Conservation genetics and habitat characteristics of
Few-flowered Club-rush (*Trichophorum planifolium*
(Spreng.) Palla), an endangered woodland sedge in
Canada

by

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Abstract

Trichophorum planifolium, like many Canadian species-at-risk, reaches the northern extent of its range in Canada, but is common farther south in the United States. Peripheral populations may be important sources of adaptive and evolutionary potential, possessing unique genetic diversity and/or adaptations to local conditions. However, such populations are typically small and isolated and may therefore be susceptible to extinction. Using microsatellite markers, we compared the level and spatial distribution of genetic diversity and environmental characteristics between the Canadian and American populations of *T. planifolium*. Overall, low diversity and high inbreeding were detected, with all populations exhibiting comparable levels. This suggests inbreeding depression is not limiting recruitment in the Canadian population, as previously hypothesized. We also found high differentiation between populations and unique environmental conditions at the northern range margin. Consequently, efforts to bolster genetic diversity in the Canadian population are not presently warranted and should be avoided in the future.

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Preface

Chapter 1 and 4 are original, unpublished, independent work by the author, V.J. Nowell. A version of Chapter 2 has been published. V.J. Nowell was the principal investigator, responsible for lab work, data analysis and manuscript preparation. S. Wang contributed to the lab work. T.W. Smith was the supervisory author on this project and was involved in revising the manuscript. V.J. Nowell was the lead investigator for Chapter 3 and was responsible for fieldwork, lab work, data analysis and manuscript composition. S. Wang contributed to the lab work. T.W. Smith was the supervisory author on this project and was involved throughout, from project development to manuscript edits.

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1 Chapter: Introduction and objectives

1.1 Introduction

1.1.1 The conservation of plant biodiversity

Plants comprise approximately 298,000 species on earth, about 3.4% of the total estimated number of eukaryotes (Mora et al., 2011). As primary producers, they are fundamental components of biodiversity and sustainability, forming the base of many ecosystems and supporting an array of other organisms. Plants also provide essential ecosystems services, influencing local and regional climate, water supply and soil stability. In addition, plants are an invaluable source of food, medicine and other materials on which humans rely.

It is estimated that about one third of all plant species are currently at risk of extinction, mainly as a consequence of human activities (Hamilton and Hamilton, 2006). Of particular concern are the numerous examples of common or widespread species that have experienced rapid and inexplicable declines and/or extinction (Kramer and Havens, 2009; Lindenmayer et al., 2011). Human population growth, as well as increased consumption, has created the pressure to develop natural habitats, expand and intensify agriculture and collect more resources from wild plants (Hamilton and Hamilton, 2006). This has resulted in overexploitation, major losses and degradation of habitat and has contributed to the spread of invasive species, pests and diseases. Furthermore, climate change, occurring as a result of the increase in greenhouse gases in our atmosphere, directly caused by human activities, is expected to result in further plant extinctions (Hamilton and Hamilton, 2006; Hawkins et al., 2008).

Populations are the basic unit of conservation for plant species, as they are typically isolated, supporting local symbionts, herbivores and parasites (Frankel et al., 1995; Primack, 2008; Allendorf et al., 2013). To identify the factors threatening populations and to develop appropriate and effective management plans, an integrative approach which combines an understanding of species' ecology and population biology is required (Heywood and Iriondo, 2003; Primack, 2008). In particular, knowledge of the level and spatial distribution of intraspecific genetic diversity is important because genetic variation plays a critical role in population viability as well as the ability of populations and species to respond to changing environmental conditions (Frankel et al., 1995; Allendorf et al., 2013).

1.1.2 Determinants of population genetic diversity and divergence

In short, population genetic diversity and differentiation are determined by the interplay between four major genetic processes: gene flow, genetic drift, mutation and natural selection. These, in turn, are affected by the size and spatial separation of populations, certain life history traits and environmental characteristics (Loveless and Hamrick, 1984; Slatkin, 1987; Ellstrand and Elam, 1993). As a result, substantial differences in genetic variation can occur among populations of the same species (Slatkin, 1987; Frankel et al., 1995; Allendorf et al., 2013).

1.1.2.1 Population size and distribution

Habitat quality is frequently variable across species' geographic ranges, influencing the abundance and distribution of populations. The central-marginal

hypothesis (Eckert et al., 2008), or abundant-centre model (Sagarin and Gaines, 2002), is a common biogeographic theory which asserts that population size and density are greatest at the geographic centre of a species' range where abiotic and biotic factors are optimal for survival and reproduction (Brown, 1984).

Conversely, populations toward the range margin are expected to be smaller and more isolated due to reduced resource availability. These are termed core and peripheral populations, respectively. Gene flow among core populations is generally high, leading to high levels of genetic diversity and low differentiation among populations. Peripheral populations, in contrast, are expected to experience limited gene flow, greater genetic drift and a higher occurrence of inbreeding, resulting in reduced genetic variation within populations and greater divergence between populations. Furthermore, isolated plant populations may adaptively diverge from one another under intermediate levels of gene flow (Lenormand, 2002).

Empirical studies have confirmed a significant positive correlation between population size and genetic variation (Leimu et al., 2006) and adaptive divergence of plant populations is well documented (Frankham et al., 2002) with so-called 'ecotypes' (Turesson, 1922) exhibiting adaptations to local conditions including soil, temperature, moisture and grazing (Frankel et al., 1995; Briggs and Walters, 1997). However, the geographic patterns of genetic diversity and differentiation predicted for core and peripheral populations are not consistently supported. In a review of 134 genetic studies in plants, Eckert et al. (2008) found that only 64.2% of studies identified reduced genetic diversity in peripheral

populations and 70.3% showed increased genetic differentiation towards the range margin. The authors also noted that the overall differences in genetic diversity between peripheral and core populations were not large (Eckert et al., 2008). These results may be attributable to differences in distribution and life-history characteristics among species (Gibson et al., 2009).

1.1.2.2 Life-history traits

In plants, life-history characteristics are a major determinant of genetic structure as individuals are relatively immobile, apart from dispersal by pollen or by seeds and/or vegetative propagules (Loveless and Hamrick, 1984; Frankel et al., 1995). Dispersal ability is inversely correlated with the magnitude of genetic differentiation among populations and positively correlated with levels of intra-population genetic diversity because it is related to gene flow (Frankham et al., 2002). In an empirical review of 155 genetic studies in plants, significant differences in gene flow (Nm) were noted among species employing different pollination mechanisms ($\chi^2 = 55.5$, $p \leq 0.001$; Govindaraju, 1988). Wind-pollinated species exhibited the highest levels of gene flow ($Nm = 2.921$), relative to animal and self-pollinating species ($Nm = 1.211$ and 0.830 , respectively; Govindaraju, 1988). These findings support earlier studies by Loveless and Hamrick (1984) that found that, in wind-pollinated species, background pollen levels can prevent differentiation over large geographic areas and lead to greater intra-population genetic diversity and heterozygosity. However, this also depends on the morphology of the species. For example, in a study of the wind-pollinated sedges *Carex plantaginea* Lam. and *Carex platyphylla* Carey, Handel

(1976) found that pollen movement was highly restricted (i.e., less than 5 m), which he suggested would lead to differentiation among isolated populations in these species.

In self-compatible plants, the effect on diversity and differentiation depends on the extent of outcrossing (Loveless and Hamrick, 1984). Predominately self-pollinating species have been found to exhibit higher levels of differentiation (F_{ST}) and lower observed heterozygosity (H_o) than predominately outcrossing or mixed-mating species (Loveless and Hamrick, 1984; Nybom, 2004).

Significant differences in measures of within-population diversity, observed heterozygosity (H_o), and among-population differentiation (F_{ST}) have also been reported for species employing different mechanisms of seed dispersal (Nybom, 2004). Species with wind and/or water or 'ingested' modes of seed dispersal exhibited high within-population genetic diversity and low population differentiation while the reverse was found in species with gravity dispersed or animal attached seeds (Hamrick and Godt, 1996; Nybom, 2004). Local seed deposition, as in species with gravity-dispersed seeds, promotes family structure and inbreeding, leading to loss of heterozygosity and increased genetic differentiation of populations (Loveless and Hamrick, 1984).

1.1.2.3 Habitat characteristics

Large and small-scale landscape features may act as barriers to dispersal by seeds or pollen, thereby restricting gene flow and influencing population genetic structure (Slatkin, 1987). For example, the Strait of Gibraltar, the narrow

strait which separates the Iberian Peninsula from North Africa, has been demonstrated to be an effective barrier to gene flow in *Carex helodes* Link, a wind-pollinated sedge, promoting increased genetic divergence among separated populations (Escudero et al., 2008). In Bog sedge (*Carex magellanica* subsp. *irrigua* (Wahlenb.) Hultén), local habitat characteristics have been invoked to explain the high genetic differentiation observed in large, continuous core populations because wind-pollination of small herbs in closed vegetation may be less effective (Kull and Oja, 2010).

1.1.3 The role of genetics in conservation

Maintenance of intraspecific genetic diversity is of major conservation concern as it has short and long-term evolutionary consequences for populations and species (Frankham et al., 2002). Loss of genetic diversity can have immediate, negative consequences for population viability causing a decline in individual reproductive fitness, termed inbreeding depression. Inbreeding depression is predominately caused by increased homozygosity, resulting from genetic drift and inbreeding, which exposes recessive deleterious alleles (Charlesworth and Willis, 2009) and has been detected in a number of fitness traits in natural plant populations, including percent germination, seed number and plant biomass (Angeloni et al., 2011). Small, isolated populations, in particular, are susceptible to inbreeding depression because they are expected to experience greater genetic drift and inbreeding than large, continuous populations (Ellstrand and Elam, 1993). Together with stochastic demographic, environmental and catastrophic factors, inbreeding depression can lead to

increased likelihood of extinction for small populations (Soulé and Mills, 1998; Brook et al., 2002). Erosion of genetic diversity can also limit the capacity of populations and species to adapt to changing environmental conditions, as evolutionary potential is dependent on the magnitude of underlying genetic diversity (Frankel et al., 1995; Frankham et al., 2002; Allendorf et al., 2013). Given that species face ever-changing environments, related to natural or anthropogenic causes, loss of genetic variation increases susceptibility of plant populations to extinction in the long-term (Frankel et al., 1995; Frankham et al., 2002; Kramer and Havens, 2009; Sgrò et al., 2010).

1.1.3.1 Informing management practices

Ideally, plant species should be conserved as naturally occurring populations within intact habitat, thereby protecting associated communities and preserving ecosystem function. However, habitats are increasingly degraded and/or population sizes are severely depleted rendering habitat protection insufficient for conservation (Kramer and Havens, 2009; Heywood, 2014). In such cases, augmentation, introduction or reintroduction may be necessary (Heywood and Iriondo, 2003; Heywood, 2014).

Augmentation seeks to counter inbreeding depression in small, genetically depauperate populations through the introduction of individuals from outside populations with the goal of increasing genetic variation within the target population to increase its adaptive capacity and facilitate long-term survival (Sgrò et al., 2010; Weeks et al., 2011; Frankham, 2015). If existing habitat is severely degraded, reintroduction or introduction of rare plants to novel sites may increase

species' survival prospects (Maunder, 1992; Guerrant and Kaye, 2007). Sites for reintroduction or introduction could include restored habitat (i.e., areas where the plant formerly occurred) or previously unoccupied habitat that could support a new population of plants (Fiedler and Laven, 1996). As global development continues, reintroduction and augmentation, together with whole-habitat restoration, are expected to become increasingly important management options due to increases in habitat loss and degradation, globalization of pests and diseases, as well as climate change (Kramer and Havens, 2009; Weeks et al., 2011).

In practice, many augmentation (Storfer, 1999) and reintroduction efforts for plant species (Godefroid et al., 2011; Drayton and Primack, 2012) have failed. Plant populations are often adapted to local conditions and initial failure of augmentation or reintroduction can occur if translocated individuals are insufficiently adapted for survival or reproduction in their new environment (Hufford and Mazer, 2003). Furthermore, if translocated individuals interbreed with the resident population, hybrid offspring may have reduced fitness relative to one or both parents, a phenomenon known as outbreeding depression (Hufford and Mazer, 2003; McKay et al., 2005; Weeks et al., 2011; Frankham, 2015).

For improved viability of restored populations, decisions should be informed by an understanding of genetics, species ecology and evolution. According to Allendorf et al. (2013), source populations for augmentation and reintroduction should possess high genetic diversity and be genetically and environmentally similar to the target population. This will minimize the risk that

translocated individuals are maladapted to their new environment and will avoid mixing adaptively differentiated populations (Allendorf et al., 2013). Alternatively, individuals from several source populations could be mixed in order to maximize the adaptive potential of the introduced population; however, this may dilute the adapted genome of the target population and should be carefully considered if the target population is genetically differentiated (Allendorf et al., 2013). Lopez et al. (2009) report that translocation of only a few individuals per generation can minimize the risks of outbreeding depression.

1.1.3.2 Identifying intraspecific conservation units

Identification of distinct intraspecific management units, or populations, is of great practical importance in conservation, facilitating efficient management and monitoring of targeted populations and guiding the allocation of limited conservation resources (Conner and Hartl, 2004; Allendorf et al., 2013). Among criteria for delineating conservation units, genetic variation is recognized as a key parameter (Frankham et al., 2002; Moritz, 2002; Conner and Hartl, 2004).

Protection of multiple distinct populations is necessary to preserve the evolutionary processes that sustain intraspecific genetic variation, minimizing extinction risk and maximizing adaptive potential of species (Moritz, 2002).

Specifically, molecular markers (e.g., microsatellites, allozymes etc.) can be used to elucidate patterns of dispersal among populations and to identify those that have experienced restricted gene flow (Crandall et al., 2000; Moritz, 2002). Genetic information should be complemented by knowledge of life history, morphology and environmental conditions (Crandall et al., 2000), reflecting

adaptive differentiation of populations (Frankham et al., 2002). Unique traits among adaptively differentiated populations may be important in the long-term persistence of species (Lesica and Allendorf, 1995). If populations exhibit large molecular differences that are concordant with differences in life history characteristics, morphological traits and/or environment or habitat type they warrant preservation and management as separate conservation units (Frankham et al., 2002; Allendorf et al., 2013).

1.1.4 Microsatellites

Microsatellite markers, also called simple-sequence repeats (SSR's), short tandem repeats (STR's) or variable number tandem repeats (VNTR's), are the most widely used DNA marker in population genetics, with applications in genome mapping, molecular ecology and conservation studies (Allendorf et al., 2013). Microsatellites are regions in the genome of many eukaryotic species consisting of several to many copies of the same short sequences (motifs), usually di-, tri- or tetranucleotides (e.g., "CGTCGTCGTCGTCGT", often written (CGT)₅).

Microsatellite markers are informative in conservation genetic studies for two main reasons; they are co-dominant and highly variable (polymorphic) (Frankham et al., 2002; Conner and Hartl, 2004). Co-dominant markers are useful as they can be used to distinguish between homozygote and heterozygote genotypes, with alleles displaying Mendelian inheritance. The high variability of microsatellites allows for assessment of relationships, structure and classification not only among populations, but also between individuals. In comparison with

other molecular markers (e.g., AFLP's and allozymes), microsatellites exhibit higher levels of heterozygosity, affording greater resolution to genetic studies, though patterns of diversity are consistent between studies employing microsatellite versus other marker types (Allendorf et al., 2013). The high variability observed in microsatellites is due to high mutation rates within microsatellite regions, chiefly as the result of slippage during DNA replication (Ellegren, 2000).

The relative utility of neutral markers (e.g., microsatellites) for conservation genetic studies versus quantitative genetics is recurrently debated in the literature (e.g., Holderegger et al., 2006; Gebremedhin et al., 2009; Kramer and Havens, 2009). It has been argued that microsatellites have limited utility because they cannot identify adaptive divergence (Holderegger et al., 2006), a critical determinant of a population's evolutionary potential (Kramer and Havens, 2009). However, microsatellites do provide valuable data on gene flow which, in itself, influences adaptation (Allendorf et al., 2013). Additionally, microsatellite markers have been shown to reveal the same patterns of population relationships as quantitative trait loci in some cases (e.g., Luikart et al., 2011).

1.1.5 Few-flowered Club-rush

Few-flowered Club-rush (*Trichophorum planifolium* (Spreng.) Palla), also known as Bashful Bulrush, is a perennial woodland species within the Cyperaceae (sedge) family. Its range extends from Massachusetts west to Ontario and south to Virginia and Kentucky with disjunct populations occurring in Missouri and southern Illinois (Figure 1.1). Globally, *T. planifolium* is secure to

apparently secure (NatureServe Rank: G4G5), but it is rare along the periphery of its distribution (Smith and Rothfels, 2007).

In Canada, there is only one known extant population of *T. planifolium*, located in a remnant natural area within one of the most highly urbanized regions in Canada (Crins et al., 2009) at the Royal Botanical Gardens (RBG) in Hamilton, Ontario. *Trichophorum planifolium* previously occurred at one other site in Canada, in the Rouge Valley near Toronto, but has not been observed in this location since 2005 (Smith and Rothfels, 2007). Owing to its limited Canadian range and an apparent decline in population size, *T. planifolium* is listed as endangered under both the Canadian *Species at Risk Act* (SARA) and the Ontario *Endangered Species Act* (COSEWIC, 2000). Recent population surveys at the Hamilton site have found that less than 2% of the population is comprised of young plants (<10 shoots) (Iwanycki et al., 2010). It has been suggested that the low recruitment observed may be due to low intrapopulation genetic diversity (Smith and Rothfels, 2007).

Trichophorum planifolium is caespitose, growing in dense, low tufts (~ 40 cm) from short rhizomes. At the base of the plant, the leaf sheaths are weathered, shredded, and light brown to a distinctive reddish-brown color (Figure 1.2). Its stems are 3-angled or round and often wiry, terminating in a single spikelet, about 5 mm long, with 4-8 flowers that occur in late April through June. *Trichophorum planifolium* exhibits characteristics of a wind-pollinated plant, lacking a showy perianth and nectaries and possessing long and exerted styles and stamens (Crins, 1989; Iwanycki et al., 2010). The fruit is a brown, 3-sided

achene that matures in mid-summer and disperses in late July to August (Iwanycki et al., 2010). A mechanism for seed dispersal has not been confirmed (Smith and Rothfels, 2007), but the matting of leaves and stems during the fruiting period may facilitate local seed dispersal (Crins, 1989). If colonies are formed in this way, nearby tufts would be genetically similar (Crins, 1989).

Up to now, molecular research with *T. planifolium* has focused on resolving the complex taxonomy of the family Cyperaceae using plastid DNA (*rbcL* and *trnL-F*) in combination with morphological analysis (e.g., Dhooge, 2005 and Muasaya et al., 2009). Besides these phylogenetic analyses, no genetic work has been published for *T. planifolium* and any information regarding the population genetics for the species has been based on inferences from observations of the species in the field.

1.2 Thesis objectives

The overarching objective of this study is to assess the genetic variability within the Canadian population and between the Canadian and American populations of *T. planifolium*, given as an urgent priority in the *Recovery Strategy for Few-flowered Club-rush/Bashful Bulrush* (*Trichophorum planifolium*) in *Canada* (Smith and Rothfels, 2007). As no species-specific markers appropriate for studies of intraspecific genetic variation have been developed for *T. planifolium*, it was first necessary to develop molecular markers. In Chapter 2, I describe the isolation and characterization of 11 polymorphic microsatellite markers for *T. planifolium*.

In Chapter 3, I present the results of a range-wide population genetic and habitat survey, which will be used to address the following research questions: 1) Is the Canadian population of *T. planifolium* genetically depauperate relative to American populations? 2) Is the Canadian population genetically differentiated from American populations? and 3) Is the Canadian population subject to different environmental conditions than elsewhere in the species' range? I subsequently provide recommendations for the management of the species in Canada.

Figure 1.1 Global distribution of Few-flowered Club-rush (*Trichophorum planifolium*) based on records obtained from online databases (Global Biodiversity Information Facility (GBIF; www.gbif.org) and Tropicos (www.tropicos.org)), herbaria (WVA, VPI, NYBG, OS, HAM, and PH), local botanists, and literature (House, 1924; Schaffner, 1934).

Figure 1.2 Characteristic red-brown leaf sheaths and growth form of Few-flowered Club-rush (*Trichophorum planifolium*). Photo courtesy of Dr. Tyler Smith.

2 Chapter: Development and characterization of 11 microsatellite primers for the sedge, *Trichophorum planifolium* (Cyperaceae)¹

2.1 Introduction

Trichophorum planifolium (Spreng.) Palla (Cyperaceae) is a woodland sedge that occurs on dry, rocky slopes in north-eastern North America. In Canada, there is only one known extant occurrence, located in a remnant natural area within one of the most highly urbanized regions in the country. Owing to its limited Canadian range and an apparent decline in population size (COSEWIC, 2000), *T. planifolium* is listed as endangered under the Canadian *Species at Risk Act* (SARA). Like many species-at-risk in Canada (Yakimowski and Eckert, 2007; Gibson et al., 2009), *T. planifolium* reaches the northern limit of its range in Canada, but is relatively common farther south. Although there is some debate over whether, or when, peripheral populations merit national concern (Gibson et al., 2009), empirical evidence suggests that they may be important reserves of genetic diversity (Eckert et al., 2008), providing adaptive and evolutionary potential for the species (Lesica and Allendorf, 1995) and facilitating species' responses to climate change (Etterson and Shaw, 2001; Parmesan, 2006; Gibson et al., 2009).

An understanding of the population genetic structure of *T. planifolium* is needed to guide management strategies for this species. At present, no

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molecular markers appropriate for studies of intraspecific genetic variation have been developed for *T. planifolium*. To this end, we isolated and characterized 11 polymorphic microsatellite loci.

2.2 Methods and results

Genomic DNA (~ 6 µg) was extracted from the silica–dried leaf tissue of one individual of *T. planifolium* collected from Daniel Boone National Forest (Kentucky, U.S.A.) in 2011 using a Nucleospin Plant II kit (Machery-Nagel, Bethlehem, Pennsylvania, U.S.A.) following the manufacturer’s protocol. The sample was submitted to the Georgia Genomic Facility at the University of Georgia (Athens, Georgia, U.S.A.) for isolation of microsatellite loci and primer development. DNA was fragmented using the Bioruptor UCD-300 sonication device (Diagenode, Denville, New Jersey, U.S.A.). Libraries compatible with Illumina TruSeqHT were prepared using the Kapa Library Preparation Kit (KR0453-v2.13; Kapa Biosystems, Wilmington, Massachusetts, U.S.A.) with custom indexes from Faircloth and Glenn (2012). Libraries were quantified with Qubit (Life Technologies, Burlington, Ontario, Canada) and sequenced using an Illumina MiSeq v3 600 cycle kit (Illumina, San Diego, California, U.S.A.). A total of 6,391,132 reads were imported and paired in Geneious 7.0.6 (Biomatters, Auckland, New Zealand). Illumina TruSeq adapters and bases with an error probability limit above 0.05 were trimmed. A de novo assembly was performed on the first 1,000,000 sequences where both reads of any pair were ≥200 bases. Consensus sequences between 200 and 420 bp were exported from Geneious as FASTA files and imported into MSATCOMMANDER 1.0.8 beta (Faircloth,

2008). A total of 721 loci with perfect di-, tri- or tetra-nucleotide repeats were designed at default minimum lengths (i.e., 8 repeats for di- and tri-nucleotide motifs, 6 repeats for tetra-nucleotide motifs) and combining loci ≤ 50 bp apart. Sixty-three CAG-tagged primer pairs for di- (9), tri- (47) and tetranucleotide (7) microsatellite loci with the greatest number of motif repeats were selected for further testing.

Loci were subsequently evaluated for amplification consistency and screened for polymorphisms with 96 samples of *T. planifolium* collected from 12 populations distributed through the range of the species in May 2014: (1) CTPP, Tarrywile Park, Connecticut; (2) MABM, Bare Mountain, Massachusetts; (3) MDDM, Dan's Mountain, Maryland; (4) MOBS, Big Spring State Park, Missouri; (5) MOSH, Sutton Hollow, Missouri; (6) NHER, Elmer G. Raymond Park, New Hampshire; (7) NYMP, Mendon Ponds Park, New York; (8) OHSC, Strait Creek Prairie Bluffs Preserve, Ohio; (9) ONRB, Royal Botanical Gardens, Ontario; (10) PAGP, Gifford Pinchot State Park, Pennsylvania; (11) VAHB, Huckleberry Trail, Virginia; and (12) WVFM, Fisher Mountain, West Virginia (Appendix A). These collections yielded substantially higher DNA quality than the samples obtained from the Kentucky population used in microsatellite isolation. Consequently, we chose to use the more recent collections of *T. planifolium* for further testing of the microsatellite loci. Voucher specimens were obtained for each population, except when population size was estimated to be below 100 individuals, or if permits did not allow destructive sampling. For sites where a voucher was not collected, a representative voucher has been assigned if available. The deposition of

vouchers is provided in Appendix A.

Reactions were carried out in 12.5 µl reaction volumes containing 2.5 µL 5X Phusion High-Fidelity Buffer (New England Biolabs, Whitby, Ontario, Canada), 0.25 µL dNTPs (10 mM), 0.625 µL untagged primer (10mM), 0.0625 µL tagged primer (10 mM), 0.5625 µL dye-labelled CAG Tag (10mM), 0.375 µL DMSO, 0.125 µL Phusion High Fidelity Polymerase (2 U/µL (New England Biolabs)), 5.5 µL ddH₂O and 2.5 µL DNA (10 ng/µL) using a T-100 Thermal Cycler (Bio-Rad, Hercules, California, U.S.A.). To obtain high quality amplification product, we used the thermocycling profile of touchdown PCR (TD-PCR) (Korbie and Mattick, 2008) with some modification. Thermal cycling began with 5 min denaturation at 95°C, followed by the touchdown phase with 15 cycles of 30 s denaturation at 95°C, 30 s annealing from 72 to 57°C (–1°C per cycle) and 30 s elongation at 72°C, followed by a generic amplification stage of 20 cycles of 30 s denaturation at 95°C, 30 s annealing at 57°C and 30 s elongation at 72°C followed by a 5-min final elongation at 72°C. Amplification products with incorporated fluorescent labels (6-FAM and VIC; Life Technologies) were pooled into groups of four and sequenced by capillary electrophoresis using a 3130xL Genetic Analyzer (Life Technologies) with the GeneScan 500 LIZ Size Standard (Life Technologies). Of the 63 primers tested, 18 exhibited consistent amplification and polymorphisms. Eleven loci that could be pooled into four genotyping runs (i.e., the fragment sizes for the primers in each run did not overlap each other) were selected (Table 2.1) and their utility for future studies of genetic diversity and structure in *T. planifolium* was evaluated.

Individual samples were genotyped using GeneMapper v.5 software (Life Technologies) and verified with manual scoring. Standard measures of intra-population genetic diversity including average number of alleles (A) and observed (H_o) and expected (H_e) heterozygosity were calculated with the R package 'adegenet' version 1.4-2 (Jombart, 2008) and 'PopGenReport' version 2.1 (Adamack and Gruber, 2014) (Table 2.2). Of the 96 samples initially screened, 16 failed at one or more loci and were excluded from the population genetic analysis. In total, 31 alleles were observed for 11 microsatellite loci in 80 individuals from 12 populations of *T. planifolium*. The number of alleles per locus ranged from two to six (overall mean = 2.82 alleles), with the highest number detected in the population at Fisher Mountain, West Virginia (WVFM). The mean H_o per site ranged from 0.00 to 0.06, whereas the mean expected H_e varied between 0.00 and 0.19. Although these values are low, they are comparable to those documented in other woodland sedges (e.g., *Carex breviculmis* R. Br. and *C. hebes* Nelmes; M'baya et al., 2013), and may be explained by limited pollen and seed dispersal among populations (Crins, 1989).

2.3 Conclusions

The primer pairs developed in this study successfully amplified 11 polymorphic microsatellite loci in populations distributed across the species' range and, as such, will be a useful tool with which to examine patterns of genetic diversity and differentiation in *T. planifolium*. An understanding of genetic variability and structure within the Canadian population, and between the

Canadian and core populations in the United States, is necessary to guide the development of effective management and monitoring protocols for the species.

Table 2.1 Characterization of 11 polymorphic microsatellite loci developed in *Trichophorum planifolium*.

Genotyping group	Locus	Repeat motif	Primer sequence (5' – 3')	Size range (bp)	A	T _a (°C)	Fluorescent dye	Genbank accession no.
Group 1	TP142	(AAT) ₁₁	F: CAGTCGGGCGTCATCACTTCATGTAGATCCGTCCC R: TGTCCTTACTCACCTAAGCCAAAG	135-145	2	53	6-FAM	KR264968
	TP174	(AAT) ₁₄	F: CTCGAGTTCAAGTCCAAGCG R: CAGTCGGGCGTCATCATCCATCAGACCGTCACCC	167-170	2	55	VIC	KR264969
	TP341	(AGC) ₁₄	F: CAGTCGGGCGTCATCATGGTGGATTCTGCATGG R: GTGCTTTATCACCAACAGCAG	299-323	4	54	6-FAM	KR264970
	TP152	(AG) ₁₅	F: CAGTCGGGCGTCATCAGTTTCAAAAGGCCAAAGTCG R: GTTTAATCACTCAGTCGTCCACC	154-156	2	55	VIC	KR264971
Group 2	TP326	(AG) ₁₆	F: GTTTGTTGCCATCAGTCAAGTGG R: CAGTCGGGCGTCATCATCTCTGCCATAGTCACTGCC	216-226	2	55	6-FAM	KR264972
	TP45	(AAG) ₁₂	F: CAGTCGGGCGTCATCACCAAGCTAGATTCGCCAAC R: TCTCCTTGGTACCCTCAG	368-382	2	54	VIC	KR264973
	TP325	(AAT) ₁₈	F: CAGTCGGGCGTCATCATCTTAAAGGTGACGTGACGG R: AAATTTGCGGAATAGCGGCG	152-171	6	54	VIC	KR264974
Group 3	TP406	(AAT) ₁₅	F: CGGATCTCTGCTGGTGATCC R: CAGTCGGGCGTCATCAGCAGAAATTCCTCCTAATCC	213-216	2	54	6-FAM	KR264975
	TP434	(AAT) ₁₃	F: CAGTCGGGCGTCATCATCTGGTCGAACAATCAGAAGAC R: GCATTGGATCAGATACCGCC	163-169	3	55	6-FAM	KR264976
Group 4	TP80B	(AAT) ₈	F: ACGCCAGTGGACCTATGTG R: CAGTCGGGCGTCATCAGAGCGGTTTATGGCCTTTC	208-220	4	55	VIC	KR264977
	TP330	(AAT) ₁₁	F: CAGTCGGGCGTCATCATTTGCCATCACAGTATTACACCG R: ACTTCCCAGAGCACCATAGC	355-358	2	55	6-FAM	KR264978

Note: T_a = theoretically optimal annealing temperature (touchdown PCR was carried out with annealing temperatures of 72° - 57°C for all loci); A = number of alleles.

Table 2.2 Results of initial primer screening and genotyping in 80 individuals from 12 populations of *Trichophorum planifolium*.

Locus	CTTP (n = 8)		MABM (n = 6)		MDDM (n = 7)		MOBS (n = 7)		MOSH (n = 6)		NHER (n = 8)		NYMP (n = 8)		OHSC (n = 6)		ONRB (n = 6)		PAGP (n = 6)		VAHB (n = 5)		WVFM (n = 7)	
	A	H _e	A	H _e	A	H _e	A	H _e	A	H _e	A	H _e	A	H _e	A	H _e	A	H _e	A	H _e	A	H _e	A	H _e
TP142	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
TP152	1.0	0.0	0.0	1.0	0.0	0.2	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	2.0	0.2
TP174	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	2.0	0.4	0.2	1.0	0.0	0.0
TP325	1.0	0.0	0.0	2.0	0.4	0.0	1.0	0.0	0.0	2.0	0.5	0.0	1.0	0.0	0.0	1.0	0.0	0.0	2.0	0.3	0.0	1.0	0.0	0.0
TP326	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	2.0	0.4	0.2	1.0	0.0	0.0
TP330	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
TP341	1.0	0.0	0.0	2.0	0.3	0.0	2.0	0.3	0.1	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
TP406	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
TP434	1.0	0.0	0.0	1.0	0.0	0.0	2.0	0.1	0.1	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
TP45	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0	2.0	0.4	0.2	1.0	0.0	0.0
TP80B	1.0	0.0	0.0	2.0	0.3	0.0	1.0	0.0	0.0	2.0	0.5	0.0	1.0	0.0	0.0	1.0	0.0	0.0	2.0	0.4	0.2	1.0	0.0	0.0
Mean	1.0	0.0	0.0	1.3	0.1	0.0	1.4	0.1	0.0	1.2	0.1	0.0	1.4	0.1	0.0	1.0	0.0	0.0	1.5	0.2	0.1	1.0	0.0	1.6

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

3 Chapter: Conservation genetics and habitat characteristics of Few-flowered Club-rush (*Trichophorum planifolium*)

3.1 Introduction

In high-latitude countries, geographically peripheral populations form a significant proportion of species-at-risk (Yakimowski and Eckert, 2007; Gibson et al., 2009). For example, in Canada, approximately 90% of threatened or endangered plant species are locally rare representatives of species that reach the northern extent of their range in Canada, but are common farther south in the United States (Yakimowski and Eckert, 2007). Concern over the investment of conservation resources into species that are locally rare, but globally common has led to debate over whether, or when, peripheral populations merit national concern (e.g., Hunter. and Hutchinson, 1994; Hampe and Petit, 2005; Gibson et al., 2009).

Peripheral populations are typically small and geographically isolated due to reduced resource availability at the range margin (Brown, 1984). As a consequence, they may be at greater risk of extirpation due to genetic (i.e., inbreeding depression) as well as stochastic demographic, environmental and catastrophic factors (Soulé and Mills, 1998; Brook et al., 2002). These risks may be exacerbated by anthropogenic threats such as habitat loss and fragmentation (Young et al., 1996). Nevertheless, peripheral populations may be important sources of adaptive and evolutionary potential for the species (Safriel et al., 1994; Channell and Lomolino, 2000; Leppig and White, 2006; Eckert et al.,

2008), harbouring unique genetic diversity and/or adaptations to conditions at the range edge (Lesica and Allendorf, 1995; Bunnell et al., 2004).

However, not all peripheral populations are genetically differentiated from core populations and merit conservation concern (Lesica and Allendorf, 1995; Leppig and White, 2006). For example, in a review of 134 genetic studies in plants, Eckert et al. (2008) found that only 70.3% of studies identified increased genetic differentiation towards the range margin. These differences may be attributable to species' specific characteristics (Gibson et al., 2009). In plants, life-history characteristics (i.e., breeding system and dispersal mechanisms) are a major determinant of population genetic structure, because they are related to gene flow (Loveless and Hamrick, 1984; Frankel et al., 1995). In this regard, knowledge of the level and spatial distribution of intraspecific genetic diversity can add valuable information to guide conservation efforts for peripheral populations, especially when the reproductive biology and ecology of the species are not well understood.

Lesica and Allendorf (1995) proposed a useful theoretical framework for evaluating the conservation value of peripheral populations, identifying isolation of populations and environmental differences as main criteria. In practice, neutral genetic markers, such as microsatellites, can be used to identify populations that have experienced restricted gene flow (i.e., are genetically isolated) (Crandall et al., 2000; Moritz, 2002). However, microsatellite markers cannot provide information on adaptive divergence of populations (Holderegger et al., 2006). Therefore, to capture variation in selection pressures across the species' range,

environmental differences among populations should be assessed (Allendorf et al., 2013). A similar approach was recently used to identify conservation priorities in the endangered African tree *Prunus africana* (Hook. f.) Kalkman (Vinceti et al., 2013).

In plant conservation, augmentation is a valuable tool to mitigate habitat loss and there is some evidence that improving population connectivity (i.e. gene flow) will increase survival of peripheral populations (e.g., Lawson et al., 2012). However, implementation of augmentation efforts needs to be carefully considered, as introduction of differentiated individuals can dilute local genetic composition and may decrease the conservation value of peripheral populations (Barrett and Kohn, 1991). Moreover, outcrossing can be deleterious, leading to hybrid offspring with reduced fitness – a phenomenon known as outbreeding depression (McKay et al., 2005; Weeks et al., 2011). Some effort has been made to develop a means of assessing outbreeding risk for management efforts (e.g., Frankham et al., 2011). In particular, the extent and mechanism of genetic differentiation among populations must be explored to evaluate genetic rescue as a potential management strategy for plant populations (Hufford and Mazer, 2003; McKay et al., 2005; Weeks et al., 2011; Frankham, 2015).

One example of a plant species that reaches the northern limit of its range in Canada, but is common further south is *Trichophorum planifolium* (Spreng.) Palla, commonly known as Few-flowered Club-rush or Bashful Bulrush. This is a perennial woodland species within the family Cyperaceae that typically occurs on dry, rocky slopes in association with hardwoods including Oaks (*Quercus* spp.)

and American Beech (*Fagus grandifolia* Ehrh.). The range of *T. planifolium* extends from Massachusetts west to Ontario and south to Virginia and Kentucky with disjunct populations occurring in Missouri and southern Illinois. The species is caespitose, growing in dense, low tufts (~ 40 cm) from short rhizomes. It is likely wind-pollinated, possessing long and exerted styles and stamens and lacking both a showy perianth and nectaries (Crins, 1989; Iwanycki et al., 2010). However, because of the low stature of *T. planifolium*, and the varied terrain on which it is usually found, outcrossing may be limited (Crins, 1989). Seeds mature in mid-summer and disperse in late July to August (Iwanycki et al., 2010). A mechanism for seed dispersal has not been confirmed (Smith and Rothfels, 2007), but it has been suggested that the matting of leaves and stems during the fruiting period may facilitate local seed dispersal and the formation of genetically similar tufts (Crins, 1989).

In general, *T. planifolium* is considered common within the central portion of its range, but rare along the periphery (Smith and Rothfels, 2007). Southern Ontario represents the northern extent of the species range where there is only one known extant locality: Cootes Paradise at the Royal Botanical Gardens in Hamilton, Ontario. *Trichophorum planifolium* was previously known from one other location in Ontario, in Rouge Valley near Toronto, but no plants have been located at this site since 2005 (Smith and Rothfels, 2007). Owing to its limited Canadian range and an apparent decline in population size (COSEWIC, 2000), *T. planifolium* is listed as endangered under the Canadian *Species at Risk Act* (SARA). It has previously been suggested that low recruitment observed at the

Hamilton site may be due to limited intrapopulational genetic diversity (Smith and Rothfels, 2007).

The main objective of our research was to assess genetic variability within the Canadian population and between the Canadian and American populations of *T. planifolium* to address the following questions: (1) Does the Canadian population of *T. planifolium* exhibit similar levels of genetic diversity to American populations? (2) Is the Canadian population genetically distinct from American populations? and (3) Are environmental conditions, in terms of soils and climate, different at the northern range edge? To address these questions, we used nuclear microsatellite markers, specifically developed for *T. planifolium*, as these are hyper-variable and can elucidate patterns of gene flow among populations. In addition, we explored differences in soil and climatic characteristics across the species' range to reflect differences in selection pressures that could lead to the adaptive divergence of populations not captured by our genetic analysis. Implications for conservation and management initiatives for the species in Canada are subsequently discussed.

3.2 Methods

3.2.1 Occurrences and genetic sampling

In spring 2014, we sampled 29 populations of *T. planifolium* across its geographic distribution (Table 3.1, Figure 3.1). Population sizes were estimated in the field and categorized as very small (<100 individuals), small (100-500), medium (501-1000) or large (>1000). To ensure a comparable sampling area was maintained between populations, we sampled individuals from within a

maximum diameter of 100 m. However, this could lead to underestimates of genetic diversity for larger populations. We also imposed a minimum distance of 1 m between sampled individuals to avoid collecting from the same clone. A total of 919 samples were obtained, with a minimum of four and a maximum of 50 samples per population (mean = 31.7). Our protocol was modified for one population (PACR), where one sample was taken at a distance of 150 m to increase sample size to seven.

Each sample consisted of 200 mg, about 20 centimeters, of leaf tissue from individual *T. planifolium* plants. After collection, samples were immediately placed on 40-50 mL of silica gel for drying and stored at room temperature prior to molecular analysis. A random subsample of 20 samples per population, except for populations where fewer than 20 samples were obtained (PACR, PASG and WVSS), were selected for genetic analysis. For these populations, all samples collected were used.

To complement our genetic analysis, we examined soil and climate conditions for populations surveyed, as microsatellite markers do not provide information on the adaptive differentiation of populations. Soil samples, composed of at least 7 sub-samples, were collected at each population (except WVMO and WVSS), with sub-samples made to rooting depth (~10 cm) immediately adjacent to *T. planifolium* plants. Composite samples were submitted to the University of Massachusetts Soil Laboratory or the University of Guelph Soil Laboratory, according to their provenance, and soil properties including pH, cation exchange capacity (CEC), organic matter (OM) and soil

nutrients (Calcium, Magnesium, Manganese, Phosphorous, Potassium, Zinc) were quantified. Climate data for all 29 populations was extracted by point from each of 19 bioclimatic variables (30-arc second resolution) obtained from the Worldclim dataset (Hijmans et al., 2005; www.worldclim.org). Soils and climate data are provided in Appendix B.

3.2.2 DNA extraction and microsatellite assays

For each individual, 20 mg of silica dried leaf tissue was ground with 3 mm-diameter stainless steel beads in a 2 mL Eppendorf tube at 30 Hz for 2 min using a TissueLyser II (Qiagen, Venlo, Limburg, Netherlands). Total genomic DNA was extracted from ground tissue using the Nucleospin Plant II Kit (Machery-Nagel, Düren, Germany). The quality and concentration of DNA obtained was verified using a Nanodrop 2000 UV-Vis Spectrophotometer (ThermoScientific, Waltham, Massachusetts, U.S.A.).

Eleven nuclear polymorphic microsatellite markers developed by Nowell et al. (in press) were used to assess the population genetics of *T. planifolium*. Amplification of microsatellite fragments was performed in 8 µL reaction volumes containing 0.416 µL primer mix (10 mM; 10:1 untagged to tagged primer), 0.192 µL dye-labelled CAG Tag (10 mM; 6-FAM or VIC, Life Technologies, Carlsbad, California, U.S.A.), 0.24 µL DMSO, 4 µL 2X Phusion High-Fidelity Master Mix with HF Buffer (New England Biolabs, Ipswich, Massachusetts, U.S.A.), 2.152 µL ddH₂O and 1 µL DNA (10 ng/µL) using a T-100 Thermal Cycler (Bio-Rad, Hercules, California, U.S.A.). Cycling conditions followed touchdown PCR (TD-PCR) (Korbie and Mattick, 2008) as in Nowell et al. (in press), as these were

optimal for the suite of primers used. PCR products were visualized on a 1% agarose gel stained with GelRed (Biotium, Hayward, California, U.S.A.) and viewed with a High Performance Transilluminator (UVP, Upland, California, U.S.A.) with a 100 bp DNA ladder (New England Biolabs) to confirm the presence and size of amplification products and absence of contamination prior to genotyping.

Amplification products were subsequently pooled into groups of four and visualized by capillary electrophoresis using a 3130xL Genetic Analyzer (Life Technologies) with the GeneScan 500 LIZ Size Standard (Life Technologies). Individual samples were genotyped using Genemapper v.5 software (Life Technologies) and verified with manual scoring.

3.2.3 Data analysis

Owing to amplification failure, only 401 individual samples could be genotyped at all 11 loci (Appendix B). These samples were used in the subsequent analysis of population genetic diversity and differentiation. One population, VASC, was excluded entirely due to amplification failure at nearly all loci, owing to poor DNA quality, possibly as a result of recent flooding (pers. obs.). All genetic analyses were performed in R ver. 3.1.2 (R Development Core Team, 2014), unless otherwise specified.

3.2.3.1 Genetic diversity

All individuals were tested for clonality via calculation of the Index of Association (I_a) and Standardized Index of Association (r_d) in the R package 'poppr' ver. 1.1.4 (Kamzar et al., 2014). Tests for departures from Hardy-

Weinberg equilibrium (HWE) were performed in the R package 'adegenet' ver. 1.4-2 (Jombart, 2008). The observed frequency of null alleles at each locus was examined following the methods of Brookfield (1996) implemented in the R package 'PopGenReport' ver. 1.6.6 (Adamack and Gruber, 2014).

Standard measures of intra-population genetic diversity were calculated for each population using the R packages 'adegenet', 'poppr', 'PopGenReport' and 'hierfstat' ver. 0.04-14 (Goudet, 2005). Genetic diversity measures included: number of private alleles (A_p), allelic richness (A_R) and total number of multi-locus genotypes. To compensate for differences in sample size, rarefaction was performed on number of multi-locus genotypes using the methods implemented 'poppr'. Additionally, we calculated observed (H_o) and expected (H_e) heterozygosity and the inbreeding coefficient (F_{is}). Confidence intervals for the inbreeding coefficient were constructed with 10,000 bootstrap replicates. We used the Linkage Disequilibrium Method in NeEstimator ver. 2.01 (Do et al., 2014) to estimate contemporary effective population size (N_e) for each population.

To compare levels of genetic diversity among populations and population size groups (given in Table 3.1) we used Kruskal-Wallis tests and analysis of variance (ANOVA). We also evaluated differences in genetic diversity among Small-sized populations using a Kruskal-Wallis test to compare the genetic diversity of the Canadian population (ONRB) while controlling for the effects of population size.

3.2.3.2 Population structure

Pairwise F_{ST} , the level of differentiation among populations (Nei, 1973), was calculated in 'adegenet' and confidence intervals were constructed with 10,000 bootstrap replicates in 'hierfstat'. To evaluate the distribution of genetic variance within and among populations, we performed analyses of molecular variance (AMOVA) in 'poppr' based on Nei's genetic distance following the methods of Excoffier et al. (1992). The statistical significance of the results was assessed with 1000 random permutations. We tested for isolation by distance (IBD) among populations by comparing Nei's genetic distances and the geographical (great-circle) distances between individuals by application of the Mantel test with 999 permutations.

Initially, we intended to use the program Structure ver. 2.3.4 (Pritchard et al., 2000) to explore the number of genetic clusters in our dataset. However, our populations did not meet the basic assumptions of the model (e.g., HWE and linkage equilibrium), as described below. As such, we elected to avoid model-based assignment protocols and instead relied on clustering of populations based on Nei's genetic distance. Hierarchical clustering was carried out using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), validated with the cophentic correlation. The Kelley-Gardner-Sutcliffe penalty function (Kelley et al., 1996) was calculated with the R package 'maptree' ver. 1.4-7 (White and Gramacy, 2012) to determine the number of clusters in the dataset.

3.2.3.3 Environmental characteristics

Hierarchical clustering was used to identify similarities in environment among populations of *T. planifolium* sampled for genetic analysis. Soil and climate datasets were first rescaled to have a mean of zero and a standard deviation of one. The Euclidian distance between the variables was subsequently calculated. We used the UPGMA clustering method, validated with the cophenetic correlation coefficient, and the Kelley-Gardner-Sutcliffe penalty function to derive an optimal number of clusters in the dataset.

3.3 Results

3.3.1 Genetic analysis

The calculated Index of Association (I_a) and Standard Index of Association (ra) were 0.34 ($p < 0.05$) and 0.035 ($p < 0.05$), respectively, suggesting a high degree of linkage disequilibrium among loci. Significant departures from HWE were detected in 17 of 28 sites (61% of sites) and only one marker, TP142, showed no departures from HWE. Other markers showed significant departures from HWE in up to 12 populations (TP325). The mean estimation of frequency of null alleles for the dataset was high, ranging from 0.12 to 0.65 per marker. However, as discussed below, this result is also consistent with high levels of inbreeding, either through self-pollination or mating among closely related individuals.

3.3.2 Genetic diversity

A total of 43 alleles were observed for 11 microsatellite loci in the 401 individuals analyzed, an average of 3.91 alleles per locus. The number of alleles per locus ranged from two (TP174, TP326, TP330 and TP45) to nine (TP325).

Eleven private alleles were detected in ten populations (for up to two loci). Allelic richness (A_R) varied across populations from 1.00 to 1.47 (mean = 1.14). In total, 97 unique multi-locus genotypes were detected among the 401 individuals, with five populations each comprised of a single multi-locus genotype. Among the 97 genotypes, only four were shared between populations: two between MOOC and MOSH, one between OHBH, OHPR and PASG and one between CTPP, MD-DM and NYTS. Overall, the number of unique multi-locus genotypes per population ranged from 1.00 to 6.30 after rarefaction. Observed heterozygosity (H_o) per site varied between 0.00 and 0.06 (mean = 0.01) while expected heterozygosity (H_e) ranged from 0.00 to 0.24 (mean = 0.07). All populations except two, MAMT and MOHC, were significantly inbred, with estimates of F_{is} ranging from -0.12 to 1.00 in the 28 populations. Genetic variation in the 28 populations is summarized in Table 3.2. Contemporary effective population size (N_e) is not reported because NeEstimator failed to yield reasonable estimates due to the low variation in our dataset.

Across all measures of genetic diversity, there were no statistically significant differences between populations (Kruskal-Wallis test: $\chi^2 = 27$, $df = 27$ $p > 0.05$) or among population size groups (A_R : $F(3, 24) = 0.488$, $p = 0.694$; MLG: $F(3, 24) = 1.008$, $p = 0.406$; H_e : $F(3, 24) = 0.506$, $p = 0.682$; H_o : $F(3, 24) = 0.452$,

$p = 0.717$) though populations at the center of the species' range and in Missouri tended to exhibit higher diversity than populations at the range edge. Within the Small-sized populations, there were no statistically significant differences between populations in terms of basic population genetic parameters (Kruskal-Wallis test: $\chi^2 = 8$, $df = 8$, $p > 0.05$).

3.3.3 Population genetic structure

The mean genetic distance between populations was high, averaging 0.80 for pairwise F_{ST} estimates. Most comparisons (356 out of 378, 94.2%) were statistically different from zero, ranging from 0.03 to 1.00 (Table 3.3). Analysis of molecular variance (AMOVA) showed that the majority of genetic variation was among populations (59.1%, $p < 0.001$), indicating significant population structure, while the variation among the individuals within populations was 32.6% ($p < 0.001$) and the variation within individuals was 8.3% ($p < 0.001$). An isolation by distance pattern of genetic structuring was detected with the Mantel test: a significant correlation was found between genetic and geographic distances between individuals ($r^2 = 0.29$, $p = 0.001$).

Among the 28 populations, five genetic clusters were identified (Figure 3.2). The cophenetic correlation was 0.69, indicating good clustering structure. In general, populations were grouped geographically. Interestingly, the Canadian population was found to have a similar genetic profile to Missouri populations. Two populations, NJKP and MDDM each represent a distinct cluster.

3.3.4 Environmental characteristics

Five climatic and four soil groups were identified among the populations of *T. planifolium* surveyed. The cophenetic correlations were 0.84 and 0.94, respectively, indicating good agreement between the raw data and the clustering structure. Of the five climate clusters, characteristics among four groups (2, 3, 4 and 5) were similar, with some variation in mean annual temperature (6.9 - 13.8 °C) (Table 3.4). These comprised the majority of observations (93.1%) and corresponded to a significant portion of the species' range (Figure 3.4). The remaining climate group (1), consisting of ONRB and NYMP, represents local climate conditions occurring at the northern range margin, and is characterized by low mean diurnal temperature variation (mean = 10.1 °C), high temperature seasonality (mean = 9.3 °C) and low annual precipitation (mean = 826 mm). Likewise, for soils, one cluster (2) corresponds to conditions with a broad distribution across the species' range, and includes the majority of observations (81.4%), while the other three groups are limited in their distribution (Figure 3.5). These groups are defined by their acidity and corresponding nutrient composition: (1) MDDM has acidic soils (pH = 4.20) with high organic matter (OM) and high levels of Phosphorous (P), Potassium (K) and Manganese (Mn), (3) PAGP and MABM have more alkaline soils (pH = 5.67) than MDDM with a high Calcium (Ca) and Magnesium (Mg) content, (4) PACR and ONRB also have more alkaline soils (pH = 5.7) than MDDM, but have higher Calcium (Ca) levels and lower levels of macro- and micronutrients than group 3 (Table 3.5).

3.4 Discussion

3.4.1 Genetic diversity

Microsatellite analysis revealed low levels of genetic variability in the 28 populations of *T. planifolium* surveyed, irrespective of size ($H_o = 0.00$ to 0.06). Though wind-pollinated species are generally expected to possess high levels of genetic variation due to high levels of intrapopulational gene flow (Loveless and Hamrick, 1984), pollen dispersal in other wind-pollinated sedges has been found to be highly restricted (e.g., *Carex plantaginea* Lam. and *Carex platyphylla* Carey; Handel, 1976). Moreover, the level of diversity detected in *T. planifolium* is comparable among closely related taxa with similar growth forms and reproductive strategies (e.g., *Trichophorum caespitosum* (L.) Hartm.: $H_o = 0.01$ to 0.04 (Godt et al., 1996), *Carex loliacea* L.: $H_o = 0.00$ to 0.12 (Kull and Oja, 2007) and *Carex magellanica* subsp. *irrigua* (Wahlenb.) Hultén: $H_o = 0.00$ to 0.03 (Kull and Oja, 2010)).

The low level of heterozygosity and high inbreeding (mean $F_{is} = 0.83$) detected support earlier predictions that outcrossing in this species may be limited. Crins (1989) suggested that the low stature of the plant, together with the varied terrain on which it is found, might preclude interpopulational gene flow. Under these conditions, the probability of self-pollination is expected to increase for wind-pollinated species (Loveless and Hamrick, 1984). Recombination is less effective in self-pollinating species, leading to greater homozygosity and uniformity within populations. Indeed, several of the populations surveyed (CTTP, MAST, OHBH, OHSC and VAHB) were entirely monomorphic. While many

species in Cyperaceae are thought to be dichogamous, including *T. planifolium* (Crins, 1989), Ford et al. (1998) suggested that geitonogamous reproduction, due to an overlap in the maturation times of male and female flowers within a spike, among spikes on an inflorescence, and/or among stems on the same genet, could explain low levels of intrapopulational variation detected in previous allozyme analyses for species in *Carex*.

The low heterozygosity detected in *T. planifolium* may also be attributable to restricted seed dispersal. While a mechanism of seed dispersal is not known for *T. planifolium*, it is well documented that plant seeds often travel only one or a few metres (Cain et al., 2000). Seed dispersal in *T. planifolium* occurs in late July and August, during which time the plants' leaves become matted. Crins (1989) suggested that this would facilitate deposition of the seeds immediately adjacent to parent plants (i.e., seeds are gravity dispersed), resulting in small spatial clumps of closely related tussocks. Like self-pollination, mating among closely related individuals limits recombination and leads to increased homogeneity of genotypes in the population (Loveless and Hamrick, 1984).

Finally, null, or non-amplified, alleles can bias estimates of allele and genotype frequencies, inflating observed homozygosity. In the present study, the probability of null alleles was high (mean = 0.36) for all loci, following the methods of Brookfield (1996). However, homozygote excess due to the presence of a null allele should be locus-specific (Fankham et al., 2013). Given that departures from HWE were not restricted to particular loci or populations in *T. planifolium*, it is unlikely that heterozygote deficiency in the dataset can be

attributed to the presence of null alleles. Instead, the ecological explanations above likely provide a better account for the low heterozygosity observed.

3.4.2 Patterns of genetic structure

The majority (59.1%) of total genetic variation was found between *T. planifolium* populations, with 32.6% contained within populations (Table 3.6). High population genetic structure (mean $F_{ST} = 0.80$) was also detected. This level of differentiation among populations indicates strong divergence and suggests little gene flow occurs among populations. These results offer further support for Crins' (1989) predictions that pollen and/or seed dispersal are limited in *T. planifolium*.

Genetic divergence was positively associated with geographic distance over a range of 1800 km (Figure 3.3), conforming to the classical isolation by distance pattern described by Wright (1943). However, Mantel's r was relatively low (0.29), suggesting that long-distance dispersal and dispersal between non-adjacent populations might take place. Analysis of population structure revealed five genetic groups among the 28 populations of *T. planifolium*, separated largely according to their geographic location. Nevertheless, some populations, namely ONRB, NYTS and NHER, did not appear to cluster according to geographic provenance, but clustered with Missouri populations located between 1100 and 1800 km away. Genetic similarity between these populations might reflect occasional long-distance dispersal events, as implied by the results of the Mantel test. Though pollen has been reported to travel up to 1000 km for wind-pollinated trees (Robledo-Arnuncio, 2011), it has been found to travel much shorter

distances (i.e., less than 5 m) for small, herbaceous species in the understory (e.g., *Carex plantaginea* and *C. platyphylla*, Handel, 1976). Instead, chance long-distance dispersal by seeds could explain gene flow patterns across larger distances. Long-distance dispersal by seeds can occur when seeds adhere to or are ingested by large or migratory animals and occasionally by wind or water (Nathan et al., 2008). In *Carex arctogena* Harry Sm., Villaverde et al. (2015) found evidence that long-distance seed dispersal, possibly mediated by birds, can explain the circumpolar distribution of the species.

Alternatively, the observed genetic relationships between populations could be explained from a historical biogeographic perspective, considering a scenario of northward colonization following the Pleistocene glaciations. Many plant species inhabiting the Great Lakes region of North America were colonized from glacial refugia in the United States, following the retreat of the Laurentide ice sheet (Delcourt and Delcourt, 1991), and an east-west division of lineages is often seen, resulting from migration from isolated refugia on either side of the southern Appalachians (Soltis et al., 2006). In *T. planifolium*, ONRB, NYTS and NHER may have originated from Ozarkian refugia while other northern populations were colonized from separate refugia in the southern Appalachians. Phylogeographic evidence supports the existence of glacial refugia in both these areas and similar post-glacial migration routes have previously been proposed for other plant species (e.g., Red maple (*Acer rubrum* L.) and American beech (*Fagus grandifolia* Ehrh.), McLachlan et al., 2005). We also noted that genetic diversity was highest in southern Appalachians and Missouri populations, which

is consistent with predictions of glacial refugia in these areas (Hewitt, 2004).

The genetic similarity between the populations in our study may also be due to inadequate sampling or poor resolving power of the microsatellite markers used. However, our analysis used comparable numbers of samples and microsatellite markers relative to other recent population genetic surveys in Cyperaceae (e.g., Clarke et al., 2013; M'baya et al., 2013) and other plants (e.g., Pandey and Rajora, 2012; Zhao et al., 2014). Nevertheless, sampling additional individuals, populations and/or using additional loci may prove valuable to clarify the genetic relationships among populations. Additional research with chloroplast DNA (cpDNA) sequences could also be useful, as these are frequently applied to determining phylogenetic relationships among plant populations (Schaal et al., 1998).

3.4.3 Environmental characteristics

Together, results of our genetic analysis and our assessment of range-wide environmental characteristics demonstrate that prerequisites necessary for adaptive diversification of *T. planifolium* populations occur. Hierarchical clustering of environmental characteristics revealed heterogeneity in soil and climate characteristics across the range of *T. planifolium*. Crins (1989) previously noted variation in micro- and macronutrient content of soil among *T. planifolium* populations in Canada. Variation in climate and soil conditions range-wide can lead to different selection pressures, promoting local adaptation (Lesica and Allendorf, 1995; Kawecki and Ebert, 2004). Moreover, we found that gene flow between *T. planifolium* populations is low, likely resulting from the limited

dispersal capacity of seeds and/or pollen. This is favourable for adaptive divergence of populations, as high gene flow can oppose the effects of local selection (Lenormand, 2002). Evidence for adaptations to local soil and climate conditions have been documented in a number of plant species, including sedges. For example, preliminary investigation of the sedge *Fimbristylis ovata* (Burm. f.) J. Kern suggests that particular populations are locally adapted to serpentine soils (Chathuranga et al., 2015), such ecotypes are the first stage toward serpentine endemism (Kruckeberg, 1951). Local adaptations can have important implications for conservation efforts and should be investigated further with classical approaches (i.e., reciprocal transplant or common garden experiments) or adaptive loci (Hufford and Mazer, 2003).

3.4.4 Conclusions and implications for conservation in Canada

Our analysis revealed low genetic diversity and high levels of inbreeding in the Canadian population of *T. planifolium*. However, all populations studied were found to exhibit comparable levels of diversity and inbreeding, even when population size was considered. In general, the frequency and intensity of inbreeding is expected to be greater in plants than in animal groups due to their sessile nature, limited dispersal capacity, the bisexuality of most species and their capacity to self-fertilize (Frankel et al., 1995). While inbreeding has been shown to negatively affect a number of fitness traits in plants (Angeloni et al., 2011), prior research has found that naturally self-pollinating species show less reduction in fitness with increased inbreeding than primarily outbreeding species (e.g., Holtsford and Ellstrand, 1990; Busch, 2005). Additionally, there is some

evidence to suggest inbred species may be capable of purging their genetic load to mitigate the fitness effects of inbreeding (Crnokrak and Barrett, 2002).

Despite high levels of inbreeding in the Canadian population of *T. planifolium*, seeds are reported to form successfully at high rates (Crins, 1989). Furthermore, in unpublished results from germination experiments, 26% of *T. planifolium* seeds germinated, with an even greater percentage germinating in treatments left outside over the winter (Iwanycki, pers. comm. 2015; Iwanycki and McGoey, unpublished report). Seedlings grew fast and flowered in the second year (Iwanycki, pers. comm. 2015). It therefore seems unlikely that the low recruitment observed at the Canadian site is attributable to inbreeding depression and, at this time, genetic rescue is not warranted. Alternatively, lack of microsite availability may be responsible for the low levels of seedling recruitment observed (Eriksson and Ehrlén, 1992). For example, Crins (1989) suggested that establishment of new *T. planifolium* colonies might be limited by the amount of light reaching the forest floor. Likewise, we observed that *T. planifolium* was generally restricted to open forests during our fieldwork. It has previously been reported that for woodland Cyperaceae there is little or no time for germination before canopy closure (Leck and Schütz, 2005). Therefore, research focused on the regeneration requirements of the species, for example the level of local disturbance, would be beneficial to clarify threats facing the Canadian population.

Considerable genetic divergence was found among the Canadian and American populations of *T. planifolium*, with the Canadian population possessing

one of only 11 private alleles detected in the 28 populations surveyed. Different environmental conditions, in terms of soils and climate, were also recorded at the Canadian site. Together, these results indicate that the Canadian population is of high conservation value (as per Lesica and Allendorf, 1995). Indeed, protection of many populations would be necessary to conserve species-level genetic diversity as it is chiefly partitioned among, rather than within, populations (see Koelling et al., 2011 or Baskauf et al., 2014). These results also suggest that risks of outbreeding depression are high with outcrossing, and future restorative efforts (i.e., augmentation) for the Canadian population should be avoided until risks to the population are evaluated in greater detail (Frankham et al., 2011). It should also be considered that mixing of populations with differing genetic backgrounds could decrease the conservation value of the Canadian population (Barrett and Kohn, 1991).

As an alternative, an *ex situ* seed bank may be developed for future restoration or reintroduction purposes. Seeds should be obtained from all ecological settings within the Hamilton site to capture maximum genetic variation in the collection (Knapp and Rice, 1994). However, to minimize demographic risks to the population, strict seed collection protocols should be followed (i.e., Guerrant et al., 2004). Should genetic diversity decline in the Canadian population, locally sourced seeds could be used to maintain variation without sacrificing local adaptiveness (Knapp and Rice, 1994). Development of a seed bank was previously proposed for *T. planifolium* in the *Recovery Strategy for Few-flowered Club-rush/Bashful Bulrush* (*Trichophorum planifolium*) in Canada

(Smith and Rothfels, 2007) and has proven useful for the restoration of other plant species (e.g., Eelgrass or *Zostera marina* L., Leschen et al., 2010).

Table 3.1 Location of sampled populations of *Trichophorum planifolium*.

Site code	Size category	Site, County, State/Province	Latitude, Longitude
CTTP	Medium	Tarrywile Park, Fairfield, Connecticut	41.37137, -73.45273
MABM	Large	Bare Mountain, Hampshire, Massachusetts	42.30173, -72.52946
MAMT	Large	Mount Toby, Franklin, Massachusetts	42.44707, -72.53892
MAST	Small	Tantiusques Reservation, Worcester, Massachusetts	42.05853, -72.13004
MDDM	Very small	Dan's Mountain State Park, Allegany, Maryland	39.56778, -78.9119
MOBS	Large	Big Springs State Park, Carter, Missouri	36.94751, -90.99314
MOBU	Small	Bunker, Reynolds, Missouri	37.53555, -91.21163
MOHC	Large	Hungry Creek, Douglas, Missouri	37.02262, -92.16771
MOOC	Small	Ottery Cemetery, Iron, Missouri	37.66425, -90.89984
MOSH	Small	Sutton Hollow, Shannon, Missouri	37.19962, -91.32581
NHER	Small	Elmer G. Raymond Park, Hillsborough, New Hampshire	42.76427, -71.36132
NJKP	Medium	Kay Environmental Preserve, Morris, New Jersey	40.76115, -74.71585
NYMP	Large	Mendon Ponds Park, Monroe, New York	43.03012, -77.57369
NYTS	Large	Taconic State Park, Columbia, New York	42.11755, -73.49718
OHBH	Small	Boch Hollow State Nature Preserve, Hocking, Ohio	39.62051, -82.40485
OHHH	Small	Hocking Hills State Park, Hocking, Ohio	39.43813, -82.53062
OHPR	Very small	Pond Run Road, Scioto, Ohio	38.6525, -83.16373
OHSC	Medium	Strait Creek Prairie Bluffs Preserve, Highland, Ohio	39.0583, -83.38857
ONRB	Small	Royal Botanical Gardens, Hamilton, Ontario	43.26561, -79.92105
PABE	Very small	Bald Eagle State Park, Centre, Pennsylvania	41.04383, -77.65401
PACR	Very small	Chestnut Ridge, Westmoreland, Pennsylvania	40.37034, -79.22753
PAGP	Medium	Gifford Pinchot State Park, York, Pennsylvania	40.07462, -76.90913
PASG	Very small	State Game Lands #242, York, Pennsylvania	40.09756, -76.9484
VAAC	Medium	Able Creek, Fluvanna, Virginia	37.80352, -78.24927
VAHB	Small	Huckleberry Trail, Montgomery, Virginia	37.18849, -80.41925
VASC	Very small	Sandy Creek, Halifax, Virginia	36.81982, -79.06654
WVFM	Large	Fisher Mountain, Pendleton, West Virginia	38.66423, -79.24038
WVMO	Large	near Moyers, Pendleton, West Virginia	38.51881, -79.37051
WVSS	Very small	Shannondale Springs, Jefferson, West Virginia	39.22215, -77.81996

Table 3.2 Basic population genetic parameters for 401 individuals in 28 populations of *Trichophorum planifolium*. Non-significant inbreeding values (F_{is}) are in bold.

Population	N	A_P	A_R	MLG	H_e	H_o	F_{is}
CTTP	16		1.00	1.00	0.00	0.00	-
MABM	14	1	1.11	3.86	0.05	0.01	0.89
MAMT	19		1.07	2.05	0.03	0.03	0.13
MAST	20		1.00	1.00	0.00	0.00	-
MDDM	19		1.28	4.42	0.15	0.02	0.85
MOBS	16	1	1.29	6.30	0.15	0.01	0.93
MOBU	11	1	1.47	5.82	0.24	0.01	0.97
MOHC	10	1	1.09	3.00	0.05	0.01	0.82
MOOC	15		1.04	2.00	0.02	0.02	-0.12
MOSH	18		1.13	4.76	0.06	0.04	0.35
NHER	15	1	1.28	3.66	0.15	0.03	0.80
NJKP	15	1	1.17	3.00	0.08	0.01	0.86
NYMP	19		1.02	1.53	0.01	0.00	1.00
NYTS	15		1.28	2.90	0.15	0.00	1.00
OHBH	13		1.00	1.00	0.00	0.00	-
OHHH	20		1.20	5.32	0.11	0.00	0.96
OHPR	15	1	1.02	1.67	0.01	0.00	1.00
OHSC	14		1.00	1.00	0.00	0.00	-
ONRB	18	1	1.02	1.56	0.01	0.00	1.00
PABE	7		1.09	3.00	0.04	0.00	1.00
PACR	5		1.06	2.00	0.03	0.00	1.00
PAGP	14		1.39	4.85	0.20	0.06	0.73
PASG	8		1.04	2.00	0.02	0.00	1.00
VAAC	13		1.09	2.77	0.05	0.00	1.00
VAHB	12		1.00	1.00	0.00	0.00	-
WVFM	18	2	1.31	6.24	0.16	0.01	0.94
WVMO	20	1	1.30	5.29	0.15	0.00	1.00
WVSS	2		1.09	2.00	0.05	0.00	1.00

Note: N = number of individuals sampled; A_P = number of private alleles; A_R = allelic richness; MLG = number of multi-locus genotypes; H_e = expected heterozygosity, H_o = observed heterozygosity; F_{is} = inbreeding coefficient.

Table 3.3 Estimates of pairwise F_{ST} among 28 populations of *Trichophorum planifolium*. Non-significant comparisons are in bold.

Population	CTTP	MABM	MAMT	MAST	MDDM	MOBS	MOBU	MOHC	MOOC	MOSH	NHER	NJKP	NYMP	NYTS	OHBH	OHPR	OHSC	ONRB	PABE	PACR	PAGP	PASG	VAAC	VAHB	WVFM	WVMO	WVSS	
CTTP		0.871	0.694	1.000	0.663	0.779	0.671	0.939	0.955	0.859	0.704	0.841	0.902	0.552	1.000	0.739	0.941	1.000	0.973	0.853	0.931	0.627	0.915	0.900	1.000	0.623	0.586	0.920
MABM			0.742	0.805	0.721	0.650	0.548	0.841	0.877	0.803	0.522	0.612	0.880	0.684	0.816	0.625	0.796	0.890	0.853	0.719	0.654	0.526	0.726	0.565	0.900	0.575	0.551	0.601
MAMT				0.896	0.636	0.745	0.629	0.873	0.901	0.834	0.686	0.746	0.806	0.527	0.810	0.694	0.778	0.932	0.911	0.742	0.719	0.603	0.698	0.776	0.913	0.584	0.620	0.595
MAST					0.807	0.725	0.600	0.934	0.951	0.842	0.727	0.845	0.981	0.736	1.000	0.734	0.965	1.000	0.976	0.922	0.930	0.505	0.950	0.796	1.000	0.691	0.657	0.922
MDDM						0.658	0.555	0.701	0.764	0.727	0.591	0.608	0.711	0.471	0.650	0.662	0.651	0.761	0.768	0.618	0.526	0.542	0.561	0.733	0.710	0.462	0.584	0.400
MOBS							0.169	0.454	0.399	0.385	0.368	0.602	0.804	0.444	0.703	0.400	0.705	0.377	0.589	0.640	0.540	0.435	0.619	0.587	0.643	0.504	0.560	0.361
MOBU								0.365	0.434	0.349	0.237	0.478	0.698	0.357	0.585	0.380	0.585	0.377	0.383	0.512	0.462	0.377	0.501	0.441	0.582	0.376	0.454	0.267
MOHC									0.816	0.672	0.570	0.764	0.926	0.530	0.907	0.514	0.884	0.759	0.860	0.844	0.839	0.557	0.852	0.811	0.879	0.576	0.664	0.718
MOOC										0.201	0.623	0.819	0.947	0.579	0.950	0.648	0.930	0.920	0.894	0.866	0.875	0.545	0.900	0.866	0.935	0.652	0.625	0.798
MOSH											0.551	0.755	0.875	0.486	0.844	0.577	0.836	0.751	0.749	0.736	0.720	0.462	0.766	0.772	0.799	0.600	0.558	0.558
NHER												0.441	0.750	0.476	0.616	0.495	0.616	0.525	0.327	0.540	0.471	0.482	0.517	0.504	0.728	0.373	0.493	0.333
NJKP													0.859	0.635	0.788	0.662	0.780	0.799	0.744	0.711	0.680	0.626	0.706	0.647	0.862	0.480	0.628	0.534
NYMP														0.651	0.961	0.784	0.929	0.983	0.960	0.796	0.896	0.686	0.895	0.900	0.979	0.690	0.666	0.842
NYTS															0.585	0.415	0.579	0.581	0.670	0.576	0.487	0.410	0.491	0.646	0.604	0.412	0.529	0.310
OHBH																0.541	1.000	0.970	0.844	0.791	0.486	0.081	0.860	1.000	0.330	0.538	0.918	
OHPR																	0.480	0.711	0.581	0.438	0.317	0.446	0.551	0.474	0.475	0.519	0.315	
OHSC																		0.969	0.946	0.787	0.646	0.489	0.055	0.837	0.955	0.336	0.546	0.799
ONRB																			0.944	0.940	0.957	0.609	0.959	0.865	1.000	0.630	0.717	0.937
PABE																				0.875	0.916	0.668	0.920	0.788	0.974	0.647	0.680	0.802
PACR																					0.753	0.481	0.723	0.773	0.929	0.464	0.416	0.745
PAGP																					0.319	0.611	0.764	0.944	0.278	0.350	0.818	0.401
PASG																						0.395	0.534	0.506	0.424	0.401	0.284	0.401
VAAC																								0.781	0.943	0.258	0.424	0.785
VAHB																									0.887	0.605	0.594	0.518
WVFM																										0.639	0.554	0.936
WVMO																											0.469	0.316
WVSS																												0.310

Table 3.4 Climate characteristics for each of five clusters of *Trichophorum planifolium* populations identified with hierarchical clustering. Bioclimatic variables are from Hijmans et al. (2005) available from www.worldclim.org.

Climate characteristic	Group 1 (mean $\pm$ SD)	Group 2 (mean $\pm$ SD)	Group 3 (mean $\pm$ SD)	Group 4 (mean $\pm$ SD)	Group 5 (mean $\pm$ SD)
Annual Mean Temperature ($^{\circ}$ C)	8.45 $\pm$ 0.07	8.2 $\pm$ 0.66	13.65 $\pm$ 0.21	13.04 $\pm$ 0.22	10.79 $\pm$ 0.77
Mean Diurnal Range ($^{\circ}$ C)	10.1 $\pm$ 0.71	11.9 $\pm$ 0.47	13.35 $\pm$ 0.07	14.02 $\pm$ 0.63	12.43 $\pm$ 0.69
Isothermality ($^{\circ}$ C)	0.03 $\pm$ 0	0.03 $\pm$ 0	0.04 $\pm$ 0	0.04 $\pm$ 0	0.03 $\pm$ 0
Temperature Seasonality ($^{\circ}$ C)	9.32 $\pm$ 0.05	8.94 $\pm$ 0.33	7.95 $\pm$ 0.08	8.72 $\pm$ 0.08	8.51 $\pm$ 0.46
Max Temperature of Warmest Month ($^{\circ}$ C)	27.55 $\pm$ 0.07	26.94 $\pm$ 0.94	31.1 $\pm$ 0.14	32.12 $\pm$ 0.43	28.99 $\pm$ 0.97
Min Temperature of Coldest Month ($^{\circ}$ C)	-8.9 $\pm$ 0.57	-10.41 $\pm$ 1.15	-4.05 $\pm$ 0.21	-7.04 $\pm$ 0.4	-7.12 $\pm$ 0.96
Temperature Annual Range ($^{\circ}$ C)	36.45 $\pm$ 0.49	37.36 $\pm$ 1.48	35.15 $\pm$ 0.07	39.1 $\pm$ 0.83	36.11 $\pm$ 0.98
Mean Temperature of Wettest Quarter ($^{\circ}$ C)	19.8 $\pm$ 0.42	12.1 $\pm$ 5.59	23.3 $\pm$ 0.42	13.08 $\pm$ 0.26	18.64 $\pm$ 2.64
Mean Temperature of Driest Quarter ($^{\circ}$ C)	-3 $\pm$ 0	-2.83 $\pm$ 0.98	3.95 $\pm$ 1.63	1.1 $\pm$ 0.23	-0.61 $\pm$ 0.96
Mean Temperature of Warmest Quarter ($^{\circ}$ C)	20.25 $\pm$ 0.21	19.39 $\pm$ 0.76	23.7 $\pm$ 0.14	23.78 $\pm$ 0.24	21.44 $\pm$ 1.05
Mean Temperature of Coldest Quarter ($^{\circ}$ C)	-3.65 $\pm$ 0.07	-3.8 $\pm$ 0.93	3.05 $\pm$ 0.35	1.1 $\pm$ 0.23	-0.61 $\pm$ 0.96
Annual Precipitation (ppm)	826 $\pm$ 12.73	1169.89 $\pm$ 68.89	1076 $\pm$ 26.87	1108.6 $\pm$ 37.83	1018 $\pm$ 35.88
Precipitation of Wettest Month (ppm)	83 $\pm$ 0	112 $\pm$ 7.19	107 $\pm$ 0	116 $\pm$ 2.92	105.91 $\pm$ 7.37
Precipitation of Driest Month (ppm)	49 $\pm$ 4.24	80.67 $\pm$ 5.52	77.5 $\pm$ 4.95	57 $\pm$ 6.89	65.27 $\pm$ 3.98
Precipitation Seasonality (ppm)	14.5 $\pm$ 3.54	9.33 $\pm$ 2.6	10.5 $\pm$ 3.54	18.8 $\pm$ 1.1	14.82 $\pm$ 3.46
Precipitation of Wettest Quarter (ppm)	234.5 $\pm$ 2.12	321.67 $\pm$ 19.15	299 $\pm$ 2.83	331 $\pm$ 8.37	298.55 $\pm$ 17.43
Precipitation of Driest Quarter (ppm)	164 $\pm$ 18.38	259.67 $\pm$ 20.41	239 $\pm$ 18.38	216 $\pm$ 26.91	208.36 $\pm$ 14.58
Precipitation of Warmest Quarter (ppm)	231.5 $\pm$ 6.36	308.67 $\pm$ 26.66	298.5 $\pm$ 3.54	281 $\pm$ 5.96	287.64 $\pm$ 14.6
Precipitation of Coldest Quarter (ppm)	170 $\pm$ 21.21	263.11 $\pm$ 21.38	241 $\pm$ 21.21	216 $\pm$ 26.91	208.36 $\pm$ 14.58

Table 3.5 Soil properties for each of four clusters of *Trichophorum planifolium* populations identified with hierarchical clustering.

Soil property	Group 1 (mean $\pm$ SD)	Group 2 (mean $\pm$ SD)	Group 3 (mean $\pm$ SD)	Group 4 (mean $\pm$ SD)
Ca (ppm)	341.0 $\pm$ 0.0	352.9 $\pm$ 275.0	1065.0 $\pm$ 200.8	1122.5 $\pm$ 321.7
CEC (meq/100g)	26.3 $\pm$ 0.0	16.0 $\pm$ 2.9	19.2 $\pm$ 1.8	12.5 $\pm$ 5.8
OM (%)	20.3 $\pm$ 0.0	6.6 $\pm$ 2.9	12.2 $\pm$ 4.2	5.1 $\pm$ 0.5
P (ppm)	15.8 $\pm$ 0.0	4.3 $\pm$ 2.0	4.0 $\pm$ 0.1	3.2 $\pm$ 3.4
Mg (ppm)	44.0 $\pm$ 0.0	64.8 $\pm$ 45.9	186.5 $\pm$ 7.8	94.1 $\pm$ 79.3
K (ppm)	160.0 $\pm$ 0.0	68.5 $\pm$ 15.5	106.5 $\pm$ 33.2	53.5 $\pm$ 19.1
Mn (ppm)	271.8 $\pm$ 0.0	30.0 $\pm$ 13.9	50.2 $\pm$ 5.2	14.9 $\pm$ 5.6
Zn (ppm)	2.8 $\pm$ 0.0	1.5 $\pm$ 0.7	3.7 $\pm$ 0.9	4.4 $\pm$ 0.2
pH	4.2 $\pm$ 0.0	5.4 $\pm$ 0.5	5.7 $\pm$ 0.2	5.7 $\pm$ 0.4

Table 3.6 Analysis of molecular variance (AMOVA) among 28 populations of *Trichophorum planifolium*. All results are significant ($p < 0.001$).

Source of variation	df	Sum of squares	Variance component	Percentage of variance
Among populations	27	168.24	0.21	59.1
Among individuals within populations	373	96.90	0.12	32.6
Within individuals	401	11.76	0.03	8.3

Figure 3.1 Location of sampled populations of *Trichophorum planifolium*.

Figure 3.2 Hierarchical clustering of *Trichophorum planifolium* populations based on Nei's genetic distance.

Figure 3.3 Relationship between genetic divergence, based on Nei's genetic distance, and geographic distances (km) among 401 individuals of *Trichophorum planifolium* (Mantel test correlation: $r^2 = 0.29$, $p = 0.001$).

Figure 3.4 Hierarchical clustering of 27 populations of *Trichophorum planifolium* based on soil properties at each site. Pink = Cluster 1; Dark blue = Cluster 2; Light green = Cluster 3; Light purple = Cluster 4.

Figure 3.5 Hierarchical clustering of 29 populations of *Trichophorum planifolium* based on local climate characteristics. Light purple = Cluster 1; Dark blue = Cluster 2; Dark green = Cluster 3; Pink = Cluster 4; Light green = Cluster 5.

4 Chapter: Conclusions and future directions

4.1 Conclusions

Our objective was to explore the level and spatial distribution of intraspecific genetic diversity in *T. planifolium*, a woodland sedge that reaches the northern limit of its range in Canada, but is common farther south in the United States. The focus of our study was to compare the level of diversity in the Canadian population relative to American populations and to explore the degree to which populations are differentiated. We successfully developed 11 novel polymorphic loci for *T. planifolium* with which to assess the population genetics of the species. In the application of these markers, combined with environmental data, we addressed the aforementioned research objectives and provided recommendations for conservation of *T. planifolium* in Canada.

Uniform low diversity and high inbreeding among the Canadian and American populations suggests that outcrossing in this species is limited, substantiating earlier predictions by Crins (1989) about the biology of the species. High levels of population differentiation offer further support for these hypotheses. Because self-pollinating species typically exhibit less fitness effects from inbreeding than regularly outbreeding species, this suggests that active genetic management of the population (i.e., genetic rescue) is not warranted at this time. This is further supported by observations that seeds are produced successfully at high rates (Crins, 1989) and germinate readily under experimental conditions (Iwanycki, pers. comm. 2015). Alternatively, limited microsite availability may be responsible for the low levels of seedling recruitment and

declines in population size observed at the Hamilton site (Eriksson and Ehrlén, 1992).

High levels of differentiation among populations in combination with evidence that populations occupy habitats with different soil and climate characteristics indicate that efforts to maximize protection of intraspecific genetic diversity for *T. planifolium* will require the protection of multiple populations (e.g., Koelling et al., 2011; Baskauf et al., 2014). In this regard, the Canadian population is of high conservation value. These results also have important implications for future management of the Canadian population in that they suggest the probability of outbreeding depression is high with outcrossing. Consequently, any future effort to bolster genetic diversity in the population should be avoided until risks are evaluated in greater detail (Frankham et al., 2011). It should also be considered that introduction of individuals with differing genetic backgrounds could reduce the conservation value of the Canadian population (Barrett and Kohn, 1991). As an alternative, an *ex situ* seed bank may be established to preserve existing local genetic diversity for use in future restoration and reintroduction initiatives. However, strict seed collection protocols should be followed to avoid posing a demographic risk to the population.

4.2 Future directions

Our analysis proved useful in addressing one of the recovery objectives set out by Smith and Rothfels (2007) in the *Recovery Strategy for Few-flowered Club-rush/Bashful Bulrush* (*Trichophorum planifolium*) *in Canada*. However, it highlights the need for additional research on the species, in particular with

regards to the species' biology and ecology. Heywood and Iriondo (2003) noted that such information is often lacking for species' of conservation concern, but is critical to the development and implementation of successful management strategies.

In particular, we found that the low recruitment observed at the Hamilton site is likely not attributable to high levels of inbreeding. Instead, ecological factors, such as microsite availability, may be limiting population growth. For example, establishment of new *T. planifolium* colonies might be constrained by the amount of light reaching the forest floor as this species appears to be restricted to open forests (Crins, 1989). Research to clarify biological factors influencing establishment of *T. planifolium* is necessary. It may be beneficial to simulate high disturbance events (e.g., fire) by removing all co-occurring vegetation to determine if *T. planifolium* germinates from the natural seed bank.

We also report preliminary evidence that the Canadian population experiences different climate and soil conditions than elsewhere in the species' range. Classical approaches (i.e., reciprocal transplant or common garden experiments) or the use of adaptive loci are necessary to examine the degree to which populations are locally adapted. These methods are useful to gain an understanding of the risks of outbreeding associated with interpopulation seed transfer. We therefore propose that future conservation initiatives for the species should include the development of an *ex situ* seed collection with locally sourced seeds. In the event that genetic diversity does decline in the Canadian

population, this would provide a source of local diversity, avoiding the risk of outbreeding depression.

Appendices

Appendix A - Deposition of voucher specimens

Table A.1 Voucher information for *Trichophorum planifolium* populations used in this study.

Site code	Latitude, Longitude	Date Collected	Collector(s)	Deposition
CTTP	41.37137, -73.45273	May 23, 2014	V. J. Nowell & T. W. Smith	DAO
MABM	42.30173, -72.52946	May 26, 2014	V. J. Nowell & T. W. Smith	DAO
MAMT	42.44707, -72.53892	May 26, 2014	V. J. Nowell & T. W. Smith	DAO
MAST	42.05853, -72.13004	May 25, 2014	V. J. Nowell & T. W. Smith	DAO
MDDM	39.56760, -78.91190	May 10, 2011	P. M. Peterson, J. M. Saarela & C. Frye	US
MOBS	36.94751, -90.99314	May 9, 2014	V. J. Nowell & T. W. Smith	DAO
MOBU	37.53555, -91.21163	May 8, 2014	V. J. Nowell & T. W. Smith	DAO
MOHC	37.02262, -92.16771	May 8, 2014	V. J. Nowell & T. W. Smith	DAO
MOOC	37.66425, -90.89984	May 9, 2014	V. J. Nowell & T. W. Smith	DAO
MOSH	37.19962, -91.32581	May 6, 2014	V. J. Nowell & T. W. Smith	DAO
NHER	42.76427, -71.36132	June 12, 2011	J. Sachs, C. Kane, P. Hines & D. Allen	NHA
NJKP	40.76115, -74.71585	May 22, 2014	V. J. Nowell & T. W. Smith	DAO
NYMP	43.03012, -77.57369	May 28, 2014	V. J. Nowell & T. W. Smith	DAO
NYTS	42.11755, -73.49718	May 27, 2014	V. J. Nowell & T. W. Smith	NYS
OHBH	39.62051, -82.40485	May 12, 2014	V. J. Nowell & T. W. Smith	OS
OHHH	39.43813, -82.53062	May 12, 2014	V. J. Nowell & T. W. Smith	DAO
OHPR	38.6525, -83.16373	May 11, 2014	V. J. Nowell & T. W. Smith	DAO
OHSC	39.0583, -83.38857	May 11, 2014	V. J. Nowell & T. W. Smith	Edge of Appalachia
ONRB	43.26561, -79.92105	June 7, 2011	P.G. O'Hara	HAM
PABE	41.04383, -77.65401	May 13, 2010	J. Kunsman	PNHP
PACR	40.37034, -79.22753	May 21, 2014	V. J. Nowell & T. W. Smith	DAO
PAGP	40.07462, -76.90913	May 17, 2014	V. J. Nowell & T. W. Smith	DAO
PASG	40.09756, -76.9484	May 7, 2013	J. Kunsman	PNHP
VAAC	37.80352, -78.24927	May 17, 2014	V. J. Nowell & T. W. Smith	DAO
VAHB	37.18849, -80.41925	May 14, 2014	V. J. Nowell & T. W. Smith	DAO
VASC	36.81982, -79.06654	May 14, 2014	V. J. Nowell & T. W. Smith	DAO
WVFM*	38.66423, -79.24038	-	-	-
WVMO*	38.51881, -79.37051	-	-	-
WVSS*	39.22215, -77.81996	-	-	-

Note: Vouchers for West Virginia populations are unavailable. However, these occurrences are documented by the West Virginia Natural Heritage Program. Deposition abbreviations follow Index Herbariorum, except Edge of Appalachia (Edge of Appalachia Preserve Office, 4274 Waggoner Riffle Road, West Union OH 45693) and PNHP (Pennsylvania Natural Heritage Program, Pennsylvania Department of Conservation and Natural Resources, Bureau of Forestry, P.O. Box 8552, Harrisburg PA 17105).

Appendix B – Climate and soil dataset

Table B.1 Raw climatic data for 29 populations of *Trichophorum planifolium*.

Population	bio1	bio2	bio3	bio4	bio5	bio6	bio7	bio8	bio9	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio18	bio19
GTPP	91	110	30	8793	272	-87	359	80	-14	202	-25	1248	116	87	8	333	285	308	284
MABM	81	122	31	9261	276	-112	388	130	-30	198	-43	1146	104	82	7	303	259	299	264
MAMT	81	127	32	9319	278	-117	395	130	-32	197	-45	1127	102	79	7	297	253	296	259
MAST	80	119	31	9056	270	-110	380	36	-30	194	-42	1226	114	89	6	326	287	302	295
MDDM	81	119	33	8352	256	-95	351	165	-32	184	-32	1069	107	70	13	311	221	295	221
MOBS	130	150	37	8828	326	-77	403	132	9	239	9	1173	120	69	17	345	262	286	262
MOBU	131	136	35	8621	318	-67	384	131	13	237	13	1082	114	53	20	324	194	281	194
MOHC	131	136	34	8735	321	-68	388	130	12	239	12	1082	118	52	19	328	202	271	202
MOOC	127	136	35	8680	316	-69	384	127	8	234	8	1096	113	55	19	326	206	285	206
MOSH	133	143	36	8739	325	-71	396	134	13	240	13	1110	115	56	19	332	216	282	216
NHER	84	120	31	9218	279	-107	386	39	-26	201	-39	1102	115	82	9	307	258	262	267
NJKP	91	121	32	8681	277	-90	367	179	-12	200	-25	1277	124	81	11	354	274	350	274
NYMP	84	106	28	9283	275	-93	368	201	-30	201	-37	817	83	46	17	236	151	236	155
NYTS	69	115	30	9089	256	-118	374	162	-41	182	-53	1171	108	78	10	321	252	325	256
OHBH	104	127	34	8821	289	-83	372	194	-16	213	-16	1027	113	61	20	319	200	305	200
OHHH	106	126	33	8778	290	-82	372	195	-14	214	-14	1035	115	62	20	319	201	303	201
OHPR	115	127	35	8489	295	-67	362	202	1	220	1	1087	114	69	16	320	233	310	233
OHSC	115	119	33	8676	294	-66	360	112	-2	222	-2	1062	115	67	17	316	217	295	217
ONRB	85	96	26	9358	276	-85	361	195	-30	204	-36	835	83	52	12	233	177	227	185
PABE	96	120	32	8950	288	-84	372	187	-24	208	-24	1004	110	61	16	308	200	297	200
PACR	80	118	32	8691	261	-101	362	168	-38	187	-38	1163	118	78	13	343	248	341	248
PAGP	112	114	31	8906	300	-63	363	207	-5	225	-5	1016	98	70	10	281	223	269	223
PASG	110	113	31	8914	296	-66	362	196	-8	223	-8	1028	100	71	10	285	225	273	225
VAAC	135	133	37	8008	310	-42	352	236	28	236	28	1057	107	74	13	301	226	301	226
VAHB	111	132	38	7650	283	-58	341	189	9	207	9	1007	100	69	12	284	211	275	211
VASC	138	134	38	7892	312	-39	351	230	51	238	33	1095	107	81	8	297	252	296	256
WVFM	99	132	36	7914	275	-77	352	180	-7	198	-7	961	100	61	15	282	185	277	185
WVMO	99	132	37	7921	274	-77	351	180	-7	199	-7	989	102	63	14	286	196	280	196
WVSS	120	125	34	8596	305	-60	365	208	6	229	6	982	98	64	13	284	201	280	201

Note: A description of each of the 19 bioclimatic layers is available from Hijmans et al. (2005) and online at www.worldclim.org.

Table B.2 Soil properties for 27 populations of *Trichophorum planifolium*. Ca = Calcium (ppm); CEC = cation exchange capacity (meq/100g); OM = organic matter (%); P = Phosphorous (ppm); Mg = Magnesium (ppm); K = Potassium (ppm); Mn = Manganese (ppm); Zn = Zinc (ppm).

Population	Ca	CEC	OM	P	Mg	K	Mn	Zn	pH
CTTP	151.0	15.2	7.5	3.6	29.0	58.0	18.7	1.6	5.1
MABM	1207.0	20.5	15.2	4.1	181.0	83.0	46.5	4.3	5.8
MAMT	418.0	16.9	8.5	7.5	58.0	63.0	46.8	1.5	5.5
MAST	255.0	15.9	13.1	5.1	58.0	100.0	39.4	1.4	5.7
MDDM	341.0	26.3	20.3	15.8	44.0	160.0	271.8	2.8	4.2
MOBS	571.0	13.2	4.5	2.1	100.0	81.0	32.3	0.6	5.8
MOBU	606.0	12.5	4.4	2.3	61.0	77.0	40.5	1.7	5.9
MOHC	398.0	11.7	3.4	5.4	58.0	62.0	18.4	0.8	5.4
MOOC	410.0	13.8	4.6	3.6	84.0	84.0	25.8	2.0	5.3
MOSH	980.0	13.4	5.9	3.0	162.0	103.0	36.6	1.2	6.4
NHER	211.0	14.1	9.2	2.6	27.0	70.0	15.9	0.8	5.7
NJKP	112.0	21.8	11.9	7.3	33.0	82.0	9.4	3.4	4.9
NYMP	824.0	12.7	5.1	2.4	148.0	61.0	14.1	0.9	6.4
NYTS	440.0	20.7	12.8	4.4	39.0	49.0	64.8	1.7	5.4
OHBH	52.0	19.9	3.9	5.4	27.0	51.0	21.5	1.0	4.7
OH HH	149.0	18.9	6.0	4.4	41.0	64.0	30.4	2.0	4.7
OHPR	67.0	16.5	4.7	5.0	35.0	65.0	27.8	1.2	4.7
OHSC	245.0	19.4	5.7	6.4	59.0	70.0	49.4	2.8	5.0
ONRB	895.0	8.4	4.7	5.6	150.2	67.0	11.0	4.5	5.4
PABE	53.0	17.7	5.7	8.2	16.0	64.0	41.7	0.9	4.9
PACR	1350.0	16.6	5.4	0.8	38.0	40.0	18.9	4.2	6.0
PAGP	923.0	17.9	9.2	3.9	192.0	130.0	53.9	3.1	5.5
PASG	568.0	18.4	4.9	2.0	187.0	50.0	19.6	1.4	5.5
VAAC	63.0	16.0	8.1	6.8	32.0	71.0	21.0	1.9	5.2
VAHB	259.0	15.9	5.8	3.9	73.0	82.0	39.4	1.5	5.2
WVFM	122.0	13.5	5.2	1.9	47.0	49.0	33.1	1.6	5.6
WVSS	809.0	13.8	4.4	1.7	51.0	50.0	14.1	1.4	5.5

Appendix C - Genetic dataset

Table C.1 Table of alleles for 11 microsatellite loci used in this study.

Locus	Alleles									
TP142	Name	1	3	5						
	Size (bp)	135	145	148						
TP152	Name	1	3	4						
	Size (bp)	145	154	156						
TP174	Name	1	2							
	Size (bp)	167	170							
TP325	Name	2	3	5	6	7	8	9	10	
	Size (bp)	151	153	159	162	165	168	171	174	
TP326	Name	3	4							
	Size (bp)	216	226							
TP330	Name	1	2							
	Size (bp)	356	358							
TP341	Name	2	3	4	7	8	9	10		
	Size (bp)	296	299	308	320	323	284	317		
TP406	Name	1	4	5	6					
	Size (bp)	198	207	213	216					
TP434	Name	9	10	11						
	Size (bp)	163	166	169						
TP45	Name	1	3							
	Size (bp)	368	382							
TP80B	Name	2	3	5	6	7	8			
	Size (bp)	208	211	217	220	223	226			

Table C.2 Genotypes for 401 individuals from 28 populations of *Trichophorum planifolium* used in the genetic analyses.

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
CTTP02	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP05	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP06	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP08	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP09	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP11	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP14	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP17	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP22	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP24	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP26	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP27	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP31	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP32	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP34	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
CTTP35	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
MABM04	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM06	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM09	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	3,3
MABM12	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM14	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM17	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM20	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM22	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM23	3,3	3,3	1,1	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM24	3,3	3,3	1,1	6,6	4,4	2,2	3,3	5,5	9,9	3,3	2,2
MABM26	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM28	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM30	3,3	3,1	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MABM32	3,3	3,3	1,1	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
MAMT02	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
MAMT06	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT11	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT12	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT13	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT14	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT15	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT16	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT18	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT19	3,1	3,3	1,1	6,6	4,3	2,2	4,4	6,5	9,9	3,1	6,5
MAMT20	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT25	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT26	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT27	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT28	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT30	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT33	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAMT34	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,2
MAMT35	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST01	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST02	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST04	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST06	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST07	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST08	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST10	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST11	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST12	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST13	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST14	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST15	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST16	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST17	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST18	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
MAST19	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST20	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST21	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST22	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MAST24	3,3	3,3	2,2	8,8	4,4	2,2	4,4	6,6	9,9	3,3	2,2
MDDM01	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM02	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM03	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM04	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM05	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM08	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM10	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM11	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM12	1,1	4,4	1,1	6,6	3,3	1,1	8,4	6,6	11,11	3,3	6,6
MDDM13	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM14	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,5
MDDM15	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MDDM16	1,1	4,4	1,1	6,6	3,3	1,1	8,8	6,6	11,11	3,3	6,6
MDDM17	1,1	3,3	1,1	6,6	4,4	2,1	4,4	6,6	9,9	3,3	6,5
MDDM18	1,1	4,4	1,1	6,6	3,3	2,2	4,4	6,6	11,9	3,3	6,6
MDDM20	1,1	4,4	1,1	6,6	3,3	1,1	8,8	6,6	11,11	3,3	6,6
MDDM21	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	11,11	3,3	6,6
MDDM23	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	9,9	3,3	5,5
MDDM25	1,1	4,4	1,1	6,6	3,3	1,1	4,4	6,6	11,11	3,3	6,6
MOBS01	3,3	3,3	2,2	3,3	4,4	1,1	4,4	5,5	9,9	1,1	6,6
MOBS02	3,3	3,3	2,2	3,3	4,4	1,1	4,4	5,5	9,9	3,3	3,3
MOBS04	3,3	3,3	2,2	3,3	4,4	1,1	4,4	5,5	9,9	1,1	6,6
MOBS07	3,3	3,3	2,2	3,3	4,4	1,1	4,4	5,5	9,9	1,1	6,6
MOBS08	3,3	3,3	2,2	7,7	4,4	1,1	4,4	6,6	9,9	1,1	3,3
MOBS09	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	6,6
MOBS10	3,3	3,3	2,2	7,7	4,4	1,1	4,4	6,6	9,9	1,1	3,3
MOBS11	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	6,6
MOBS13	3,3	3,3	2,2	3,3	4,4	1,1	4,4	5,5	9,9	1,1	3,3

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
MOBS14	3,3	3,3	2,2	7,7	4,4	1,1	4,4	6,6	9,9	1,1	3,3
MOBS15	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	3,3
MOBS17	3,3	3,3	2,2	6,3	4,4	1,1	10,10	5,5	9,9	1,1	6,6
MOBS18	3,3	3,3	2,2	6,3	4,4	1,1	4,4	5,5	9,9	1,1	3,3
MOBS19	3,3	3,3	2,2	3,3	4,4	1,1	4,4	5,5	9,9	1,1	3,3
MOBS21	3,3	3,3	2,2	3,3	4,4	1,1	4,4	5,5	9,9	1,1	6,6
MOBS24	3,3	3,3	2,2	3,3	4,4	1,1	4,4	5,5	9,9	1,1	3,3
MOBU03	3,3	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
MOBU07	3,3	3,3	2,2	6,6	4,4	1,1	9,9	6,6	9,9	1,1	8,8
MOBU11	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	3,3
MOBU12	3,3	3,3	2,2	9,9	4,4	1,1	9,9	6,6	9,9	1,1	8,8
MOBU14	3,3	3,3	2,2	6,6	4,4	1,1	9,9	6,6	9,9	1,1	8,8
MOBU20	3,3	3,3	2,2	9,9	4,4	1,1	9,9	6,6	9,9	1,1	8,8
MOBU32	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	8,3
MOBU33	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	3,3
MOBU36	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	3,3
MOBU39	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	3,3
MOBU48	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	3,3
MOHC01	3,3	3,3	2,2	6,6	4,4	1,1	4,4	1,1	9,9	1,1	6,6
MOHC03	3,3	3,3	2,2	6,6	4,4	1,1	4,4	1,1	9,9	1,1	6,6
MOHC05	3,3	3,3	2,2	6,6	4,4	1,1	4,4	1,1	9,9	1,1	6,6
MOHC11	3,3	3,3	2,2	6,6	4,4	1,1	9,9	1,1	9,9	1,1	6,6
MOHC22	3,3	3,3	2,2	6,6	4,4	1,1	9,9	1,1	9,9	1,1	6,6
MOHC27	3,3	3,3	2,2	6,6	4,4	1,1	9,9	1,1	9,9	1,1	6,6
MOHC28	3,3	3,3	2,2	6,6	4,4	1,1	9,9	1,1	9,9	1,1	6,6
MOHC30	3,3	3,3	2,2	6,6	4,4	1,1	9,9	5,1	9,9	1,1	6,6
MOHC40	3,3	3,3	2,2	6,6	4,4	1,1	9,9	1,1	9,9	1,1	6,6
MOHC48	3,3	3,3	2,2	6,6	4,4	1,1	9,9	1,1	9,9	1,1	6,6
MOOC01	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC03	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC04	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC07	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC10	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
MOOC12	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC16	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC21	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC24	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC26	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC28	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC29	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC32	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC34	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOOC40	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH01	3,3	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH03	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH04	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH05	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH07	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH09	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH11	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH13	3,3	3,3	2,2	6,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH14	3,3	3,3	2,2	8,6	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH16	3,3	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH17	3,3	3,3	2,2	3,3	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH18	3,3	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH21	3,3	3,3	2,2	8,6	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH22	3,3	3,3	2,2	8,8	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH24	3,3	3,3	2,2	8,8	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH36	3,3	3,3	2,2	8,6	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH45	3,3	3,3	2,2	8,8	4,4	1,1	4,4	6,6	9,9	1,1	5,5
MOSH51	3,3	3,3	2,2	8,8	4,4	1,1	4,4	6,6	9,9	1,1	5,5
NHER01	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	6,6
NHER02	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	6,6
NHER03	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	6,6
NHER04	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	6,6
NHER05	3,3	3,3	1,1	6,2	4,4	1,1	4,4	5,5	9,9	3,3	5,5

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
NHER06	3,3	3,3	2,2	7,6	4,4	1,1	4,4	5,5	9,9	3,3	6,6
NHER07	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	6,6
NHER10	3,3	3,3	1,1	6,2	4,4	1,1	4,4	5,5	9,9	3,3	5,5
NHER11	3,3	3,3	2,2	7,7	4,4	1,1	4,4	5,5	9,9	3,3	6,6
NHER14	3,3	3,3	1,1	6,2	4,4	1,1	4,4	5,5	9,9	3,3	5,5
NHER15	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
NHER16	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
NHER17	3,3	3,3	1,1	6,2	4,4	1,1	4,4	5,5	9,9	3,3	5,5
NHER18	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
NHER19	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
NJKP01	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP02	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP04	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP05	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP06	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP07	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP09	3,3	3,3	1,1	3,3	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP10	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP11	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP12	3,3	3,3	1,1	6,3	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP13	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP14	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP15	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NJKP25	3,3	4,4	2,2	5,5	4,4	2,2	4,4	6,4	9,9	3,3	2,2
NJKP31	3,3	3,3	1,1	6,6	4,4	1,1	4,4	5,5	11,11	3,3	2,2
NYMP01	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP02	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP03	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP08	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP09	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP11	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP12	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP13	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
NYMP14	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP15	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP18	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP19	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP24	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP25	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP26	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP27	1,1	3,3	1,1	7,7	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP28	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP32	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYMP33	1,1	3,3	1,1	6,6	4,4	2,2	8,8	6,6	9,9	3,3	5,5
NYTS01	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
NYTS04	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS08	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS09	1,1	3,3	1,1	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS10	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS12	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS16	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS17	1,1	3,3	1,1	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS20	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS22	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS25	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
NYTS27	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
NYTS28	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
NYTS29	1,1	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
NYTS34	1,1	3,3	2,2	6,6	4,4	1,1	4,4	6,6	9,9	1,1	6,6
OHBH01	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH03	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH06	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH08	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH12	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH13	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH18	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
OHBH22	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH23	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH27	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH29	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH32	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHBH33	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHHH01	3,3	3,3	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHHH02	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHHH03	3,3	3,3	1,1	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH04	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH07	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH08	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH09	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH10	3,3	3,3	1,1	3,3	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHHH11	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH12	3,3	3,3	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHHH13	3,3	3,3	1,1	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH16	3,3	3,3	2,2	3,3	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHHH17	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHHH18	3,3	3,3	2,2	3,3	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHHH19	3,3	3,3	2,1	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH21	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH22	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHHH23	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH26	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	1,1	6,6
OHHH30	3,3	3,3	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
OHPR04	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHPR05	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHPR07	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHPR09	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHPR10	5,5	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHPR11	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHPR12	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
OHRP14	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHRP15	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHRP17	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHRP19	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHRP20	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHRP21	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHRP22	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHRP30	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
OHSC01	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC05	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC06	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC09	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC11	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC15	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC16	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC18	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC19	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC22	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC25	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC27	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC29	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
OHSC35	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	1,1	6,6
ONRB01	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB02	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB04	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB05	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB06	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB07	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB08	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB09	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB11	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB12	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB13	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
ONRB15	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB18	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB22	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB24	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB25	3,3	3,3	2,2	6,6	4,4	1,1	2,2	5,5	9,9	3,3	5,5
ONRB27	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
ONRB28	3,3	3,3	2,2	6,6	4,4	1,1	4,4	5,5	9,9	3,3	5,5
PABE02	3,3	3,3	1,1	6,6	4,4	2,2	3,3	6,6	9,9	3,3	5,5
PABE04	3,3	3,3	1,1	6,6	4,4	2,2	3,3	6,6	9,9	3,3	5,5
PABE06	3,3	3,3	1,1	6,6	4,4	2,2	3,3	6,6	9,9	3,3	5,5
PABE07	3,3	3,3	1,1	6,6	4,4	2,2	3,3	6,6	9,9	3,3	5,5
PABE10	3,3	3,3	1,1	6,6	4,4	2,2	3,3	5,5	9,9	3,3	5,5
PABE19	3,3	3,3	1,1	6,6	4,4	2,2	3,3	6,6	9,9	3,3	5,5
PABE25	3,3	3,3	1,1	11,11	4,4	2,2	3,3	6,6	9,9	3,3	5,5
PACR01	3,3	3,3	1,1	11,11	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PACR02	3,3	3,3	1,1	11,11	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PACR03	3,3	3,3	1,1	11,11	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PACR05	3,3	3,3	1,1	11,11	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PACR07	3,3	3,3	1,1	8,8	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PAGP01	3,3	3,3	1,1	6,6	4,3	2,2	4,4	6,6	11,9	3,1	7,5
PAGP03	3,3	3,3	2,2	8,8	3,3	2,2	4,4	6,6	9,9	1,1	6,6
PAGP05	3,3	3,3	2,2	8,8	3,3	2,2	4,4	6,6	9,9	1,1	6,6
PAGP07	3,3	3,3	2,2	8,8	3,3	2,2	4,4	6,6	9,9	1,1	6,6
PAGP08	3,3	3,3	2,1	6,6	3,3	2,2	4,4	6,6	9,9	1,1	7,7
PAGP13	3,3	3,3	2,2	8,8	3,3	2,2	4,4	6,6	9,9	1,1	6,6
PAGP17	3,3	3,3	2,2	8,8	3,3	2,2	4,4	6,6	9,9	1,1	6,6
PAGP21	3,3	3,3	2,2	8,8	3,3	2,2	4,4	6,6	9,9	1,1	6,6
PAGP22	3,3	3,3	2,2	8,8	3,3	2,2	4,4	6,6	9,9	1,1	6,6
PAGP25	3,3	3,3	1,1	8,8	4,4	2,2	4,4	6,6	9,9	3,3	5,5
PAGP27	3,3	3,3	1,1	8,8	4,4	2,2	4,4	6,6	9,9	3,3	5,5
PAGP29	3,3	3,3	1,1	8,8	4,4	2,2	4,4	6,6	9,9	3,3	5,5
PAGP31	3,3	3,3	2,1	8,8	4,3	2,2	4,4	6,6	9,9	3,1	6,5
PAGP34	3,3	3,3	2,2	6,6	3,3	2,2	4,4	6,6	9,9	1,1	6,6

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
PASG01	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PASG02	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PASG04	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PASG05	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PASG06	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PASG07	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PASG08	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	6,6
PASG11	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	9,9	3,3	5,5
VAAC01	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC03	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC07	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC09	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC11	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC15	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC17	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC18	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC21	3,3	3,3	2,2	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC25	3,3	3,3	2,2	6,6	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC30	3,3	3,3	2,2	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC31	3,3	3,3	2,2	8,8	4,4	2,2	4,4	5,5	9,9	3,3	2,2
VAAC35	3,3	3,3	2,2	6,6	4,4	2,2	8,8	5,5	9,9	3,3	2,2
VAHB01	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB02	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB03	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB07	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB09	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB17	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB19	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB22	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB25	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB28	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB29	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6
VAHB31	3,3	4,4	2,2	6,6	4,4	2,2	4,4	6,6	9,9	1,1	6,6

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
WVFM01	3,3	4,4	1,1	9,6	4,4	1,1	4,4	6,6	9,9	3,3	6,6
WVFM04	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	10,10	3,3	6,6
WVFM05	3,3	3,3	2,2	9,9	4,4	1,1	4,4	6,6	9,9	3,3	6,6
WVFM09	3,3	3,3	1,1	6,6	4,4	1,1	4,4	6,6	9,9	3,3	6,6
WVFM11	3,3	3,3	1,1	6,6	4,4	1,1	4,4	6,6	9,9	3,3	6,6
WVFM13	3,3	3,3	1,1	9,9	4,4	1,1	4,4	5,5	9,9	3,3	6,6
WVFM19	3,3	3,3	1,1	7,7	4,4	1,1	7,7	6,6	10,10	3,3	6,6
WVFM20	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	11,10	3,3	6,6
WVFM21	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	10,10	3,3	6,6
WVFM22	3,3	3,3	1,1	6,6	4,4	1,1	4,4	6,6	10,10	3,3	6,6
WVFM26	3,3	3,3	1,1	6,6	4,4	1,1	4,4	6,6	10,10	3,3	6,6
WVFM27	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	10,10	3,3	6,6
WVFM28	3,3	3,3	1,1	6,6	4,4	1,1	4,4	6,6	10,10	3,3	6,6
WVFM29	3,3	3,3	1,1	6,6	4,4	2,2	4,4	6,6	10,10	3,3	6,6
WVFM31	3,3	3,3	1,1	6,6	4,4	1,1	4,4	6,6	9,9	3,3	6,6
WVFM32	3,3	3,3	1,1	6,6	4,4	1,1	4,4	6,6	10,10	3,3	6,6
WVFM34	3,3	3,3	1,1	6,6	4,4	1,1	4,4	6,6	10,10	3,3	6,6
WVFM35	3,3	3,3	1,1	3,3	4,4	1,1	4,4	6,6	10,10	3,3	6,6
WVMO02	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO04	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO05	3,3	3,3	2,2	9,9	4,4	2,2	4,4	5,5	9,9	1,1	5,5
WVMO06	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO08	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO09	3,3	4,4	1,1	10,10	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO10	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO12	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO13	3,3	4,4	1,1	10,10	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO14	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO18	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO21	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO22	3,3	4,4	1,1	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO24	3,3	4,4	1,1	6,6	4,4	2,2	4,4	5,5	9,9	3,3	5,5
WVMO29	3,3	4,4	2,2	9,9	4,4	2,2	4,4	6,6	9,9	1,1	5,5

	TP142	TP152	TP174	TP325	TP326	TP330	TP341	TP406	TP434	TP45	TP80B
WVMO30	3,3	4,4	1,1	9,9	4,4	2,2	4,4	5,5	9,9	1,1	5,5
WVMO32	3,3	4,4	2,2	9,9	4,4	2,2	4,4	6,6	9,9	1,1	5,5
WVMO34	3,3	4,4	2,2	9,9	4,4	2,2	4,4	6,6	9,9	3,3	5,5
WVMO35	3,3	3,3	1,1	9,9	4,4	2,2	4,4	6,6	9,9	1,1	6,6
WVMO37	3,3	3,3	1,1	9,9	4,4	2,2	4,4	6,6	9,9	1,1	6,6
WVSS03	1,1	3,3	2,2	6,6	4,4	2,2	4,4	4,4	9,9	3,3	7,7
WVSS04	3,3	3,3	2,2	6,6	4,4	2,2	4,4	4,4	9,9	3,3	7,7

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