168	4672575	Essex
AF03	3.5	-	331155	4672584	Essex
AG01	30.6	-	336027	4665787	Essex
AG02	1.2	-	336027	4665783	Essex
AG03	1.4	-	336032	4665781	Essex
AG04	-	193	336029	4665785	Essex
AG05	25.7	-	336021	4665783	Essex
AG06	30.6	-	336023	4665777	Essex
AG07	23.3	-	336010	4665773	Essex
AG08	-	105	336011	4665774	Essex
AG09	76	-	336006	4665754	Essex
AG10	31	-	336012	4665764	Essex
AG11	57.7	-	336003	4665749	Essex
AG12	25.8	-	336010	4665752	Essex

AG13	50	-	336006	4665748	Essex
AG14	-	115	336003	4665724	Essex
AG15	-	125	336008	4665726	Essex
AG16	18.3	-	336015	4665763	Essex
AG17	21.7	-	336021	4665765	Essex
AG18	20.9	-	336024	4665766	Essex
AH01	5.8	-	335625	4666193	Essex
AH02	1	-	335619	4666191	Essex
AH03	1.1	-	335638	4666195	Essex
AH04	5.1	-	335647	4666202	Essex
AH05	8.3	-	335645	4666181	Essex
AH06	4.7	-	335653	4666186	Essex
AS01	5.6	-	348931	4654473	Essex
AV01	1.6	-	388192	4730539	Lambton
AV02	12.5	-	388196	4730536	Lambton
AV03	6.2	-	388209	4730537	Lambton
AV04	8.4	-	388189	4730528	Lambton
AV05	10.3	-	388194	4730527	Lambton
AW01	43.5	-	421658	4728360	Lambton
AW02	-	140	421642	4728416	Lambton
AW03	9.7	-	421652	4728411	Lambton
AW04	12	-	421646	4728405	Lambton
AW05	19.5	-	421655	4728399	Lambton
AW06	17	-	421651	4728395	Lambton
AW07	12	-	421653	4728385	Lambton
AW08	20.5	-	421650	4728381	Lambton
AW09	4.5	-	421644	4728378	Lambton
AW10	21	-	421664	4728366	Lambton
AW11	16	-	421675	4728366	Lambton
AW12	21	-	421684	4728360	Lambton
AW13	7	-	421709	4728360	Lambton
AW14	54	-	421774	4728354	Lambton
AW15	3.5	-	421804	4728335	Lambton
AW16	17.4	-	421792	4728347	Lambton
AW17	-	140	421688	4728350	Lambton
AW18	16	-	421664	4728361	Lambton
AY01	10.9	-	359667	4615970	Essex
AY02	35.5	-	359669	4615968	Essex
AY03	6.9	-	359663	4615958	Essex
AY04	8.7	-	359676	4615948	Essex

AY05	11.7	-	359677	4615963	Essex
AY06	32.3	-	359678	4615963	Essex
AY07	13.1	-	359681	4615966	Essex
AY08	19	-	359671	4615972	Essex
AY09	8.2	-	359790	4615837	Essex
AY10	6.2	-	359805	4615826	Essex
AY11	13.8	-	359820	4615833	Essex
AY12	13.9	-	359808	4615846	Essex
AY13	14.8	-	359809	4615847	Essex
AY14	4.5	-	359822	4615872	Essex
AY15	4.6	-	359813	4615874	Essex
AY16	-	125	359807	4615860	Essex
AY17	8.9	-	359887	4615831	Essex
AY18	12.9	-	359875	4615831	Essex
AY19	9.8	-	359880	4615826	Essex
AY20	21.1	-	359882	4615833	Essex
AY21	16.6	-	359895	4615841	Essex
AY22	18.9	-	359900	4615834	Essex
AY23	-	130	359891	4615852	Essex
AY24	16.6	-	359898	4615830	Essex
AY25	3.5	-	359979	4616051	Essex
AY26	2.4	-	359980	4616046	Essex
AY27	7.4	-	359987	4616024	Essex
AY28	6.5	-	359996	4616036	Essex
AY29	2.2	-	360000	4616042	Essex
AY30	8.3	-	359973	4616028	Essex
AY31	6.6	-	360030	4616021	Essex
AY31	8.2	-	359966	4616030	Essex
AY32	14.9	-	359962	4616038	Essex
AY33	8.8	-	360031	4616024	Essex
AY34	1.5	-	360026	4616021	Essex
AY35	6.5	-	360030	4616020	Essex
AY36	2.7	-	360033	4616010	Essex
AY38	5.3	-	360028	4616008	Essex
AY39	2.5	-	360030	4616011	Essex
AY40	1.7	-	360027	4616022	Essex
AY41	13.4	-	360025	4616045	Essex
AY42	7.6	-	360023	4616047	Essex
AY43	4.8	-	360027	4616042	Essex
AY44	5.6	-	360036	4616054	Essex

AY45	-	195	360044	4616056	Essex
AY46	7.3	-	360064	4616064	Essex
AY47	4.9	-	360078	4616070	Essex
AY48	1.8	-	360089	4616041	Essex
AY49	6.2	-	360091	4616025	Essex
AY50	3.6	-	360078	4616014	Essex
AY51	2.8	-	360072	4616004	Essex
AY52	14.5	-	360091	4616019	Essex
AY53	3.4	-	360089	4616031	Essex
AY54	4.1	-	360139	4616014	Essex
AY55	2.3	-	360140	4616017	Essex
AY56	2.9	-	360135	4616012	Essex
AY57	7.1	-	360258	4616046	Essex
AY58	15.6	-	360256	4616052	Essex
AY59	-	155	360260	4616047	Essex
AY60	1.2	-	360260	4616044	Essex
AY61	5.7	-	360274	4616035	Essex
AY62	1.3	-	360275	4616033	Essex
AY63	18.7	-	360278	4616029	Essex
AY64	3.6	-	360279	4616028	Essex
AY65	11.8	-	360296	4616061	Essex
AY66	6	-	360087	4616051	Essex
BB01	5.6	-	355356	4673745	Essex
BB02	6.2	-	355359	4673755	Essex
BB03	3.2	-	355357	4673761	Essex
BC01	1.1	-	342100	4657191	Essex
BC02	-	30	342102	4657186	Essex
BC03	-	45	342097	4657181	Essex
BC04	-	60	342101	4657181	Essex
BC05	2.1	-	342098	4657181	Essex
BC06	-	135	342100	4657182	Essex
BC07	-	150	342099	4657182	Essex
BC08	-	58	342101	4657182	Essex
BC09	-	50	342099	4657182	Essex
BC10	-	120	342101	4657182	Essex
BC11	3	-	342101	4657182	Essex
BC12	-	160	342098	4657182	Essex
BC13	-	140	342098	4657183	Essex
BC14	-	130	342100	4657183	Essex
BC15	-	176	342100	4657184	Essex

BC16	-	173	342100	4657183	Essex
BC17	-	130	342100	4657183	Essex
BC18	-	53	342102	4657179	Essex
BC19	19.3	-	342100	4657183	Essex
BC20	-	160	342102	4657181	Essex
BC21	-	140	342104	4657183	Essex
BC22	-	60	342100	4657181	Essex
BC23	8.5	-	342106	4657182	Essex
BC24	15.1	-	342104	4657181	Essex
BC25	20.5	-	342104	4657184	Essex
BC26	69.9	-	342106	4657184	Essex
BC27	22.3	-	342123	4657178	Essex
BD01	6.2	-	341071	4658691	Essex
BD02		115	341071	4658691	Essex
BD03		190	341071	4658691	Essex
BD04	5.7		341071	4658691	Essex
BD06	10.3		341071	4658691	Essex
BD07	7.8		341071	4658691	Essex
BD08	6.1		341071	4658691	Essex
BD09	6.2		341071	4658691	Essex
BD10	5.7		341071	4658691	Essex
BD11	22.6		341071	4658691	Essex
BD12	26.1		341071	4658691	Essex
BD13	9.8		341071	4658691	Essex
BD14	18.7		341071	4658691	Essex
BD15		55	341071	4658691	Essex
BD16	25.8		341071	4658691	Essex
BD18	5.9		341071	4658691	Essex
BD19	18.9		341071	4658691	Essex
BD20	17.5		341071	4658691	Essex
BD21	24.3		341071	4658691	Essex
BD23	26.7		341071	4658691	Essex
BD24	28.7		341071	4658691	Essex
BD25		45	341071	4658691	Essex
BD30	5.5		341071	4658691	Essex
BD31		27	341071	4658691	Essex
BD32	38.2		341071	4658691	Essex
BK01	21	-	386930	4722356	Lambton
BO01	24.5	-	391432	4732364	Lambton
BO02	-	150	391428	4732389	Lambton

BO03	5.9	-	391426	4732387	Lambton
BO04	3.3	-	391430	4732389	Lambton
BO05	1.6	-	391440	4732386	Lambton
BO06	10	-	391438	4732373	Lambton
BO07	15.6	-	391442	4732368	Lambton
BO08	14	-	391433	4732369	Lambton
BO09	34.5	-	391428	4732361	Lambton
BO10	13.2	-	391414	4732351	Lambton
BO11	18	-	391431	4732355	Lambton
BO12	28.5	-	391437	4732358	Lambton
BP01	30	-	391296	4732742	Lambton
BP02	12	-	391299	4732740	Lambton
BP03	1.4	-	391292	4732749	Lambton
BQ01	11.3	-	391889	4732574	Lambton
BR01	3.4	-	371643	4649562	Essex
BR02	2.9	-	371647	4649570	Essex
BR03	5.3	-	371647	4649571	Essex
BR04	1.6	-	371649	4649572	Essex
BR05	4.9	-	371643	4649565	Essex
BR06	2.1	-	371644	4649567	Essex
BR07	3.8	-	371644	4649567	Essex
BR08	3.4	-	371645	4649564	Essex
BR09	3.7	-	371648	4649566	Essex
BR10	2.2	-	371656	4649566	Essex
BR11	-	150	371654	4649565	Essex
BR12	-	120	371656	4649567	Essex
BR13	-	160	371643	4649568	Essex
BR14	4.2	-	371642	4649563	Essex
BR15	1.3	-	371640	4649563	Essex
BR16	29.2	-	371645	4649554	Essex
BR17	-	195	371643	4649554	Essex
BR18	-	140	371650	4649554	Essex
BR19	-	160	371655	4649556	Essex
BR20	-	105	371655	4649558	Essex
BR21	-	75	371651	4649557	Essex
BR22	-	150	371652	4649552	Essex
BR23	1.4	-	371653	4649553	Essex
BR24	3.8	-	371654	4649552	Essex
BR25	-	120	371648	4649552	Essex
BR26	3.8	-	371649	4649555	Essex

BR27	-	80	371648	4649550	Essex
BR28	-	105	371647	4649549	Essex
BR29	-	95	371648	4649545	Essex
BR30	-	80	371646	4649542	Essex
BR31	-	130	371649	4649546	Essex
BR32	2.1	-	371654	4649549	Essex
BR33	3.2	-	371656	4649548	Essex
BR34	1.9	-	371652	4649549	Essex
BR35	-	105	371659	4649554	Essex
BR36	2.2	-	371658	4649555	Essex
BR37	2.3	-	371655	4649556	Essex
BR38	-	125	371657	4649552	Essex
BR39	1.8	-	371657	4649548	Essex
BR40	4.9	-	371656	4649553	Essex
BR41	-	130	371658	4649549	Essex
BR42	-	75	371661	4649546	Essex
BR43	-	90	371661	4649547	Essex
BR44	-	130	371661	4649545	Essex
BR45	1.7	-	371661	4649545	Essex
BR46	-	195	371659	4649544	Essex
BR47	-	70	371659	4649544	Essex
BR48	1.5	-	371661	4649544	Essex
BR49	5.8	-	371657	4649542	Essex
BR50	1.6	-	371658	4649541	Essex
BR51	6.2	-	371657	4649544	Essex
BR52	-	85	371659	4649542	Essex
BR53	23.7	-	371652	4649533	Essex
BR54	-	1.6	371659	4649536	Essex
BR55	6.9	-	371659	4649538	Essex
BR56	7.6	-	371657	4649539	Essex
BR57	-	145	371658	4649539	Essex
BR58	-	165	371657	4649539	Essex
BR59	1.4	-	371664	4649534	Essex
BR60	1.2	-	371663	4649537	Essex
BR61	2.3	-	371665	4649534	Essex
BR62	2.6	-	371666	4649534	Essex
BR63	23.2	-	371667	4649536	Essex
BR64	3.3	-	371664	4649539	Essex
BR65	2.1	-	371664	4649536	Essex
BR66	2.7	-	371669	4649535	Essex

BR67	2.8	-	371663	4649538	Essex
BR68	-	150	371663	4649539	Essex
BR69	-	160	371662	4649537	Essex
BR70	-	190	371660	4649534	Essex
BR71	-	85	371662	4649538	Essex
BR72	1.9	-	371664	4649533	Essex
BS01	4.1	-	374031	4645125	Essex
BS02	3.7	-	374031	4645126	Essex
BS03	2.6	-	374035	4645125	Essex
BS04	37.4	-	374029	4645123	Essex
BT01	9.1	-	374042	4644613	Essex
BT02	8.1	-	374044	4644599	Essex
BT03	12.5	-	373950	4644900	Essex
BT04	6.8	-	374004	4644763	Essex
BT05	1	-	374006	4644759	Essex
BT06	2.9	-	374008	4644758	Essex
BT07	5.3	-	374004	4644757	Essex
BT08	3.6	-	374001	4644763	Essex
BT10	3.4	-	374006	4644756	Essex
BT11	7.4	-	374001	4644754	Essex
BT12	12.8	-	374014	4644751	Essex
BT13	0.8	-	374010	4644754	Essex
BT14	0.9	-	374007	4644757	Essex
BT15	1.8	-	374015	4644750	Essex
BT16	0.9	-	374008	4644750	Essex
BT17	5.6	-	374009	4644746	Essex
BT18	4.2	-	374008	4644749	Essex
BT19	4.4	-	374012	4644749	Essex
BT20	4.9	-	374014	4644748	Essex
BT21	3.2	-	374010	4644752	Essex
BT22	1.8	-	374020	4644749	Essex
BT24	4.3	-	374004	4644746	Essex
BT25	3.9	-	374009	4644752	Essex
BT26	4.7	-	374009	4644747	Essex
BT27	10	-	374003	4644728	Essex
BT28	-	143	374011	4644746	Essex
BT29	21.3	-	373991	4644760	Essex
BU01	4.6	-	374276	4643896	Essex
BU02	5.4	-	374275	4643902	Essex
BU03	-	132	374278	4643888	Essex

BU04	-	143	374278	4643893	Essex
BU05	-	83	374271	4643888	Essex
BU06	22.4	-	374274	4643859	Essex
BV01	8.9	-	374643	4642896	Essex
BV02	3.4	-	374670	4642825	Essex
BW01	19.3	-	374792	4642551	Essex
BW02	19.8	-	374805	4642563	Essex
BX01	9.5	-	367826	4684616	Essex
CA01	7	-	403106	4748005	Lambton
CA02	5.5	-	403108	4747963	Lambton
CA03	20	-	403080	4748016	Lambton
CA04	16.5	-	403072	4748009	Lambton
CA05	14	-	403082	4748028	Lambton
CA06	5.8	-	403092	4748039	Lambton
CB01	14	-	420201	4728911	Lambton
CB02	14	-	420200	4728931	Lambton
CB03	11.2	-	420201	4728941	Lambton
CB04	13	-	420205	4728950	Lambton
CB05	13	-	420215	4728954	Lambton
CB06	10.6	-	420225	4728955	Lambton
CB07	13	-	420233	4728957	Lambton
CB08	9.2	-	420247	4728957	Lambton
CC01	-	195	431738	4741899	Lambton
CC02	2	-	431738	4741896	Lambton
CC03	1.9	-	431737	4741904	Lambton
CC04	-	185	431738	4741910	Lambton
CC05	15.8	-	431736	4741924	Lambton
CC06	7.5	-	431734	4741930	Lambton
ES01	26	-	346144	4630815	Essex
ES02	-	132	346145	4630812	Essex
ES03	-	85	346148	4630821	Essex
ES04	-	104	346153	4630821	Essex
ES05	28.2	-	346143	4630821	Essex
ES07	7.4	-	346131	4630826	Essex
ES08	12.8	-	346135	4630830	Essex
ES09	11.2	-	346131	4630831	Essex
ES10	33.7	-	346121	4630835	Essex
ES11	9.2	-	346122	4630828	Essex
ES13	-	130	346123	4630834	Essex
ES14	20.2	-	346144	4630801	Essex

ES15	4.6	-	346147	4630804	Essex
ES16	20.9	-	346144	4630802	Essex
ES17	7.6	-	346153	4630802	Essex
ES18	4.3	-	346138	4630801	Essex
ES19	2.7	-	346140	4630802	Essex
ES20	2.2	-	346138	4630801	Essex
ES21	4.8	-	346151	4630806	Essex
ES22	5.5	-	346151	4630802	Essex
ES23	1.8	-	346154	4630801	Essex
ES24	1.5	-	345960	4630799	Essex
ES25	1.8	-	345909	4630811	Essex
ES26	4.4	-	345906	4630815	Essex
ES27	2.3	-	345908	4630815	Essex
ES28	9.8	-	346140	4630802	Essex
ES29	4.2	-	345901	4630809	Essex
ES30	4.4	-	345905	4630815	Essex
ES32	15.3	-	345898	4630814	Essex
ES33	2.6	-	345894	4630813	Essex
ES34	20.2	-	345898	4630816	Essex
ES35	12.4	-	345897	4630814	Essex
ES37	4.2	-	345900	4630812	Essex
ES38	11.2	-	345896	4630814	Essex
ES39	19.3	-	345892	4630814	Essex
ES40	5.6	-	345892	4630809	Essex
ES41	6.2	-	345890	4630813	Essex
ES42	9	-	345893	4630813	Essex
ES43	11.6	-	345904	4630737	Essex
ES44	10.9	-	345901	4630740	Essex
ES46	7.7	-	345897	4630742	Essex
ES47	3	-	345895	4630742	Essex
ES48	2.4	-	345892	4630740	Essex
ES49	4.1	-	345897	4630739	Essex
ES50	18.5	-	345820	4630598	Essex
ES51	3.2	-	345822	4630604	Essex
ES52	6.4	-	345825	4630594	Essex
ES53	21	-	345822	4630592	Essex
ES55	3.3	-	345820	4630604	Essex
ES56	33.1	-	345812	4630609	Essex
ES57	5.8	-	345813	4630600	Essex
ES58	4.8	-	345816	4630603	Essex

ES59	24.1	-	345817	4630603	Essex
ES60	14	-	345801	4630616	Essex
ES61	8.9	-	345794	4630614	Essex
ES62	7.1	-	345802	4630617	Essex
ES63	12.9	-	345801	4630616	Essex
ES64	2.4	-	345798	4630610	Essex
ES65	21.1	-	345790	4630593	Essex
ES66	27.8	-	345807	4630586	Essex
ES67	37.8	-	345805	4630581	Essex
ES68	31.7	-	345804	4630580	Essex
ES69	3	-	345797	4630577	Essex
ES70	11.6	-	345789	4630585	Essex
ES71	12.5	-	345790	4630597	Essex
ES72	16	-	345783	4630598	Essex
ES73	18.9	-	345787	4630586	Essex
ES74	25.1	-	345783	4630595	Essex
ES75	31.3	-	345770	4630608	Essex
ES76	7.3	-	345751	4630570	Essex
ES77	7.1	-	345749	4630569	Essex
ES78	19.9	-	345756	4630546	Essex
ES79	10.7	-	345801	4630617	Essex
DB01	13.4	-	617348	4853940	Vaughn
DB02	12.4	-	617357	4853942	Vaughn
DB03	12	-	617400	4853953	Vaughn
DB04	10.2	-	617521	4853990	Vaughn
DC01	16.8	-	620851	4844331	Vaughn
DA01	64.2	-	500789	4735631	Elgin