Accuracy and reliability of microscopic characteristics to identify *Typha species* and their hybrids

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ABSTRACT

Accuracy and reliability of microscopic characteristics to identify *Typha species* and their hybrids

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Advanced generation/backcrossed (non-F1) hybrids can be challenging to identify when their traits are similar to those of parental taxa, F1 hybrids, or both. This is particularly evident in the North American hybrid zone involving *Typha latifolia*, *T. angustifolia*, F1 *T.* × *glauca* and non-F1 hybrids. Cattails are challenging to differentiate based on gross morphological characteristics. Microscopic characteristics in female inflorescences have not been previously studied to differentiate parental taxa from non-F1 hybrids. To investigate whether researchers can use microscopic floret and bracteole characteristics for taxonomic identification, I compared pistillate flower length, bracteole length and width, and bracteole colour among taxa. I found that floret and bracteole characteristics can be useful for identifying *T. latifolia* but cannot accurately differentiate *T. angustifolia* and F1 *T.* × *glauca* from non-F1 hybrids. Further, a flowering bias can lead to the underestimation of the frequency of *T. latifolia* when using floral characters to examine the relative abundance of cattail taxa.

Keywords: *Typha angustifolia*, *Typha latifolia, Typha* × *glauca*, invasive species, advanced-generation hybrids, backcrossed hybrids, morphology, bracteole, species identification

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Chapter 1: General Introduction

1.1 Species identification

Accurate species identification is fundamental to understanding biodiversity because it enables researchers to differentiate between species and assess their abundance and distribution (Khuroo et al., 2007; Costello et al., 2015). Correct species identification is crucial for biodiversity conservation as conservation organizations, national environmental policies, and international agreements depend on the assumption that species are accurately identified (Garnett & Christidis, 2017; Thomson et al., 2018). Cryptic species are organisms that are morphologically indistinguishable but are genetically distinct. Challenges in taxonomy include delimiting cryptic species, which, if left unidentified, may lead to inaccurate estimates of species richness (misidentifications and over- or under-estimation).

Distinguishing between plant species is a fundamental task in biology, and various methods exist to achieve this. Plant morphology refers to the study of the form and structure of plants, encompassing both the external features (gross morphology) and the internal structures (microscopic morphology). Gross morphology consists of distinct traits visible to the naked eye. These characteristics include but are not limited to aspects of plants such as stems, leaves, flower structure, fruit type and roots (Stützel & Trovuó, 2013; Kumar et al., 2019; Susetyarini et al., 2020). For example, gross morphological characteristics such as the structures of pistillate and staminate flowers can differentiate between *Amaranthus rudis* and *Amaranthus tuberculatus* (Pratt & Clark, 2004). However, it can be difficult to distinguish between closely related species that share similar characteristics. For example, *Potamogeton clystocarpus* is

morphologically indistinguishable from *Potamogeton pusillus* and *Potamogeton foliosus* based on some gross morphological traits (e.g., presence or absence of basal tubercles on fruits; Whittall et al., 2004). However, microscopic characteristics can sometimes be more useful for differentiating closely related species or subspecies. Microscopic morphology describes minute anatomical characteristics. Micromorphological traits of leaves, such as epidermal cell size, have been used to differentiate multiple taxonomic groups, including ferns (Shah et al., 2018; Shah et al., 2019) and bryophytes (Câmara & Kellogg, 2010; Khan et al., 2020). However, in some situations, morphological overlap occurs, and genetic data are more reliable for distinguishing taxa (Duellmand & Venegas, 2005).

Genetic markers have emerged as the most effective tools to differentiate species (Li et al., 2015). Various technologies, including SNPs and RAD sequencing (Ousmael et al., 2023; Silva et al., 2020) can be used to study molecular differentiation among plant species in addition to more traditional genetic markers that include AFLPs (Amplified Fragment Length Polymorphisms; Medrano et al., 2020), PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism; Park et al., 2014), nuclear microsatellites (Muñoz-Pajares et al., 2020), and DNA barcoding (Li et al., 2015). Morphological overlap can complicate species identification (Whittall, 2004), and in these circumstances, genetic information can be beneficial when distinguishing between species. For example, genetic differentiation enabled the identification of cryptic species of a rose, *Potentilla delphinensis*, which is morphologically indistinguishable from *Potentilla grandiflora* and *Potentilla thuringiaca* (Nicolè et al., 2007). Although genetic data can provide a more accurate method of identifying

species, it can be time-consuming and cost-prohibitive, requiring specialist equipment. Developing an accurate and reliable morphological analysis could provide inexpensive and rapid species identification.

Interspecific hybridization occurs when two species are genetically unique and interbreed, producing offspring called hybrids. Hybrids can complicate species identification due to trait overlap with parental taxa. For example, some species of willows (*Salix*; *Salicaceae*) can hybridize, and both parental and intermediate morphological characteristics were observed in hybrid plants (Hardig et al., 2000). Hybrids can sometimes be differentiated from parental taxa based on microscopic morphology (Feldberg et al., 2011; Abbott et al., 2013). For example, *Quercus* × *dysophyll*, a hybrid between *Quercus crassipes* and *Quercus crassifolia*, can be difficult to identify based on gross morphology alone. However, leaf micromorphological characters can easily differentiate the parental species from their hybrids (López-Caamal et al., 2017). In some cases, it can become challenging to differentiate hybrid and parental taxa if they share both gross and microscopic morphological traits. F1 hybrids (first-generation hybrids) often have intermediate morphology between their parent species. If F1 hybrids are fertile, they can breed with each other to produce advanced-generation hybrids. F1 hybrids can sometimes breed with one or both parental species to produce backcrossed hybrids. Advanced-generation and backcrossed hybrids (hereafter non-F1 hybrids) may exhibit broad variation in morphological characters, including characteristics resembling F1 hybrids, parental taxa, or a combination of both, making identification more challenging (Mercure &

Bruneau, 2008). Such variability in morphology can pose challenges for researchers tasked with species identification.

1.2 Identification of cryptic invasive hybrids

One reason we are interested in identifying hybrids is their potential to exhibit novel traits that can contribute to invasiveness (Ellstrand & Schierenbeck, 2000; Rieseberg et al., 2007), potentially resulting in threats to native communities and challenges for conservation and management (Vilà et al., 2000; Blair et al., 2010). The success of invasive hybrids can often be attributed to heterosis, also known as hybrid vigour, which is when hybrids demonstrate performance gains surpassing those of parental species. Heterotic hybrids, particularly F1 hybrids, can exhibit accelerated growth rates, increased biomass, and rapid adaptation to novel habitats (Pyšek et al., 2009; Arnold & Martin, 2010; Hovick et al., 2012; Parepa et al., 2013; Hovick & Whitney, 2014). While heterotic hybrids can be advantageous in crop breeding (Li et al., 2018), they can also pose concerns as they may outcompete and displace native and parental species, thereby promoting the invasiveness of hybrids (Ellstrand & Schierenbeck, 2000; Barker et al., 2019). While first-generation hybrids may exhibit heterosis, the benefits often diminish in subsequent generations (Johansen-Morris & Latta, 2006). Introgression, the process by which genetic material from one species is incorporated into the gene pool of another through repeated backcrossing, poses a threat to the genetic integrity of local parental populations in hybrid zones (Abbott et al., 2013; Barker et al., 2019). Therefore, accurate identification of invasive hybrids is important due to their threat to native and parental species, which can have significant ecological implications.

The emergence of invasive hybrids can significantly threaten ecosystem balance and ecological integrity in habitats worldwide, raising concerns about their long-term effects and the preservation of native biodiversity (Lishawa et al., 2019; Sierszen et al., 2012). Wetlands are one ecosystem specifically vulnerable to invasions, with some of the most invasive taxa being plant wetland invaders (Zedler & Kercher, 2004). Wetlands are areas of land saturated or flooded with water, either permanently or seasonally (Tiner, 2017). Wetland ecosystems play vital ecological roles and offer numerous benefits (Sierszen et al., 2012). They serve as habitats for a diverse array of plant and animal species, including migratory birds (Richter & Azous, 2000), amphibians (Petranka et al., 2003), fish (Beesley et al., 2014), and insects (Batzer & Wissinger, 1996). Wetlands are crucial ecosystems that provide various services, such as water filtration (Coveney et al., 2002) and nutrient cycling (Fennessy et al., 2008). However, climate change can lead to increased water temperatures and changes in rainfall patterns and water flow, negatively impacting wetlands. Invasive hybrids can have a competitive advantage over native species in these altered conditions, leading to a decline in the overall health of the wetlands (Flanagan et al., 2015). One example of an invasive hybrid with a competitive advantage over native and parental species is *Spartina anglica*, an allopolyploid hybrid capable of invading salt marshes and becoming a dominant species (Thompson, 1991).

1.3 *Typha* system

Some of the most impactful wetland invaders worldwide are members of the *Typha* genus (cattails), a group of perennial freshwater emergent aquatic plants. *Typha* plants are monoecious, meaning they have both male and female florets on the same

plant. The male florets are located in the upper portion of the inflorescence, while the female florets are located in the lower portion. *Typha* plants can reproduce sexually and are self-fertile, with the male florets releasing pollen and fertilizing the female florets (Grace & Harrison, 1986). Cattails also propagate asexually through clonal reproduction via rhizomes, horizontal underground stems that spread out from the parent plant. New shoots and roots emerge from these rhizomes, allowing the plant to form dense, interconnected stands (McNaughton, 1966).

Typha plants are ecosystem engineers, which are essential to many wetland habitats. They reduce nutrient loads, pollution, and harmful elements (metal and excess nutrients). They are also used for bioremediation and offsetting global carbon dioxide emissions (Bansal et al., 2019). However, in some regions, *Typha* can be a problematic invader, such as eastern North America, where *Typha spp.* form an extensive hybrid zone. *Typha* × *glauca* is an invasive hybrid of native *Typha latifolia* and *Typha angustifolia*, likely introduced from Europe centuries ago (Ciotir et al., 2013; Ciotir & Freeland, 2016). The formation of F1 *T.* × *glauca* occurs asymmetrically following the pollination of *T. angustifolia* by *T. latifolia* (Pieper et al., 2017). *Typha* × *glauca* can interbreed to produce advanced-generation hybrids and backcross to both parent species (Pieper et al., 2017). Overall, *T.* × *glauca* are more common than *T. latifolia,* with *T. angustifolia* being relatively uncommon in the Great Lakes region (Pieper et al., 2020; Kirk et al., 2011; Freeland et al., 2013). F1 *Typha* × *glauca* are heterotic, highly invasive, and dominate wetlands through excessive litter formation (Larkin et al., 2012), high rates of clonal growth (Bunbury-Blanchette et al., 2015), and greater height (Zapfe & Freeland, 2015). There is evidence for hybrid breakdown in non-F1 hybrids (Bhargav

et al., 2022), but it remains abundant in some regions (Kirk et al., 2011; Tangen et al., 2022). Hybrid *T.* × *glauca* negatively impacts wetlands and agricultural systems (Vaccaro et al., 2009; Larkin et al., 2012). Invasive hybrid *Typha* plants can significantly reduce the diversity of native plant and animal communities by outcompeting native species (Angeloni et al., 2006; Frieswyk & Zedler, 2007; Wilcox et al., 2008; Farrer & Goldberg, 2009; Tuchman et al., 2009) altering plant-community structure (Lishawa et al., 2010), and modifying the physical structure of vegetation (Lishawa et al., 2017). Hybrid *T.* × *glauca* is problematic from a management perspective in many places, such as the Great Lakes, Lake Winnipeg watershed, Everglades, and Prairie Pothole Region (Luscz et al., 2015), whereas *T. latifolia* and *T. angustifolia* are present in these regions but are generally not a management concern (Bansal et al., 2019).

Genetic tests have been developed to identify *Typha spp.* and their hybrids, which are the most reliable way to identify cattail species. Genetic markers used to differentiate *Typha* spp*.* and their hybrids include PCR-RFLPs, microsatellites, and SNPs (Snow et al., 2010; Kirk et al., 2011; Chambers et al., 2023; Aleman et al., 2024). While genetic markers can differentiate F1 hybrids from non-F1 hybrids, we cannot differentiate backcrossed hybrids from advanced-generation hybrids (Boecklen & Howard, 1997)**.** Genotyping can be more accurate at identifying species than morphology, mainly when cryptic hybrids or non-flowering plants are present. However, genetic tests may be cost-prohibitive (Singh et al., 2008).

1.4 Morphology of *Typha*

Morphology, as opposed to genetics, can provide practical advantages such as cost-effectiveness, accessibility, and applicability for field-based studies, particularly in

circumstances where genetic analysis may be impractical. However, hybridization can complicate species identification when using gross morphology. In general, *Typha latifolia* and *T. angustifolia* can be differentiated based on leaf width, the gap between staminate and pistillate spikes, and the width of their spikes (Bansal et al., 2019; Finkelstein, 2003; Grace & Harrison, 1986; Kirk et al., 2011; Krattinger, 1975; Smith, 1967; Snow et al., 2010). *Typha latifolia* has broad leaves, no spike gap, and relatively wide spikes (Smtih, 2000; Geddes et al., 2021; Tangen et al., 2022). *Typha angustifolia* has narrow leaves, large gap, and narrow spikes (Smith, 2000; Geddes et al., 2021; Tangen et al., 2022). F1 *T.* × *glauca* are generally intermediate in these characteristics (Grace & Harrison, 1986; Kirk et al., 2011; Smith, 2020; Snow et al., 2010; Tangen et al., 2022). However, non-F1 hybrids (advanced-generation and backcrossed hybrids) have overlapping measurements for these characters with parental species (Geddes et al., 2021; Tangen et al., 2022) and thus complicate identification based on gross morphological characters.

Microscopic characteristics can assist in identifying *Typha latifolia* and *T. angustifolia* due to the differences in leaf, pollen, and floral spike characters between *Typha spp.* and hybrids (Smith, 1967; Smith, 2000). Microscopic evaluation of leaf cross sections examining leaf-apex angle (Kim et al., 2003; Wasko et al., 2021), leaf-lamina margin, and vascular bundles (McManus et al., 2002; Wasko et al., 2021) have been used to distinguish between *T. latifolia* and *T. angustifolia* and their hybrids. Hybrid plants could be either F1s or non-F1 hybrids and would require additional validation to be useful for studying hybrid zones. Pollen can distinguish *T. angustifolia* and *T. latifolia*: Tetrad pollen is the only type of pollen that *T. latifolia* produces, and *T. angustifolia* only

produces monad pollen (Smith, 2000; Finkelstein, 2003; Selbo & Snow, 2004). However, *T.* × *glauca* produces a mixture of pollen arrangements, including dyads and triads (Smith, 2000; Finkelstein, 2003; Selbo & Snow, 2004). While pollen morphology can effectively differentiate between *T. latifolia* and *T. angustifolia*, it cannot distinguish between F1 *T.* × *glauca* and non-F1 hybrids.

Other microscopic characteristics that have been used to differentiate *Typha spp*. and their hybrids are the bracteoles associated with pistillate florets found within the female inflorescence. Bracteoles refer to small, modified leaves or leaf-like structures along a plant's flowering stalk or inflorescence. In Smith's (2000) key, characters such as the presence or absence of bracteoles and their characteristics are utilized to distinguish between *T. latifolia*, *T. angustifolia* and F1 *T.* × *glauca*. Smith (2000) found that bracteoles are generally accurate for differentiating *T. latifolia* (lacks bracteoles), *T. angustifolia* (dark bracteoles that are larger than the adjacent stigmas), and F1 *T.* × *glauca* (lighter bracteoles that are smaller than the surrounding stigmas; Smith, 2000). However, it is unknown whether florets and bracteoles can reliably differentiate non-F1 hybrids from parental species and whether they can more reliably enable species identification than gross morphological characters.

1.5 Objectives

In this study, I hypothesized that using Smith's (2000) bracteole-based key for distinguishing cattail taxa, bracteoles can facilitate accurate and reliable identification of *Typha latifolia*, *T. angustifolia*, and F1 *T.* × *glauca*. The methodology for testing this hypothesis involves a comparative analysis of samples from known parental species

and hybrids (F1s and non-F1s) based on genetic data. I further hypothesize that additional characteristics might improve the reliability of female floret-based characters for species identification. I predict that measuring the pistillate flower length, the length and width of bracteoles of *Typha* spp. and their hybrids, and the colour of bracteoles in *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids will reveal distinct differences among them. Further, these measurements will show that *T. angustifolia* has wider bracteole widths than F1 *T.* × *glauca*, with non-F1 hybrids displaying intermediate widths. Lastly, *T. angustifolia* will have dark bracteoles, F1 *T.* × *glauca* will have lighter bracteoles, and non-F1 hybrids will exhibit intermediate or variable colouration. To validate these predictions, I will analyze samples collected at different times within the growing season and from different geographical regions in central and eastern Canada. The rationale behind these measurements stems from the observed differences in spike widths suggesting potential taxonomic differences in pistillate flower and bracteole length among *Typha spp.* Further, *T. angustifolia* has wider bracteole widths than F1 *T.* × *glauca* (Smith, 2000), and non-F1 hybrids could potentially display intermediate bracteole widths. Lastly, *T. angustifolia* has dark bracteoles, and F1 *T.* × *glauca* has lighter bracteoles (Smith, 2000). If we can quantify these colour differences, non-F1 hybrids may exhibit different colouration. In this study, I expanded the comparison of Smith's (2000) key to include non-F1 hybrids. To determine the accuracy and reliability of bracteole- and floret-based taxonomic identification, I used samples that had been genotyped using species-specific PCR-RFLP and microsatellite markers. Using this approach, I hoped to validate Smith's (2000) key, thereby supporting a readily available method for biologists conducting ecological monitoring and conservation of *Typha spp.*

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Chapter 2: Accuracy and reliability of microscopic characteristics to identify *Typha species* and their hybrids.

2.1 Introduction

Plant morphology and taxonomy involve examining plant structures, encompassing the external features (gross morphology) and the internal structures (microscopic morphology). Gross characteristics have traditionally been fundamental in discriminating plant species (Linnaeus, 1758; Stace, 1989; Pratt & Clark, 2004; Esfandani-Bozchaloyi & Zaman, 2018). These characteristics include but are not limited to aspects of plants such as stems, leaves, inflorescence, flower structure, and fruit type (Stützel & Trovuó, 2013; Kumar et al., 2019; Susetyarini et al., 2020). In some cases, microscopic characteristics are more accurate than gross morphological characteristics for distinguishing taxa (Rashid et al., 2017; Amina et al., 2020). For example, micromorphological traits of leaves, such as epidermal cell size, have been used in taxonomic studies of multiple taxonomic groups, including ferns (Shah et al., 2018; Shah et al., 2019) and bryophytes (Câmara & Kellogg, 2010; Khan et al., 2020). Gross morphology is essential for preliminary observations, but microscopic characteristics often achieve more accurate identification and taxonomic discrimination than gross morphological features. The presence of hybrids introduces complexity to taxonomic identification, often exhibiting intermediate or overlapping characteristics that may cause inaccurate estimates of species richness (over- or underestimating or misidentifications).

Typha spp. are perennial freshwater aquatic plants that grow in wetlands (Smith, 1967). The increasing dominance of *Typha spp.* in North America can be attributed to an extensive hybrid zone formed by the hybridization between native *T. latifolia* and *T. angustifolia,* which was likely introduced from Europe (Ciotir et al., 2013; Ciotir & Freeland, 2016). *Typha angustifolia* and *T. latifolia* interbreed to produce the F1 hybrid *T.* × *glauca*. Interspecific hybridization in *Typha* is asymmetric, with *T. latifolia* as the paternal parent and *T. angustifolia* as the maternal parent (Pieper et al., 2017). F1 *T.* × *glauca* are partially fertile, can breed with each other to produce advanced-generation hybrids, and can sometimes breed with one or both parental species to produce backcrossed hybrids (hereafter non-F1 hybrids). Hybrid *Typha* swarms comprise parental species, F1 and non-F1 hybrids (Freeland et al., 2013; Kirk et al., 2011; Pieper et al., 2020; Travis et al., 2011). F1 *T.* × *glauca* is highly invasive and dominates wetlands through excessive litter formation (Larkin et al., 2012), high rates of clonal growth (Bunbury-Blanchette et al., 2015), and greater height (Zapfe & Freeland, 2015). However, these advantages of hybrid cattails diminish in subsequent hybrid generations (Bunbury-Blanchette et al., 2015; Bhargav et al., 2022). Non-F1 hybrids are common in some regions. For example, they comprised 57% of surveyed cattails in the Prairie Pothole Region (Tangen et al., 2022) and approximately 22% of surveyed cattails in eastern North America (Kirk et al., 2011). *Typha* × *glauca* is highly invasive in wetlands, and reliably identifying the occurrence of hybrid cattails is needed.

Hybrid cattails can be challenging to identify due to overlapping morphological characteristics between hybrids – particularly non-F1 hybrids and parental taxa. A diagnostic key of gross and microscopic morphological characteristics by Smith (2000)

was proposed to distinguish *Typha latifolia*, *T. angustifolia*, and F1 *T.* × *glauca*. Generally, *T. angustifolia* and *T. latifolia* can be differentiated based on leaf width, the gap between staminate and pistillate spikes, and the width of their spikes (Smith, 1967; Krattinger, 1975; Grace & Harrison, 1986; Smith, 2000; Finkelstein, 2003; Snow et al., 2010; Kirk et al., 2011; Bansal et al., 2019). *Typha latifolia* has broad leaves, no gap, and wide spikes. *Typha angustifolia* has narrow leaves, a large gap, and narrow spikes (Smith, 2000; Tangen et al., 2022). F1 hybrids are generally intermediate in these characteristics (Grace & Harrison, 1986; Snow et al., 2010; Kirk et al., 2011; Smith, 2020; Tangen et al., 2022), but these characters overlap between F1 hybrids, non-F1 hybrids, and parental species (Geddes et al., 2021; Tangen et al., 2022).

Microscopic characteristics, including pollen and floret traits, can differentiate *Typha latifolia*, *T. angustifolia*, and F1 *T.* × *glauca* (Smith, 2000). Pollen traits can distinguish taxa based on whether pollen occurs in tetrads, monads, or a mix (Smith, 2000; Finkelstein, 2003; Selbo & Snow, 2004). Additionally, floral structures within the pistillate spikes have been reported as taxonomically informative: *Typha latifolia* lacks bracteoles, *T. angustifolia* has dark bracteoles that are larger than the adjacent stigmas, and F1 *T.* × *glauca* has lighter bracteoles that are smaller than the adjacent stigmas (Smith, 2000). However, Smith's (2000) key does not include non-F1 hybrids. Gross morphological characteristics such as leaf width and spike gap are insufficient for distinguishing non-F1 hybrids from their parental taxa (Geddes et al., 2021; Tangen et al., 2022), and this raises the question of whether these floret traits can be used to differentiate these non-F1 hybrids from *T. latifolia*, *T. angustifolia*, and F1 *T.* × *glauca.*

Microscopic characters present a potentially cost-effective, accurate, and reliable solution for taxon identification in the *Typha* hybrids zone of Canada. However, they must be verified across hybrid classes, particularly non-F1 hybrids. *Typha species* and hybrids can be differentiated based on molecular markers, including PCR-restriction fragment length polymorphisms (RFLP), microsatellites, and SNPs (Snow et al., 2010; Kirk et al., 2011; Chambers et al., 2023; Aleman et al., 2024). While genetic markers can differentiate F1 hybrids from non-F1 hybrids, they cannot differentiate backcrossed hybrids from advanced-generation hybrids (Boecklen & Howard, 1997). Genotyping can provide a more accurate identification of the species than morphology, mainly when cryptic hybrids or non-flowering plants are present. However, genetic tests may be cost-prohibitive and time-consuming (Selbo & Snow, 2004; Snow et al., 2010; Krik et al., 2011).

In this study, I hypothesized that using Smith's (2000) bracteole-based key for distinguishing cattail taxa, bracteoles can facilitate accurate and reliable identification of *Typha latifolia*, *T. angustifolia*, and F1 *T.* × *glauca*. The methodology for testing this hypothesis involves a comparative analysis of samples from known parental species and hybrids (F1s and non-F1s) based on genetic data. I further hypothesize that additional characteristics might improve the reliability of female floret-based characters for species identification. I predict that measuring the pistillate flower length, the length and width of bracteoles of *Typh*a spp. and their hybrids, and the colour of bracteoles in *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids will reveal distinct differences among them. Further, these measurements will show that T. angustifolia has wider bracteole widths than F1 *T.* × *glauca*, with non-F1 hybrids displaying intermediate widths. Lastly,

T. angustifolia will have dark bracteoles, F1 *T.* × *glauca* will have lighter bracteoles, and non-F1 hybrids will exhibit intermediate or variable colouration. To validate these predictions, I will analyze samples collected at different times within the growing season and from different geographical regions in central and eastern Canada. This study thereby investigates whether a readily available method based on flower morphology can allow for the rapid differentiation of taxa, which, in turn, will facilitate projects concerned with monitoring and conserving *Typha spp.* and wetlands. This method only applies to flowering cattails, as it relies on the observation of floral traits. Studies aimed to understand the relative frequency of taxa at a site must know whether all taxa are equally likely to flower. To address this objective, I compare the proportions of flowering taxa to randomly collected cattails in southern Ontario.

2.2 Methods

2.2.1 Sample Collection

A total of 292 female inflorescences were collected by me and others as part of other projects from Ontario and the Prairie Pothole Region in Canada (Fig. 2-1). From June 21 to July 12, 2022, I collected an average of 2.4 cattail material from seven sites in Peterborough Ontario, totalling 17 samples. Locations were recorded to the nearest 2 m using the Bad Elf Flex (West Hartford, Connecticut). In all cases, a segment of a leaf (~10 cm) from each plant was removed and placed into a labelled coin envelope for taxonomic verification via genotyping. Leaf samples were desiccated and stored in re-sealable bags containing Sorbead orange silica beads. On September 14-15, I returned to the same field sites to collect the fruits and placed them in labelled paper bags to dry. In all cases, the achenes were detached from the inflorescence stalk, placed into

resealable plastic bags, and refrigerated at 4 °C. Additional samples from other geographic regions were contributed by others. Between August 19 and August 24, 2022, Joyee (2024) collected an average of 3.3 cattail samples were collected from 18 sites across the western Prairie Pothole Region, totalling 66 samples. From June 24 to August 20, 2019, Bhargav et al. (2022) collected an average of 3 cattail samples from 5 sites in Peterborough, Ontario, totalling 25 samples. Lastly, between June 15 and August 4, 2021, and during the summer of 2022, Melvin (2024) collected an average of 16.5 cattail samples from 11 sites in Southern central Ontario, totalling 184 samples.

2.2.2 DNA extraction and genotyping

From each sample, approximately 50 mg of desiccated leaf tissue was ground into a semi-fine powder using a Retsch® MM300 mixer mill (Retsch; Haan, Germany). DNA was then extracted from each sample using EZNA extraction kits (Omega Biotek; Norcross, United States) following the manufacturer's protocol for extracting from dried leaf samples. The extracted DNA was then stored in a freezer at −18 °C.

For samples collected from Peterborough, Ontario (2019), DNA was amplified at four microsatellite loci to differentiate *Typha latifolia*, *T. angustifolia*, F1 *T.* × *glauca* or non-F1 hybrid (TA3, TA5, TA8, and TA20; Tsyusko-Omeltchenko et al., 2003; Bhargav et al., 2022). For samples collected from Peterborough, Ontario (2022), western Prairie Pothole Region, and Southern Central Ontario, up to four Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and one microsatellite marker were used to differentiate *T. latifolia*, *T. angustifolia*, F1 *T.* × *glauca* or non-F1 hybrid (advanced-generation or backcross hybrids; Tsyusko-Omeltchenko et al., 2003; Kirk et al., 2011; Chambers et al., 2023; Table 2-1). Five species-specific genetic marker loci

should provide sufficient power to discriminate between non-F1 hybrids, F1 hybrids and parental species (Boecklen & Howard, 1997). PCR-RFLP is a technique having a species-specific single nucleotide polymorphism (SNP) within the PCR amplicons. This SNP determines whether the restriction enzyme will cut the DNA fragment at a particular site, resulting in different fragment lengths for different species. Microsatellites consist of short, repetitive DNA motifs and have high levels of polymorphism, which allows for differentiating between closely related taxa.

DNA was amplified using a Mastercycler thermocycler (Eppendorf; Hamburg, Germany). Cycling conditions comprised an initial denaturation of 94 °C for two minutes; 35 cycles of DNA amplification with 45 sec at 94 °C, 45 sec annealing (Table 2-1), and 60 sec at 68 °C; and a final extension of 68 °C for two minutes. PCR amplification of the four PCR-RFLP loci (PhyC, AseI, BtsCI, BsrI) and a microsatellite locus (TA3) were visualized using gel electrophoresis (1% agarose gel for PCR-RFLP, 2% agarose gel for microsatellite and 1× TBE buffer), with a 100 bp DNA Ladder for reference and controls. Amplifications of PCR-RFLP were incubated with the appropriate RE. One × rCutSmart buffer (New England Biolabs; Ipswich, United States), three U (AciI), four U (BtsCI, AseI), or eight U (BsrI) of the PCR product with 5U (AciI, BsrI, AseI) or 8U (BtsCI) of the enzyme, and ddH2O to a final volume of ten µL were used for each RE incubation. The manufacturer's instructions were followed for inactivation times and temperatures, but the incubation period was extended to 1.5 hours since the shorter time frame suggested occasionally led to partial digests. Table 2-1 shows the primers, PCR conditions, enzymes, and expected band sizes for the restriction enzyme digests and the microsatellite locus. Plants with only *Typha latifolia* or *T. angustifolia* alleles were

categorized as *T. latifolia* or *T. angustifolia*, respectively. Plants categorized as F1 *T.* × *glauca* were heterozygous for *T. latifolia* and *T. angustifolia* alleles at all loci. A minimum of two loci was used to categorize a plant as a non-F1 hybrid if they were heterozygous for *T. latifolia* and *T. angustifolia* alleles at some but not all loci (Fig. 2-2; Fig. 2-3).

2.2.3 Bracteole numbers across inflorescence positions

I conducted a pilot study to understand the morphological characteristics of bracteoles and their potential implications for identification. I aimed to investigate if the occurrence of bracteoles was more common in different sections of the inflorescence and to determine if there was a difference in the number of bracteoles between F1 *Typha* × *glauca* and non-F1 hybrids. It is important to note that each floret can have at most one bracteole, with the possibility that not every floret has a bracteole. Moreover, the count of bracteoles over an equal number of florets is subject to variation across different taxa (Smith, 2000). The number of bracteoles was counted per 20 (n = 40) and 10 (n = 27) florets in hybrid samples (Fig. 2-4) in different sections (top/distal, middle, and bottom/proximal) of intact female inflorescence samples. Upon finding no discernible differences in bracteole count per 20 florets, subsequent samples were analyzed per 10 florets. Florets were sampled from the middle section of the female inflorescence for the remaining samples with intact inflorescences (see results).

To analyse and capture images of florets and bracteoles, a dissecting microscope (NIKON-SZ800; Tokyo, Japan) was equipped with a Nikon D5000 DSLR camera (Tokyo, Japan). A Fiber-Lite Mi-150 Illuminator (Dolan-Jenner, Boxborough, MA) was used to control for brightness and set it to 100% unless otherwise specified.

2.2.4 Bracteole-based taxon identification

To determine the accuracy of using bracteoles to identify *Typha spp.*, 244 samples were scored on the presence/absence and colour of their bracteoles and later compared to genetic data. The key developed by Smith (2000) was used to differentiate between *T. latifolia*, *T. angustifolia*, and hybrid cattails (n.b., using the bracteole-based key no attempt was made to distinguish between F1 and non-F1 hybrids; Fig. 2-4). A sample was categorized as *T. latifolia* if it had no bracteoles, as *T. angustifolia* if it had darker, wider bracteoles than the adjacent stigmas, and as *T.* × *glauca* if it had lighter-colored, narrower bracteoles than the adjacent stigmas. A small sample of florets (10-20) per inflorescence were used when scoring bracteoles. All samples were scored blind (samples were coded such that no taxonomic information was available before examination), and then a third party compared bracteole-based and genetically-based identifications.

2.2.5 Floret measurements

The assignment of plants used for measurements was based on genetic data. Measurements of pistillate flower lengths and the lengths and widths of bracteoles were made to identify characteristics that might provide additional taxonomic resolution. The length of pistillate flowers and bracteole length were chosen because gross morphology suggests that *Typha latifolia* has wide spikes, while *T. angustifolia* and F1 *T.* × *glauca* have narrow spikes, and non-F1 hybrids have overlapping spike lengths (Smith, 2000; Geddes et al., 2021; Tangen et al., 2022). Bracteole width measurements were taken since *T. angustifolia* has wider bracteoles while *T.* × *glauca* has narrower bracteoles (Smith, 2000). Images of a microscope objective micrometer calibration slide glass

stage with four graduated scales DIV 0.01mm (SANON) and florets were uploaded to Fiji (Schindelin et al., 2012; version 2.15.1), a distribution of ImageJ (Rasban, 2014; version 1.54h 15). Images of the calibration slide were taken at all magnifications (1x, 2x, 3x, and 4x). These were used to obtain pixel length measurements and convert them into metric distances. To ensure accuracy and precision while reducing errors, ten measurements were taken from the calibration slide, from each 0.1 mm line to the following 0.1 mm line, and the average distance of pixels per 0.1 mm division was calculated. The average number of pixels was then converted to 0.1 mm for calculating floret measurements. Images of the pistillate flower length, bracteole length, and bracteole width were captured at various magnifications, ensuring clarity. The magnification settings of each image were used to facilitate precise measurements using the corresponding scale. By applying this calibrated scale to sample pictures taken at the same magnification, measurements of the pistillate flower length, bracteole length, and bracteole width were taken for 25 samples of *T. latifolia, T. angustifolia*, F1 *T.* × *glauca*, and 22 non-F1 hybrids (Appendix 3-6). A more detailed guide on calibrating microscopy-generated images can be found in Appendix 7.

2.2.6 Bracteole Colour

This study aimed to assess the potential of quantifying bracteole colour for distinguishing *Typha angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids. By utilizing ImageJ/Fiji and Adobe Photoshop (version 25.2.0; Adobe Inc.; San Jose, CA), I performed colour calibration, image manipulation, and analysis to quantify the colour of bracteoles (see statistical analysis). Applying these tools allowed me to standardize

colour, ensuring consistency across images and enhancing the precision of my quantitative analyses.

The colour of bracteoles was measured for 25 *Typha angustifolia*, 25 F1 *T.* × *glauca* and 21 non-F1 hybrids. Non-F1 hybrids were further classified into two groups: those identified as *T. angustifolia* (*n* = 10) and those identified as F1 *T.* × *glauca* (*n* = 11) (Table 2-2). The colour calibration chart Calibrite ColorChecker Classic Mini (X-Rite; Grand Rapids, United States) assisted with colour calibration. Each of the 24 colour patches on the ColorChecker was captured under the microscope using the same conditions as the sample measurements. Individual patches were then reconstructed in Adobe Photoshop in the same placements as on the colour grid. I uploaded the reconstructed ColorChecker into ImageJ/Fiji, and colour calibration was performed using the IJP-Color plugin (version 0.12.1; Sacha, 2022). Sample photos were then calibrated against the calibrated ColourChecker and uploaded to Adobe Photoshop. I outlined the bracteole tip and recorded the average colours of all the pixels within the outlined region. Appendix 9 provides more detailed steps for colour calibration.

2.2.7 Repeatability assessment

Repeatability analysis aims to assess the reliability of data performed by different raters. In this study, another rater and I conducted bracteole-based identification using Smith's (2000) key on 135 samples. This repeatability analysis aimed to evaluate the consistency and agreement between our bracteole-based identification methods, ensuring that taxonomic identification is reliable.

2.2.8 Proportions of flowering cattail by taxon

The utility of female inflorescence characteristics for taxonomic identification depends on the proportion of flowering plants in natural populations. The flowering frequency, i.e., the proportion of shoots that produce an inflorescence each season, may vary among different taxa. However, this aspect has not been assessed yet for cattails. If the proportion of shoots that flowered per year varied among each taxon, distinct proportions of taxa would be observed between the two types of collection (randomly collected *Typha* plants and flowering collected *Typha* plants). Investigating the utility of female inflorescence characteristics for taxonomic identification can provide valuable insights into the variation and distribution of different cattail species within natural populations.

The proportion of plants that flower for each cattail taxon was determined by comparing the frequency of taxa in two types of sample collection (random and flowering types) from the same geographical region. Pieper et al. (2020) sampled 547 *Typha* plants in Ontario, regardless of whether they had flowered. Transects were laid out at 3-m intervals, perpendicular to each site's shoreline (wetlands) or road (ditches). In that study, inflorescences were not collected. Despite this study covering a wider range of sites, only sites within the same geographic region as other samples in the dataset were used. Three sites in Ontario were sampled: Cornwall, Pickering, and Kingston. An average of 184 plants (± 3.2 SD) from these locations were collected (Pieper et al., 2020). Although in their study, they did not differentiate between F1 *T.* × *glauca* and non-F1 hybrids, access to the molecular data enabled me to determine the proportions of *T. latifolia*, *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids. Flowering

Typha plants were collected by Melvin (2024), and H. Wilcox genotyped a subset of 152 plants, sampled flowering cattails between 3 and 7 transects along a water-depth gradient.

2.2.9 Statistical analyses

Floret measurements

I used analysis of variance (ANOVA) and Tukey's HSD comparisons to examine differences in floret measurements (including pistillate flower length, bracteole length, and bracteole width) among species. In the one-way ANOVA model, I designated the dependent variables as the floret measurements (pistillate flower length, bracteole length, and bracteole width), and taxon was the independent variable with four levels: *Typha latifolia*, *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids. Because *T. latifolia* lacks bracteoles, only three levels for taxon were considered for the analysis of bracteole length: *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids. To examine whether time of collection early (June/July) versus late (August/September) - influenced these floral traits, I performed a 2-way ANOVA with the same effects specified above for the one-way analyses and time of collection and the interaction between time of collection and taxon included as additional independent variables. All statistical analysis was conducted using R (v. 4.3.2; R core Team 2020); within R Studio (v. 2023.12.1; Posit Team, 2023). I calculated the significance of fixed effects using the ‘Anova_test’ function and assessed differences among species using the ‘tukey_hsd’ function; both functions provided by the ‘rstatix’ package (v. 0.7.2; Kassambara, 2023).

Perceived lightness

Perceived lightness, a key aspect of colour perception, was determined using a custom R script developed in RStudio. This script, found in Appendix 10, first converted the RGB values to linear RGB using the sRGB colour space transformation. It then calculated the luminance component using established formulas based on the linear RGB values. Finally, a non-linear transformation was applied to obtain the perceived lightness values for each colour (Myndex, 2024). I used analysis of variance (ANOVA) and Tukey's HSD comparisons to examine the differences in perceived lightness. In the one-way ANOVA model, I designated the dependent variables as the perceived lightness, and taxon was the independent variable with three levels: *Typha angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids. To assess the differences in perceived lightness across taxa, I performed a Tukey's HSD test using linear regression, with lightness as the dependent variable and taxon as the independent variable.

Repeatability statistic

Calculating repeatability statistics between two raters is important for ensuring measurement reliability, validity, and consistency. The Cohen's kappa is a statistic used for interrater reliability testing between two raters. Cohen's (1960) kappa is a robust statistic useful for interrater reliability testing between two raters. In this study, 184 samples from Southern Central Ontario were scored using bracteoles by myself and another rater (Melvin, 2024), and we recorded our identification as either *Typha latifolia, T. angustifolia*, or *T.* × *glauca*. Cohen's (1960) Kappa was calculated as:

$$\kappa = \frac{\Pr(o) - Pr(e)}{1 - \Pr\,(e)}.$$

Pr(o) represents the actual observed agreement, and Pr(e) represents the chance agreement. Actual observed agreement consists of the sample size of the

number of observations made across which raters are compared. The chance agreement is obtained through the following formula:

$$Expected\ (Chance)Agreemnt\ = \frac{\left(\frac{cm^1 \times rm^1}{n}\right) + \left(\frac{(cm^2 \times rm^2)}{n}\right)}{n}.$$

Where cm^1 represents column 1 marginal, cm^2 represents column 2 marginal, rm^1 represents row 1 marginal, rm^2 represents row 2 marginal, and n represents the number of observations (not the number of raters).

Proportions of flowering cattail plants by taxon

To determine the proportion of plants that flower for each cattail taxon, I compared the frequency of taxa in two types of samples (flowering versus random). A chi-square test of independence was used to examine the relation between the proportion of flowering taxa between the two samples (Melvin, 2024; Pieper et al., 2020) to test if the proportions differed. The data was organized into a contingency table (Table 2-3). Analysis was conducted using R within R Studio, and a chi-square test was applied using the ‘chisq.test’ function from the ‘rstatix’ package.

2.3 Results

2.3.1 Bracteole numbers across inflorescence positions

I found similar numbers of bracteoles per sample of florets across the upper, middle and lower segments of female inflorescences. The number of bracteoles across these segments differed by only 1 or 2 per sample (Fig. 2-5; Appendix 1; Appendix 2). Notably, the bracteole count remained consistent throughout the entirety of the inflorescence, with the specific section sampled having minimal influence on the observed variations.

2.3.2 Taxon identification

Two hundred forty-four samples were identified using both genetic and bracteole-based techniques. The genetic analysis revealed that 35 samples were *Typha latifolia*, 52 were *T. angustifolia*, 134 were F1 *T.* × *glauca*, and 23 were non-F1 hybrids. When comparing genotype-based identifications with bracteole identifications, I found an overall 93% agreement between genetic analysis and bracteole-based identification. I found that bracteole-based identifications were accurate for identifying *T. latifolia*. None of the samples I identified as *T. latifolia* from the absence of bracteoles were incorrectly identified based on genetic data. Similarly, bracteole-based identifications were successful in identifying F1 *T.* × *glauca* plants. I correctly identified all 134 genetically-confirmed F1 *T.* × *glauca* samples as F1 hybrids based on the presence of narrow, light-coloured bracteoles. For *T. angustifolia* plants, bracteole-based identification was correct for 50/52 plants. The two incorrectly identified *T. angustifolia* plants were misidentified as F1 *T.* × *glauca*. Lastly, bracteole-based identifications led to the incorrect identification of almost half of the non-F1 hybrid plants (10 of 23 samples). Of the ten misidentified non-F1 hybrids, I incorrectly identified nine as *T. angustifolia* and one as *T. latifolia*. To summarize, 96% of *T. latifolia* plants, 79% of *T. angustifolia* plants, and 96% of hybrids (F1s plus non-F1s) were correctly identified using bracteole-based characters compared to the genetic data (Table 2-4).

2.3.3 Floret measurements

Data for measurements of pistillate flowers, as well as the length and width of bracteoles, are represented as boxplots in Fig. 2-6 and Table 2-5. The results of the ANOVA test examining differences in the length of the pistillate flower among *Typha*

latifolia, *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids yielded significant differences between the groups ($F_{3, 88}$ = 7.03, *df* = 4, *p* > 0.001). The mean floret length for *T. latifolia* (6.94 mm ± 3.25 SD), was 28% greater than the mean floret length of *T. angustifolia* (4.59 ± 2.42 SD; Tukey HSD *p* = 0.0003; Table 2-6; Fig. 2-6a), 41% greater than for F1 *T.* × *glauca* (4.92 ± 3.11 SD, Tukey HSD *p* = 0.003; Table 2-6; Fig. 2-6a), and 31% greater than non-F1 hybrids (5.29 ± 2.36 SD, Tukey HSD *p* = 0.03; Table 2-6; Fig. 2-6a).

The results of the ANOVA test examining variation in bracteole length among taxon did not yield significant differences between the groups ($F_{2, 65}$ = 0.012, *df* = 3, *p* < 0.05; Fig. 6b). The overall mean bracteole lengths for *T. angustifolia*, F1 *T.* × *glauca,* and non-F1 hybrids was 4.35 mm (± 2.35 SD; Fig. 2-6b)**.**

The results of the ANOVA test examining variation in bracteole width among taxon yielded significant differences between the groups ($F_{2, 65}$ = 24.8, *df* = 3, *p* < 0.001). F1 *T.* × *glauca* had narrower bracteoles than *T. angustifolia* and non-F1 hybrids. F1 *T.* × *glauca* (mean bracteole width = 0.05 ± 0.02 SD) was 45% narrower than that of *T. angustifolia* (0.09 ± 0.04; Tukey HSD *p* < 0.0001; Table 2-7; Fig. 2-6c), and then that of non-F1 hybrids (0.09 ± 0.03; Tukey HSD *p* = <0.0001; Table 2-7; Fig. 2-6c).

Measurements of bracteole length and width differed depending on the time of collection (early/late; Fig. 2-7). I did not find any significant differences for pistillate flower length across early versus late collection times ($F_{3, 88}$ = 0.937, *df* = 3, *p* > 0.05; Fig. 2-7a). I did find significant differences for bracteole length ($F_{2, 65}$ = 4.350, *df* = 3, *p* < 0.05; Fig. 2-7b) and bracteole width ($F_{2, 65}$ = 13.0, *df* = 3, *p* < 0.001; Fig. 2-7c). Tukey's HSD Test revealed that the means significantly differed between early and late

collection within taxon for bracteole length for *Typha angustifolia* and F1 *T.* × *glauca* (Table 2-8) and in the bracteole width for *T. angustifolia* (Table 2-9). This indicates that the mean values tended to be smaller during the early collection period and larger during the late collection period. Non-F1 hybrids had no significant differences within taxon for either pistillate flower length, bracteole length, or bracteole width.

2.2.4 Bracteole colour

In general, *Typha angustifolia* had darker bracteoles than F1 *T.* × *glauca*. A colour gradient that spanned the range of bracteole colours for *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids identified as *T. angustifolia* or F1 *T.* × *glauca* is shown in Fig. 2-8). Although colours clearly differed across taxon, bracteole colours for non-F1 hybrids were highly variable and similar to those of the other taxon (Fig. 2-8). Linear regression analysis revealed significant differences in perceived lightness among different taxonomic groups (Fig. 2-9). The model showed a strong overall effect of taxon on average lightness ($F_{2, 68} = 49.9$, $p < 0.001$). The average lightness of F1 *T.* × *glauca* (74.58 ± 3.98 SD) was significantly higher than that of *T. angustifolia* (54.89 ± 4.10 SD; Tukey HSD $p = <0.0001$) and non-F1 hybrids (63.04 ± 11.28 SD; Tukey HSD $p = <0.0001$). However, Fig. 2-9 shows overlap in perceived lightness among taxa, the lightness of non-F1 hybrids similar to that of either *T. angustifolia* or F1 *T.* × *glauca* hybrids, leading to potential challenges in accurately distinguishing between these taxonomic groups based solely on perceived lightness.

2.3.5 Repeatability

In our study, we used Cohen's kappa to evaluate the level of agreement between two raters who categorized *Typha latifolia*, *T. angustifolia*, F1 *T.* × *glauca*, and non-F1

hybrids based on the bracteole characteristics. The value of κ was 0.919. I found the identification accuracy of non-F1 hybrids was lowest, but this value of κ still corresponds with 'almost perfect' (McHugh, 2012) agreement between raters.

2.2.6 Proportion of flowering shoots among taxa

The difference in the frequency of *Typha latifolia* among randomly collected *Typha* plants versus samples of flowering shoots suggests a potential flowering bias among taxa. Pieper et al. (2020) found a significantly higher occurrence of *Typha latifolia* in their collection of randomly-sampled shoots, regardless of whether it had an inflorescence presence. Their samples from Ontario included 101 (18%) *Typha latifolia* shoots, 80 (15%) *T. angustifolia shoots*, 315 (58%) F1 *T.* × *glauca* shoots*,* and 51 (9%) non-F1 hybrid shoots. Our genotyping of Melvin (2024) samples of flowering shoots revealed a strikingly different frequency of taxa with many fewer *T. latifolia* (only seven shoots, or 5%), a similar percentage of *T. angustifolia* (24 shoots, 16%), more F1 *T.* × *glauca* (110 shoots, 72%) and a similar percentage of non-F1 hybrid (11 shoots, 7%). The chi-square test of independence revealed a significant difference in the frequencies of the four taxa between the randomly collected versus flowering-shoot samples (X^2 = 19.75, *df* = 3, *n* = 699, *p* = <0.0001). Therefore, there was a difference in the relative frequencies of plants representing each taxon based on the collection of flowering versus randomly collected shoots.

2.3 Discussion

This study does not support the hypothesis that bracteole and floret length, and bracteole colour characteristics can reliably distinguish *Typha latifolia*, *T. angustifolia*, and their hybrids. While bracteole characters can indicate the range of taxa present at a

site, they do not appear more informative than other more easily observed gross morphological characteristics. Non-F1 hybrids bracteole characteristics often led to their confusion with *T. angustifolia* (for 39% of the samples). Moreover, pistillate flowers, bracteole sizes and colours overlap in their measurements between non-F1s and the other taxa, meaning that these two quantitative characters do not appear to be useful for improving the reliability of Smith's (2000) key in regions where non-F1 hybrid classes occur. However, the absence of bracteoles appears reliable for identifying *T. latifolia*: I misidentified only one *T. latifolia* sample (4%). However, the difference in the flowering frequency among different taxa results in *T. latifolia* being under-represented in sampling, which solely relies on traits that require the plant to produce flowers. Below, I interpret these findings in the context of existing literature, explore the implications, and offer insights into the broader significance of this study.

2.3.1 Floret and bracteole morphology

Given the overlapping measurements observed between cattail taxa regarding pistillate flower and bracteole length and width, relying solely on these traits may not provide accurate taxonomic identification. *Typha latifolia* tended to exhibit larger pistillate floret lengths than other taxa, whereas *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids had overlapping floret lengths. In addition, overlapping bracteole lengths were seen for *T. angustifolia*, F1 *T.* × *glauca* and non-F1 hybrids. Similarly, although F1 *T.* × *glauca* hybrids displayed narrower bracteole widths in comparison to non-F1 hybrids and *T. angustifolia*, the inability to distinguish between F1 and non-F1 plants using bracteoles alone undermines the reliability of this trait. Moreover, non-F1 hybrids were often identified as *T. angustifolia*, reducing my confidence in this metric for

distinguishing cattail taxa. I conclude that these floret and bracteole traits are not conclusive. Other studies have observed that F1 hybrid characteristics, typically intermediate and diagnostic in F1 hybrids, lose their utility when non-F1 hybrids are present. Tests using gross and microscopic morphological characteristics on *Rosa blanda*, *R. rugosa,* and their hybrids showed that the majority of characters are intermediate for F1 hybrids, whereas non-F1 hybrids had characteristics that were similar to parental trait values, making identification using morphology unreliable for these *Rosa* taxa (Mercure & Bruneau, 2008). It may be useful for future studies to explore whether combining traits could enhance the discriminatory capacity beyond using single traits.

The time of collection is an important factor to consider when collecting samples for taxonomic identification. Measurements of my samples varied depending on when the samples were collected. Compared to later collection times, floret and bracteole measurements from samples collected earlier in the flowering season were significantly smaller across taxa for all traits except bracteole width. Increases in morphological characters during the growing season are not surprising and have been noted for other taxonomic studies based on morphological characters (e.g., leaf width and length in the seagrass *Halodule wrightii*; Sordo et al., 2011). As a result, the increase in size for the morphological characteristics can be attributed to the fluctuations in trait sizes over time within cattail taxa, which could complicate the utility of such measures for differentiating cattail taxa, mainly if samples were collected at different times. However, regardless of sampling time, measurements among taxa overlapped, indicating that these microscopic traits are uninformative for taxonomic identification.

2.3.2 Colour of bracteoles

The colour of bracteoles proved not to be an informative tool for distinguishing between *Typha spp.* and their hybrids, as bracteoles of non-F1 hybrids had similar colouration as *T. angustifolia* and F1 *T.* × *glauca*. Measuring colour using digital images has been done in the study of animal coloration impact on mate selection (Bergman & Beehner, 2008), determining vegetation cover (Luscier et al., 2006; Laliberte et al., 2007; Liu et al., 2011) and coral reef health (Leiper et al., 2009). However, colour has not been extensively used for taxonomic identification. Smith (2000) described *T. angustifolia* as having dark-coloured bracteoles and F1 *T.* × *glauca* as having lighter-coloured bracteoles. My study supported that finding, but non-F1 hybrids can have bracteoles that are similar in colour to *T. angustifolia* or F1 *T.* × *glauca*, meaning that non-F1 hybrids cannot be reliably differentiated from F1s or *T. angustifolia* based on bracteole colour. This further emphasizes the limitations of bracteole-based identification, and the presence of non-F1 hybrids complicates the ability to utilize colour as a distinguishing characteristic among cattail taxa.

2.3.3 Conclusions in accurately and reliability identifying non-F1 hybrids

Despite a high level of repeatability observed between two raters and some agreement between genetic and bracteole identification, the inability of bracteoles to enable differentiation of non-F1 hybrids from parental taxa suggests limitations in their utility for taxonomic identification. The kappa statistic for inter-rater reliability, a measure of agreement between two raters that considers the possibility of agreement occurring

by chance, indicated ‘almost perfect (McHugh, 2012) reliability of using bracteoles for identification. However, although the level of agreement was consistent between the two raters, the bracteoles do not appear to enable the identification of non-F1 hybrids. *Typha latifolia* and F1 *T.* × *glauca* were frequently identified correctly compared to genetic data. Non-F1 hybrids had the lowest correct identifications, indicating potential challenges in distinguishing them accurately solely based on morphological characteristics. Non-F1 hybrids have overlapping morphological characteristics with *Typha latifolia*, *T. angustifolia* and F1 *T.* × *glauca*, making identifying them challenging without genetic data. Genetics have been used to complement the assessment of gross morphological characteristics commonly used in *Typha spp.* identification (Kuehn & White, 1999; Smith, 2000; Geddes et al., 2021; Wasko, 2022; Tangen et al., 2022). However, these traits are unreliable or untested for non-F1 hybrids. For the microscopic characters examined here, non-F1 hybrids have overlapping measurements with F1 hybrids and parental species, as seen with these gross morphological characters (Geddes et al., 2021; Tangen et al., 2022). The utility of bracteoles used for identification can vary among species. For example, bracteole number, size, shape, structure, and orientation helped identify species in the genus *Amaranthus,* but found only to help identify a few species in the genus *Amaranthus* (Costea & Tardif, 2003). Smith's (2000) key proves effective in situations where non-F1 hybrids are absent or when a representative proportion of plants are flowering; however, challenges arise in regions where non-F1 hybrids are present, compounded by the non-flowering of a significant proportion of *T. latifolia* plants. This research suggests that genetic methods

may be the only reliable way to differentiate *T. angustifolia,* F1 *T.* × *glauca* from non-F1 hybrids and accurately represent *T. latifolia*.

2.3.4 Implications

Taxonomic identification of *Typha latifolia*, *T. angustifolia* and their hybrids using bracteole characteristics is flawed partly because of the frequent misidentification of non-F1 hybrids as either *T. angustifolia* or F1 hybrids. The common occurrence of non-F1 hybrids across regions in North America exacerbates this unreliability. Non-F1 hybrids comprised approximately 25% of the sampled plants in three sites in southeastern Ontario (Pieper et al., 2020). Other studies have also reported a high prevalence of non-F1 hybrids around the Great Lakes (Travis et al., 2010; Kirk et al., 2011). Similarly, non-F1 hybrids constitute approximately a fifth of plants in New Brunswick, Nova Scotia, and Quebec (Kirk et al., 2011). Further west, Tangen et al. (2022) reported that non-F1 hybrids are more common than F1 *Typha* × *glauca* in the Prairie Pothole Region where they were found in over 80% of wetlands, constituting about 57% of plants. The difficulty of correctly identifying cattail taxa solely based on bracteole characteristics is increased by the widespread prevalence of non-F1 hybrids in various regions across Canada, and genetic methods are needed where non-F1 hybrids are present.

A second limitation of using the bracteole identification method is that it only applies to flowering plants, and the proportion of flowering plants varies among taxa. The choice of sampling methodology is crucial, as relying solely on flowering plants for sampling may introduce bias in conclusions about the distribution of *Typha latifolia*

compared to more random sampling approaches. Pieper et al. (2020) collected their samples randomly: leaves were collected every 3 m along a transect regardless of whether the plants were flowering. In contrast, Melvin (2024) sampled only flowering shoots every 3 m (or as close as possible) along a transect. Pieper et al. (2020) uncovered a much higher incidence of *Typha latifolia* in their dataset using a random sampling approach. This discrepancy in taxon frequencies emphasizes *T. latifolia* shoots flower far less frequently than other taxa, meaning that they would be under-represented in taxonomic assessments based on floral characters such as bracteoles.

In conclusion, bracteole-based identification can help identify *Typha latifolia* and indicate the presence of hybrids. However, its utility is limited in areas that include non-F1 hybrids. In areas where non-F1 hybrids are uncommon, bracteole-based identification may provide useful information for taxonomic identification. However, when genotyping has been conducted, it has been observed that there are always some non-F1 hybrids present. Non-F1 hybrids can be difficult to identify through bracteole characteristics, while genetic markers provide a more definitive way of classification. Accurate identification is necessary for monitoring invasive species and understanding their ecological impacts. Using bracteole-based identification could lead to under-representing *T. latifolia*, and genetic methods are the most reliable means of identification for non-F1 hybrids. Incorrect identification can lead to misinterpretations of species distributions, potentially resulting in underestimation or overestimation of invasion extent.

Table 2-1: Specifications of restriction enzyme (RE) digest reactions for different enzymes.

Restriction Enzyme & Primer Pair	PCR Annealing Temperature (°C)	Total Reaction Volume (µl)	RE Amount (U) and Volume (µl)	PCR Product per digest sample (µl)	Incubation Temperature (°C) & Time (min)	Heat Inactivation Temperature (°C) & Time (min)	Expected band sizes with and without digests
BstCl (BstClF, BstClR)	53	10	15U (0.75µl)	4	50°C for 1 hr	80°C for 20 min	Amplified product size ~ 1077 bp Digest (Both) ** = [727bp] [350bp]
AciI (PhyCF, PhyCR)	52	25	5U (0.5µl)	10	37°C for 1 hr	80°C for 20 min	Digest (Lat) = [382bp] [109bp]
TA3F/TA3R	57 ***	N/A	N/A	N/A	N/A	N/A	Amplified product size: Ang = [210bp] Lat = [190bp] Hybrid = [210bp][190bp][260bp]
AseI (AseIF, AseIR)	58.5	10	5U (0.5µl)	4	37°C for 1 hr	65°C for 20 min	Amplified product size ~ 1252 bp Digest (Ang)* = [862bp] [390bp]
BsrI (BsrIF, BsrIR)	56	10	5U (0.5µl)	4	65°C for 1 hr	80°C for 20 min	Amplified product size ~ 1183bp Digest (Ang)= [876bp] [307bp]

* Digest produces 3 bands; smallest fragment differs between species: *T. angustifolia* ~ [500bp]; *T. Latifolia* ~ [400bp]

** Digest produces 2 bands for *T. angustifolia* ~ [800 bp] [350 bp]. *T. latifolia* produces 3 bands next to each other between 400bp - 250bp.

*** Run on a 2% gel for one hour.

Table 2-2: Summary table of samples used in bracteole colour analysis.

Taxon ID	Location	Number of Samples
Typha angustifolia	Peterborough, ON (2022)	13
Typha angustifolia	Prairie Pothole Region	12
F1 *T.* × *glauca*	Prairie Pothole Region	22
F1 *T.* × *glauca*	Peterborough, ON (2019)	3
Non-F1 hybrids	Peterborough, ON (2022)	4
Non-F1 hybrids	Peterborough, ON (2019)	4
Non-F1 hybrids	Prairie Pothole Region	4
Non-F1 hybrids	Southern central Ontario	10

Table 2-3: Contingency tables and procedure for Chi-Square test.

Collection Type	Taxonomic Identification	Observed Frequency	Expected Frequency
Random	*T. latifolia*	7	23
	T. angustifolia	24	22
	F1 *T.* × *glauca*	110	92
	Non-F1 hybrids	11	13
Flowering	*T. latifolia*	101	84
	T. angustifolia	80	91
	F1 *T.* × *glauca*	315	332
	Non-F1 hybrids	51	48

Table 2-4: Summary table of genetic-based taxonomic identification compared to bracteole-based taxonomic identification *Typha latifolia*, *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids.

Genetic-based Taxonomic Identification	Percent scored as *T. latifolia* based on bracteole identification	Percent scored as *T. angustifolia* based on bracteole identification	Percent scored as F1 *T.* × *glauca* based on bracteole identification
Typha latifolia	100% (35)	0%	0%
Typha angustifolia	0%	96% (50)	4% (2)
F1 Typha × *glauca*	0%	0%	100% (134)
Non-F1 hybrids	4% (1)	39% (9)	57% (13)

Table 2-5: Variation in quantitative morphological traits in pistillate flower length (PFL; mm) bracteole length (BL; mm), and bracteole width (BW; mm) for *Typha latifolia*, *T. angustifolia*, F1 *T.*× *glauca* and non-F1 hybrids via bracteole identification (mm).

		Taxon ID											
		T. latifolia			*T. angustifolia*			*F1 T* × *glauca*			Non-F1 hybrids		
Morphological characteristic		Mean	Min.	Max.	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
	PFL (mm)	4.59	0.57	8.29	4.59	0.57	8.29	4.92	0.52	9.74	5.29	0.88	8.40
	BL (mm)	N/A	N/A	N/A	4.38	0.53	7.97	4.31	0.46	9.45	4.34	0.62	7.14
	BW (mm)	N/A	N/A	N/A	0.09	0.03	0.17	0.05	0.02	0.08	0.09	0.01	0.15

Table 2-6: Summary of Tukey's post-hoc tests of pairwise comparisons of pistillate flower length among taxon following a one-way ANOVA model analysis. Significant *P*-values are indicated in bold.

Pairwise comparison	Difference of means	95% confidence level		*P*
		Lower bound	Upper bound	
F1-A	0.330	-1.131	1.791	0.9343
L-A	2.356	0.895	3.816	**0.0003**
NF1-A	0.695	-0.833	2.224	0.6340
L-F1	2.026	0.565	3.486	**0.0026**
NF1-F1	0.365	-1.163	1.894	0.9236
NF1-L	-1.660	-3.189	-0.132	**0.0278**

Table 2-7: Summary of Tukey's post-hoc tests of pair-wise comparisons of bracteole width among taxon following a one-way ANOVA model analysis. Significant P-values are indicated in bold.

Pairwise comparison	Difference of means	95% confidence level		*P*
		Lower bound	Upper bound	
F1-A	-0.044	-0.060	-0.027	**<0.0001**
NF1-A	-0.004	-0.021	0.012	0.8005
NF1-F1	0.039	0.022	0.056	**<0.0001**

Table 2-8: Summary of Tukey's post-hoc tests of pair-wise comparisons of bracteole length among taxon following a two-way ANOVA model analysis examination of whether the timing of collection influenced morphological variations. Significant P-values are indicated in bold.

Pairwise comparison	Difference of means	95% confidence level		*P*
		Lower bound	Upper bound	
F1:Early-A:Early	-0.9887	-3.3961	1.4187	0.8324
NF1:Early-A:Early	1.4826	-0.7122	3.6775	0.3628
A:Late-A:Early	3.8506	1.8602	5.8410	**<0.0001**
F1:Late-A:Early	3.2820	1.7575	5.6579	**<0.0001**
NF1:Late-A:Early	2.4713	1.1350	5.4291	**<0.0001**
NF1:Early-F1:Early	4.8393	0.1172	4.8255	0.3414
A:Late-F1:Early	4.6964	2.6745	7.0040	**<0.0001**
F1:Late-F1:Early	4.6964	2.5686	6.8242	**<0.0001**
NF1:Late-F1:Early	4.2707	1.9611	6.5803	**<0.0001**
A:Late-NF1:Early	2.3679	0.4423	4.2936	**0.0075**
F1:Late-NF1:Early	2.2251	0.3401	4.1091	0.1155
NF1:Late-NF1:Early	1.7993	-0.2879	3.8865	0.1301
F1:Late-A:Late	-0.1429	-1.7842	1.4985	0.9998
NF1:Late-A:Late	-0.5686	-2.4396	1.3024	0.9470
NF1:Late-F1:Late	-0.4257	-2.2539	1.4023	0.9832

Table 2-9: Summary of Tukey's post-hoc tests of pair-wise comparisons of bracteole width among taxon following a two-way ANOVA model analysis examination of whether the timing of collection influenced morphological variations. Significant P-values are indicated in bold.

Pairwise comparison	Difference of means	95% confidence level		*P*
		Lower bound	Upper bound	
F1:Early-A:Early	-0.0165	-0.0518	0.0188	0.7438
NF1:Early-A:Early	0.0419	0.0097	0.0740	0.0039
A:Late-A:Early	0.0732	0.0440	0.1023	**<0.0001**
F1:Late-A:Early	0.0109	-0.1770	0.0394	0.8719
NF1:Late-A:Early	0.0428	0.0113	0.0742	**0.0023**
NF1:Early-F1:Early	0.0583	0.0238	0.0928	**<0.0001**
A:Late-F1:Early	0.0896	0.0579	0.1214	**<0.0001**
F1:Late-F1:Early	0.0274	-0.0038	0.0585	0.1178
NF1:Late-F1:Early	0.0592	0.0254	0.0931	**<0.0001**
A:Late-NF1:Early	0.0313	0.0031	0.0595	**0.0212**
F1:Late-NF1:Early	-0.0310	-0.0586	-0.0034	**0.0191**
NF1:Late-NF1:Early	0.0009	-0.0297	0.0315	0.9999
F1:Late-A:Late	-0.0623	-0.0863	-0.0382	**<0.0001**
NF1:Late-A:Late	-0.0304	-0.0578	-0.0030	**0.0212**
NF1:Late-F1:Late	0.0319	0.0051	0.0587	**0.0107**

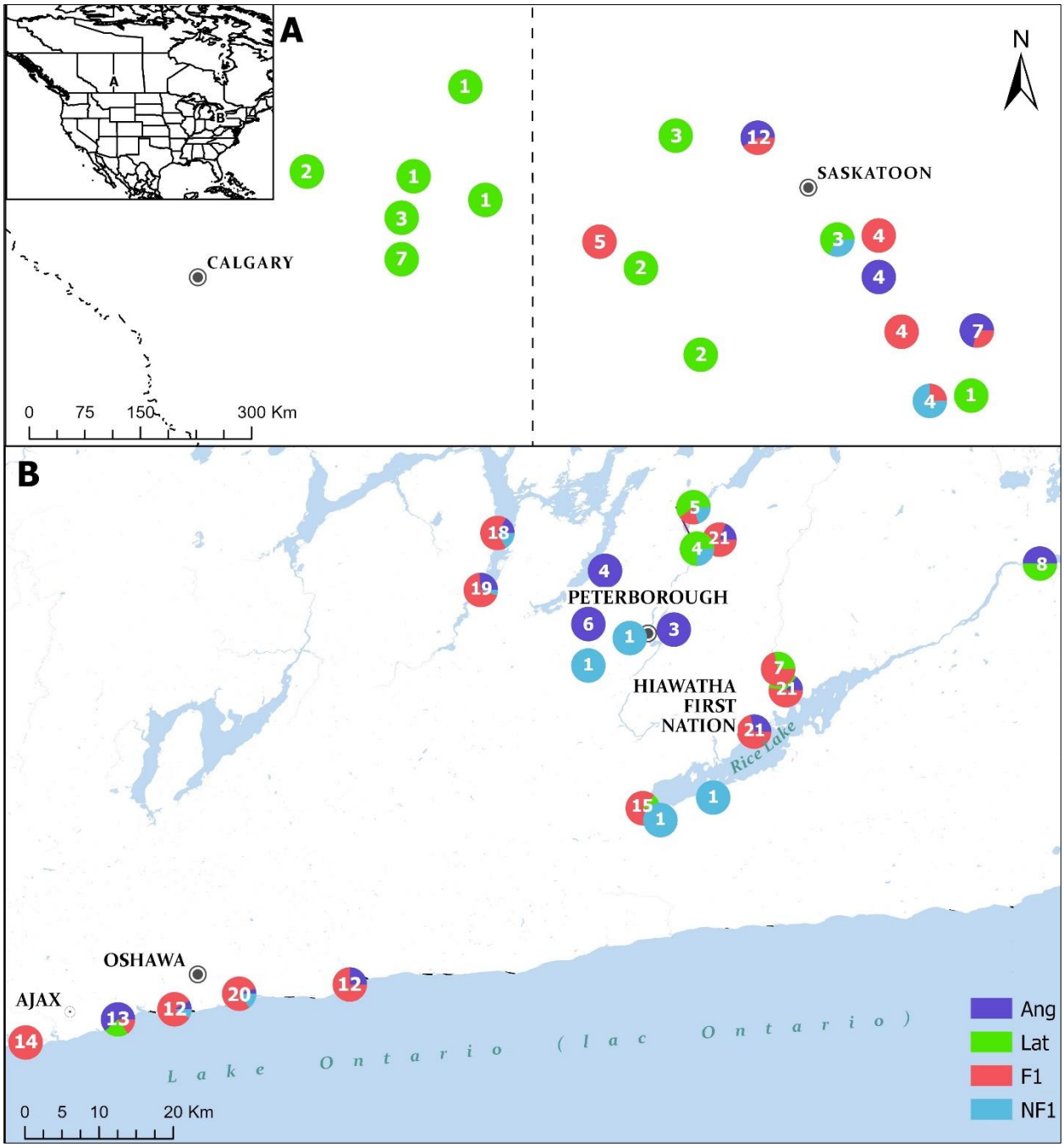

Figure 2-1: Locations and taxonomic frequencies of samples used in bracteole identification. Taxonomic identifications from these locations are based on previously generated PCR-RFLP and microsatellite markers. “Ang” = *Typha angustifolia*, “Lat” = *Typha latifolia*, “F1” = F1 *Typha* × *glauca*, “NF1” = non-F1 hybrids. Pie charts represent the proportion of taxa at each site, and the number in the centre represents the total number of samples.

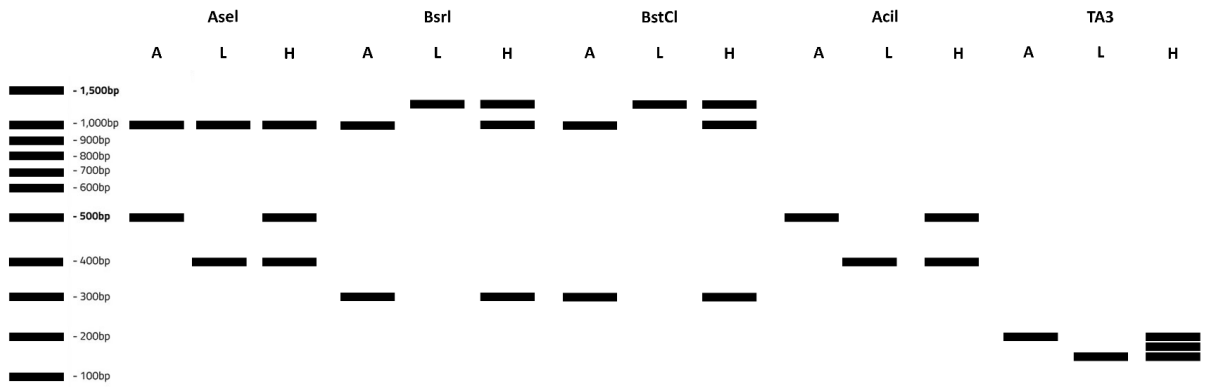

Figure 2-2: Diagram depicting the species-specific PCR-RFLP polymorphisms (AseI, BsrI, BstCI, AciI) or the PCR allele sizes (TA3). “A” shows the pattern expected for a *Typha angustifolia* identification, “L” = shows the pattern expected for a *Typha latifolia* identification, and “H” shows the pattern expected for a *Typha* × *glauca* identification.

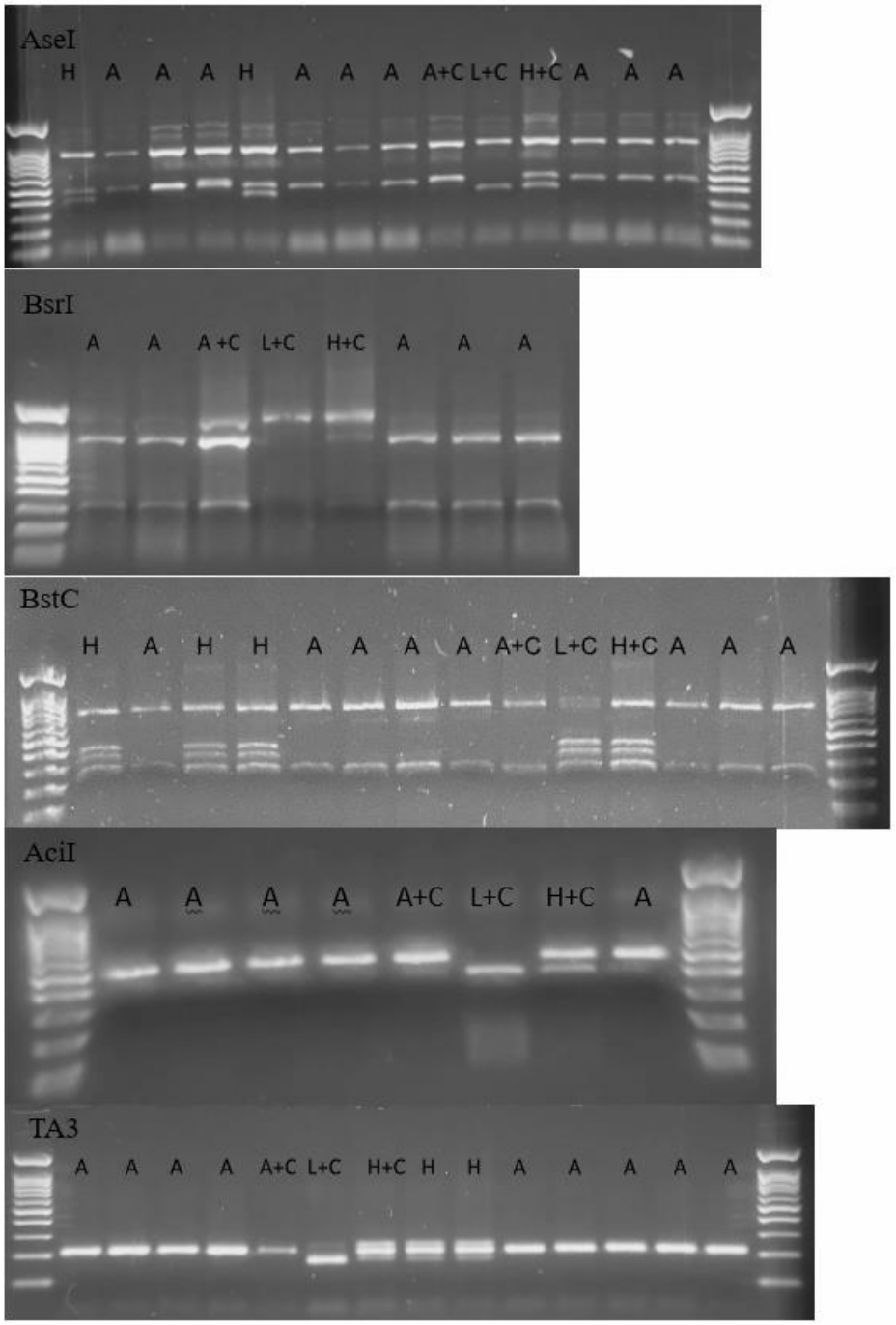

Figure 2-3: Gel images for the species-specific PCR-RFLP polymorphisms (AseI, BsrI, BstCI, AciI) or the microsatellite allele sizes (TA3). “A” shows the pattern expected for a *Typha angustifolia* identification, “L” = shows the pattern expected for a *Typha latifolia* identification, and “H” shows the pattern expected for a *Typha* × *glauca* identification. For each locus, samples with known genotypes (*T. angustifolia*, *T. latifolia*, F1 *T. x glauca*) were included as controls for each image; control samples denoted by a “+C”,

with “A+C” = *Typha angustifolia* control, “L+C” = *Typha latifolia* control; “H+C” = *Typha* × *glauca* control.

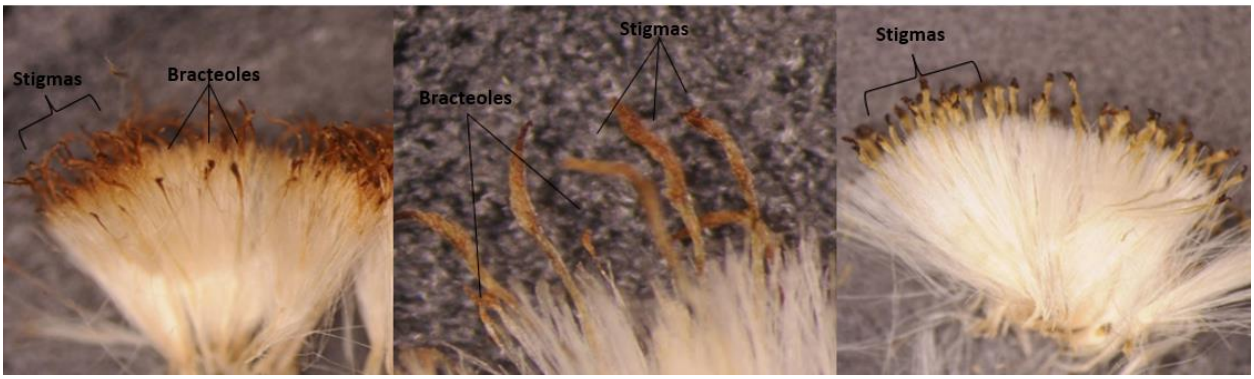

Figure 2-4: Magnified pistillate florets from *Typha angustifolia* (left), *T. x glauca* (middle), and *T. latifolia* (right) show some of the features examined in this study. *Typha angustifolia* exhibits darker bracteoles wider than the adjacent stigmas, while *Typha* × *glauca* displays lighter-coloured bracteoles narrower than the adjacent stigmas. *Typha latifolia* lacks bracteoles.

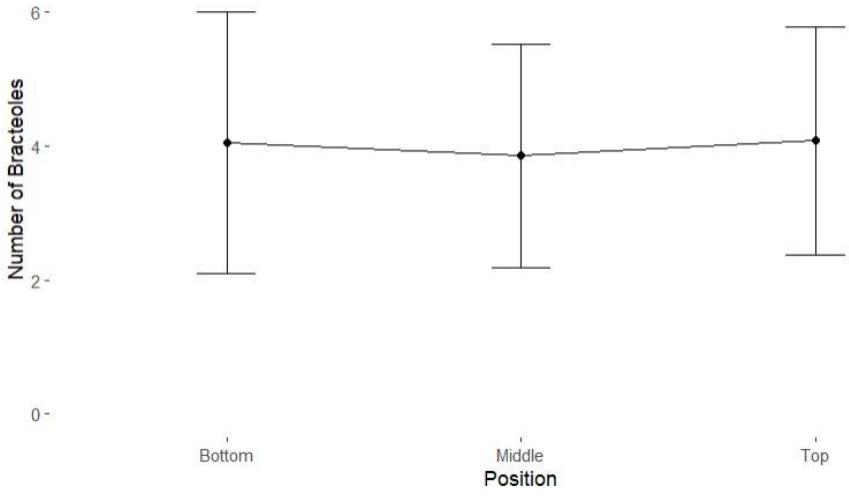

Figure 2-5: Interaction diagram representing the count of bracteoles per 10 (first ten florets for samples with 20 florets) for *T. x glauca*.

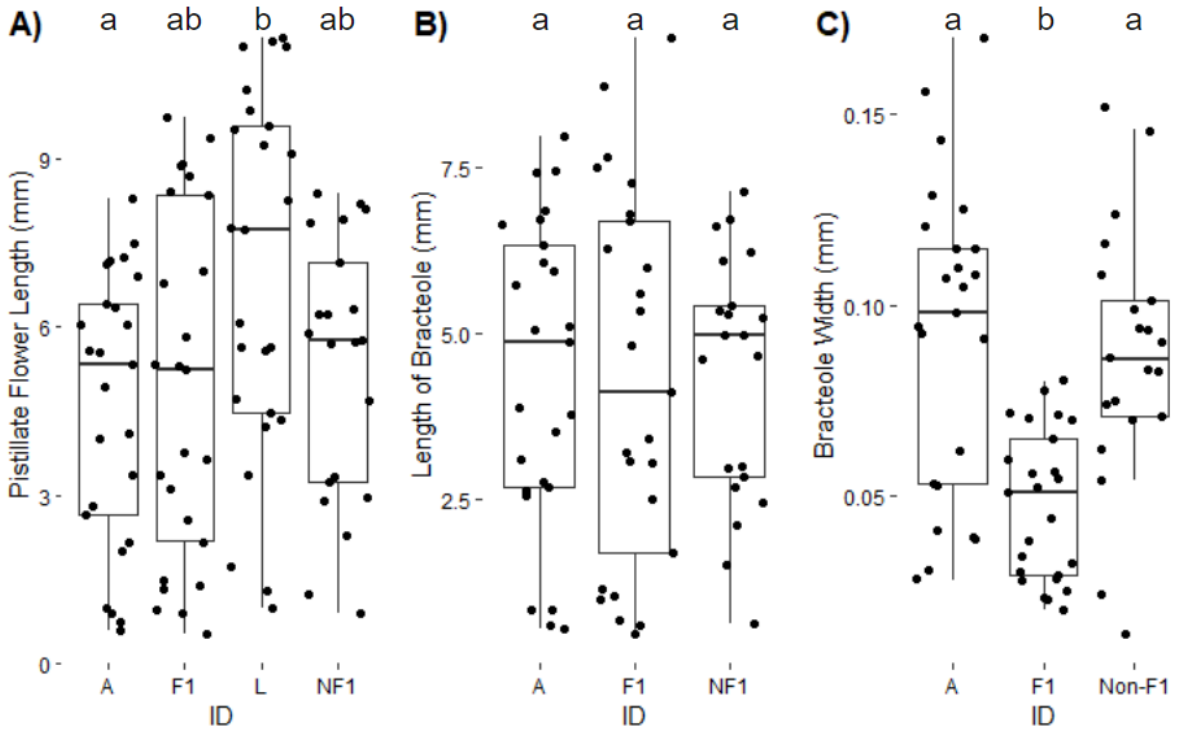

Figure 2-6: Boxplot comparisons between **A)** pistillate flower length (mm) of *Typha latifolia*, *T. angustifolia*, F1 *Typha* × *glauca* and non-F1 hybrids based on genetic identification, **B)** bracteole length, and **C)** bracteole width for *T. angustifolia*, F1 *T.* × *glauca* and non-F1 hybrid. Letters above each box indicate pairwise groupings.

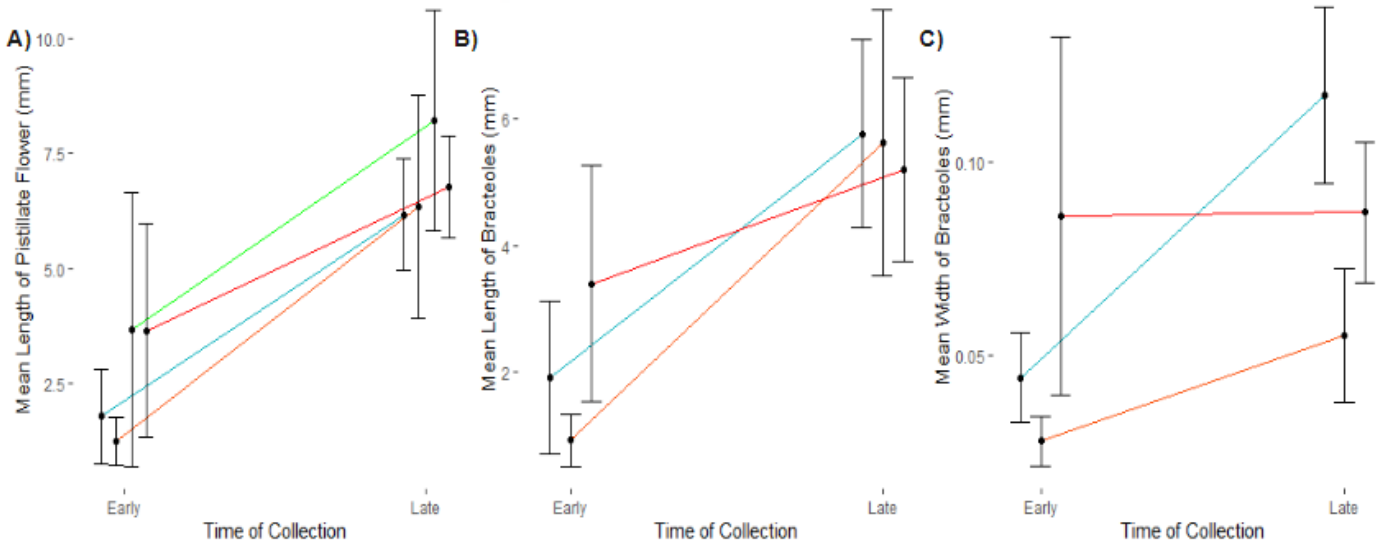

Figure 2-7: Line graphs by taxon for mean measurements of **A)** pistillate flowers, **B)** bracteole length, and **C)** bracteole width by time of collection. The green line corresponds to *Typha latifolia*, the blue line to *T. angustifolia*, the orange line to F1 *T.* × *glauca*, and the red line to non-F1 hybrids. Whiskers correspond to minima and maxima.

Figure 2-8: A colour gradient generated from the lightest to the darkest colour obtained

from the tip of the bracteoles for **a)** *T. angustifolia*, **b)** non-F1 hybrids identified as *T. angustifolia,* **c)** F1 *T.* × *glauca*, and **d)** non-F1 hybrids identified as F1 *T.* × *glauca.*

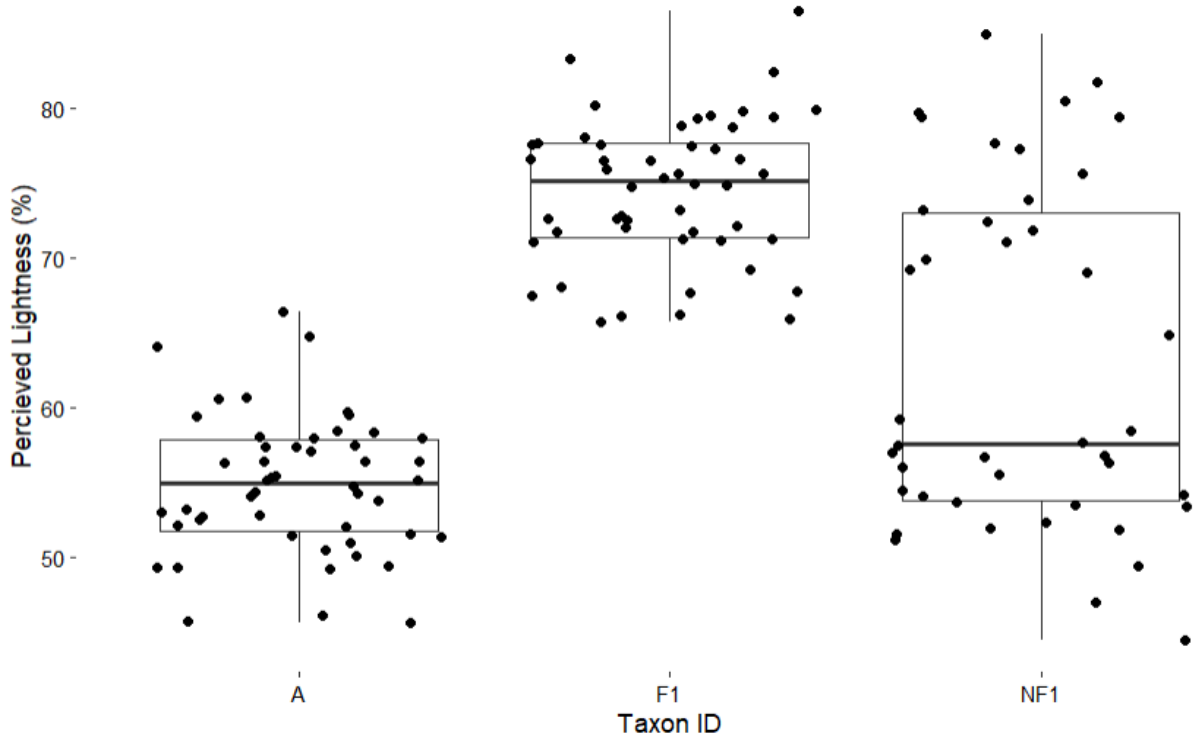

Figure 2-9: Boxplot comparisons for perceived lightness between *T. angustifolia* (A), F1 *T.* × *glauca* (F1) and non-F1 hybrid (NF1).

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Chapter 3: General Discussion

3.1 Summary of findings

My thesis investigated whether bracteole-based identification can accurately and reliably differentiate *Typha latifolia*, *T. angustifolia*, F1 *T.* × *glauca,* and non-F1 hybrids. The absence of bracteoles can accurately identify *T. latifolia*, but the presence of bracteole cannot accurately differentiate *T. angustifolia* and F1 *T.* × *glauca* from non-F1 hybrids. Using measurements of the pistillate flower length and bracteole length and width, I found that *T. latifolia* exhibits significantly larger means than other taxa, regardless of the time of collection. On the other hand, *T. angustifolia*, F1 *T.* × *glauca,* and non-F1 hybrids had comparable means for all these characteristics, regardless of the time of collection. In addition, the colour of bracteoles does not prove to be an informative characteristic, as non-F1 hybrids exhibit similar colouration to *T. angustifolia* or F1 *T.* × *glauca*. Further, data from randomly collected *Typha* plants revealed a much higher incidence of *T. latifolia* when compared to flowering plants. This study showed that bracteole-based identification can lead to bias in the distribution of taxa. Incorrect identification and biases from this method can result in misinterpretations of species distributions, potentially leading to underestimation or overestimation of the extent of invasion.

3.2 Contribution to knowledge

Under the microscope, minute morphological features enable researchers to delineate subtle differences that may not be readily apparent to the naked eye. Traditionally, microscopic features such as pollen and leaf morphology have been

instrumental in distinguishing between closely related species and their hybrids. However, microscopic traits in the seeds, stem, and roots can also be diagnostic when differentiating between taxa (Rewald et al., 2012; Sukhorukov et al., 2013), where traditionally used microscopic traits are uninformative.

Microscopic characteristics have been previously employed to differentiate between *Typha species* and their hybrids. This includes the shape of the leaf-lamina margin (McManus et al., 2002), the number of vascular bundles within each zone of fibres at the leaf-lamina margin (McManus et al., 2002), and the presence-or-absence of enlarged epidermal cells alongside the vascular bundles (McManus et al., 2002), and the arrangement of mesophyll cells connecting adaxial and abaxial leaf surfaces. However, this has yet to be tested on known F1 and non-F1 hybrids (McManus et al., 2002; Wasko et al., 2021). In my research, I have made a novel contribution to the understanding of using bracteole-based identifications for *Typha latifolia*, *T. angustifolia*, F1 *T.* × *glauca*, and non-F1 hybrids. However, my findings reveal limitations in this method that bracteoles alone are sufficient for accurate species identification because of widespread non-F1 hybrids. By comparing genetic-based identification to bracteole-based identification and examining morphological variations in measurements and colour among different taxa and hybrid types, I have revealed complexities of differentiating non-F1 hybrids from *T. angustifolia* and F1 *T.* × *glauca*. This method is, therefore, of limited utility in regions where non-F1 hybrids are prevalent. Furthermore, my research highlights potential biases in this method, as it requires flowering plants for bracteole-based identification, which may underestimate the amount of *T. latifolia*.

3.3 Future Directions

Future research could explore predictive modelling techniques widely used in various fields, including ecology, biology, and environmental science, for species identification (Almeida et al., 2020) and species distribution (Thuiller et al., 2003). In identifying species, machine learning algorithms such as regression trees are used for predictive modelling. Machine learning has been employed for the identification of lung cancer cell types, bird and frog calls, and tree species based on leaf shape outline (Martín-Gómez et al., 2022; Zhou et al., 2002; Acevedo et al., 2009; Barré et al., 2017). These applications have all yielded encouraging results with high identification accuracy. Regression trees, a decision tree algorithm, can be systematically analyzed to identify each group by observing reliable morphological traits. Regression trees have been created to identify the hybrid zone between Largemouth Bass and Spotted Bass (Godbout et al., 2011). By implementing predictive models, species identification can create sophisticated analytical methodologies for accurate identification.

3.4 Limitations and implications

Bracteole-based identification can be useful in differentiating *Typha latifolia* and in detecting hybrids. However, its usefulness may be limited if a region has non-F1 hybrids or lacks flowering plants. In regions where non-F1 hybrids are uncommon, this method may be useful for taxonomic identification. However, this method may underrepresent *T. latifolia*, as they flower less than other taxa. It is necessary to understand the limitations of this method and the potential for misidentification between non-F1 hybrids to *T. angustifolia* and F1 *T.* × *glauca*. Accurate and reliable identification

is important to monitor the spread of invasive species and understand their impact on the environment. Inaccurate identification may lead to incorrect interpretations of species distributions, which may cause the extent of invasion to be overestimated or underestimated. Therefore, genetic methods would be necessary to provide a more conclusive identification due to the misidentification and the unreliability of bracteole characteristics for identifying non-F1 hybrids.

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Appendices

Appendix 1: Number of Bracteoles per 20 florets in each section (top, middle, bottom) of the female inflorescence (samples from southern Ontario).

Sample ID	Collection Date	ID	1st Top	2nd Top	1st Middle	2nd Middle	1st Bottom	2nd Bottom
BL-101	June 25, 2021	Hybrid	1	2	3	3	4	3
BL-102	June 25, 2021	Hybrid	4	5	5	4	5	5
BL-303	June 25, 2021	Hybrid	5	5	5	5	5	6
BL-401	June 25, 2021	Hybrid	4	4	3	3	5	6
BL-402	June 25, 2021	Hybrid	4	4	2	5	5	4
BL-403	June 25, 2021	Hybrid	8	8	6	5	5	6
BL-501	June 25, 2021	Hybrid	5	4	4	4	6	7
BL-502	June 25, 2021	Hybrid	6	4	5	4	5	5
BL-602	June 25, 2021	Hybrid	3	3	2	4	3	4
BL-603	June 25, 2021	Hybrid	3	2	3	2	2	2
BL-701	June 25, 2021	Hybrid	4	5	4	4	5	5
BL-702	June 25, 2021	Hybrid	7	7	5	6	4	8

BL-703	June 25, 2021	Hybrid	7	8	7	6	8	6
BS3-101	July 5, 2021	Hybrid	2	1	3	3	5	4
BS3-102	July 5, 2021	Hybrid	3	4	3	3	4	4
BS3-103	July 5, 2021	Hybrid	3	4	3	4	4	3
BS3-202	July 5, 2021	Hybrid	3	2	4	3	3	3
BS3-303	July 5, 2021	Hybrid	2	3	3	4	3	4
CC-101	July 1, 2021	Hybrid	2	1	2	1	2	2
CC-102	July 1, 2021	Hybrid	3	3	2	1	1	2
CC-103	July 1, 2021	Hybrid	2	2	3	2	2	2
CC-301	July 1, 2021	Hybrid	2	2	2	2	1	1
CC-302	July 1, 2021	Hybrid	2	1	1	2	1	3
CC-303	July 1, 2021	Hybrid	1	1	2	2	1	1
IR-3-001	June 23, 2021	Hybrid	2	2	3	2	2	3
IR-3-002	June 23, 2021	Hybrid	3	4	4	3	4	4
IR-4-001	June 23, 2021	Hybrid	3	2	2	3	4	3

IR-4-002	June 23, 2021	Hybrid	2	3	3	3	3	2
IR-4-003	June 23, 2021	Hybrid	1	2	2	2	2	2
IR-5-003	June 23, 2021	Hybrid	2	3	3	3	3	2
IR-501	June 23, 2021	Hybrid	1	1	1	1	3	4
IR-6-001	June 23, 2021	Hybrid	3	3	3	3	2	3
IR-6-003	June 23, 2021	Hybrid	3	4	4	4	4	5
IR-OP-A	June 23, 2021	Hybrid	2	2	2	2	1	2
PLI-201	June 16, 2021	Hybrid	4	3	2	3	4	3
PLI-301B-dark	July 21, 2021	Hybrid	4	3	4	3	3	4
PLI-301B-light	July 21, 2021	Hybrid	3	2	3	3	3	2
PLI-305	June 16, 2021	Hybrid	2	3	2	3	3	2
PLI-402	July 21, 2021	Hybrid	4	5	4	3	3	4
PLI-501	July 21, 2021	Hybrid	4	2	3	2	3	3

Appendix 2: Number of Bracteoles per 10 florets in each section (top, middle, bottom) of the female inflorescence (samples from southern Ontario).

Sample ID	Collection Date	ID	1st Top	1st Middle	1st Bottom
BL-503	June 25, 2021	Hybrid	4	2	3
CC-501	July 1, 2021	Hybrid	6	5	5
CC-OP-001	July 1, 2021	Hybrid	4	4	3
PL3-101	July 22, 2021	Hybrid	4	5	4
PL3-102	July 22, 2021	Hybrid	6	3	6
PL3-103	July 22, 2021	Hybrid	5	6	6
PL3-201	July 22, 2021	Hybrid	4	3	5
PL3-202	July 22, 2021	Hybrid	6	4	4
PL3-203	July 22, 2021	Hybrid	4	5	4
PL3-4-001	June 29, 2021	Hybrid	8	4	5
PL3-403	July 22, 2021	Hybrid	3	4	5
PL3-5-001	July 22, 2021	Hybrid	6	8	7
PL3-5-002	June 29, 2021	Hybrid	6	5	6

PL3-5-003	June 29, 2021	Hybrid	6	7	5
PL3-6-001	June 29, 2021	Hybrid	7	7	8
PL3-6-002	June 29, 2021	Hybrid	3	4	5
PL3-6-003	June 29, 2021	Hybrid	3	3	5
PL3-9-002	July 22, 2021	Hybrid	3	5	5
WSB-1-001	June 30, 2021	Hybrid	9	8	8
WSB-1-002	June 30, 2021	Hybrid	5	7	6
WSB-2-001	June 30, 2021	Hybrid	8	7	6
WSB-2-002	June 30, 2021	Hybrid	4	3	4
WSB-3-003	June 30, 2021	Hybrid	6	4	4
WSB-4-001	June 30, 2021	Hybrid	8	6	7
WSB-4-002	June 30, 2021	Hybrid	5	5	4
WSB-4-003	June 30, 2021	Hybrid	5	5	4
WSB-2-003	June 30, 2021	Hybrid	5	3	4

Appendix 3: *Typha latifolia* measurements of the pistillate flower.

Length of Pistillate (mm)			
Sample ID	Sample 1	Sample 2	Average
BS3-402	4.320	4.381	4.351
BS3-403	3.463	3.227	3.345
BS3-501	4.179	4.769	4.474
BS3-601	9.941	9.228	9.585
BS3-OP-01	1.605	1.814	1.710
Dun1	6.766	6.340	6.553
Dun2	7.861	7.607	7.734
NB2	5.902	5.352	5.627
NB5	9.494	9.032	9.263
Ken2	9.623	9.823	9.723
Ken3	11.034	10.983	11.009
Mon1	8.492	8.014	8.253
NB6	9.486	10.276	9.881
Omega1	9.233	8.970	9.102
Red1	4.731	4.720	4.726
Red2	5.519	5.612	5.566
Red3	11.298	10.921	11.110
Spon1	9.684	9.382	9.533
Spon2	10.449	10.059	10.254
Spon3	7.565	7.991	7.778

Spon4	10.900	11.110	11.005
Spon6	11.217	11.119	11.168
Spon7	4.117	4.309	4.213
Stet5	5.964	6.185	6.075
Stet4	5.798	5.512	5.655

Appendix 4: *Typha angustifolia* measurements of the pistillate flower length, bracteole length and width.

	Length of Pistillate Flower (mm)			Length of Bracteoles (mm)			Width of Bracteoles (mm)		
Sample ID	Sample 1	Sample 2	Average	Sample 1	Sample 2	Average	Sample 1	Sample 2	Average
7LBA1	7.122	7.236	7.179	6.840	6.420	6.630	0.102	0.094	0.098
7LBA2	5.306	5.200	5.253	5.685	5.906	5.796	0.122	0.151	0.137
7LBA3	6.687	6.578	6.633	6.107	6.116	6.112	0.137	0.147	0.142
7LBA9	6.896	5.833	6.365	6.644	6.007	6.326	0.101	0.119	0.110
Craic4	6.354	6.467	6.411	5.809	6.323	6.066	0.166	0.083	0.125
FBA3	7.348	7.633	7.491	7.508	7.363	7.436	0.104	0.110	0.107
FBA4	7.122	7.394	7.258	6.994	6.439	6.717	0.091	0.090	0.091
FBA5	9.773	9.498	9.636	7.164	7.152	7.158	0.079	0.138	0.109
FBA6	6.820	6.991	6.906	7.401	7.413	7.407	0.106	0.234	0.170
FBA7	8.283	8.306	8.295	7.493	8.447	7.970	0.144	0.044	0.094
FBA8	7.311	7.411	7.361	6.784	5.741	6.263	0.780	0.133	0.457
Red3-1	5.815	6.243	6.029	5.971	5.866	5.919	0.193	0.119	0.156
Red3-2	4.641	4.678	4.660	3.939	3.929	3.934	0.115	0.106	0.111
Red3-3	6.454	6.288	6.371	5.819	5.830	5.825	0.122	0.115	0.119
Red3-4	4.258	3.918	4.088	3.534	4.178	3.856	0.087	0.198	0.143
Red3-5	5.514	5.575	5.545	4.945	5.148	5.047	0.106	0.123	0.115
Red3-6	7.925	8.069	7.997	5.148	5.146	5.147	0.190	0.171	0.181
Red3-7	5.781	6.312	6.047	5.002	5.173	5.088	0.126	0.083	0.105
Reg3	5.191	5.482	5.337	4.017	5.685	4.851	0.111	0.074	0.093

Reg4	4.018	3.611	3.815	3.466	3.829	3.648	0.229	0.256	0.243
Reg5	5.042	4.825	4.934	2.978	2.354	2.666	0.142	0.074	0.108
Reg6	4.130	3.893	4.012	3.771	3.763	3.767	0.099	0.142	0.121
TVRA1	6.986	6.075	6.531	5.655	5.763	5.709	0.171	0.182	0.177
TVRA3	5.659	5.511	5.585	5.605	5.815	5.710	0.122	0.108	0.115
TVRA4	6.795	7.461	7.128	7.323	6.376	6.850	0.120	0.137	0.129

Appendix 5: F1 *T.* × *glauca* measurements of the pistillate flower, length, and width of bracteoles.

	Length of Pistillate Flower (mm)			Length of Bracteoles (mm)			Width of Bracteoles (mm)		
Sample ID	Sample 1	Sample 2	Average	Sample 1	Sample 2	Average	Sample 1	Sample 2	Average
Dav1	6.986	7.033	7.010	5.551	5.639	5.595	0.065	0.078	0.072
Dav2	7.108	6.575	6.842	8.140	8.255	8.198	0.098	0.096	0.097
Dav3	8.778	8.148	8.463	8.766	7.912	8.339	0.065	0.050	0.058
Dav4	9.053	8.778	8.916	7.476	7.504	7.490	0.063	0.055	0.059
Kin2-1	9.391	10.086	9.739	9.229	9.662	9.446	0.077	0.064	0.071
Kin2-2	8.857	9.878	9.368	8.567	8.868	8.718	0.063	0.066	0.065
Kin2-3	12.568	11.625	12.097	5.299	5.494	5.397	0.083	0.074	0.079
Kin2-4	7.791	7.110	7.451	5.466	5.894	5.680	0.102	0.126	0.114
Kin2-5	9.547	8.330	8.939	7.593	7.680	7.637	0.062	0.060	0.061
MJ2-1	7.577	7.807	7.692	7.390	7.461	7.426	0.154	0.134	0.144
MJ2-2	7.817	7.022	7.420	6.667	6.206	6.437	0.198	0.084	0.141
MJ2-3	8.623	8.788	8.706	7.228	7.273	7.251	0.069	0.070	0.070
MJ-2-4	8.551	8.174	8.363	7.673	7.628	7.651	0.064	0.095	0.080
Red4-1	7.065	6.995	7.030	6.308	5.709	6.009	0.054	0.042	0.048
Red4-2	5.930	5.284	5.607	4.314	3.937	4.126	0.037	0.046	0.042
Red4-3	5.981	5.821	5.901	5.573	5.371	5.472	0.059	0.068	0.064
Red4-4	5.346	5.248	5.297	3.562	4.669	4.116	0.074	0.038	0.056
Red4-5	5.793	4.694	5.244	5.970	5.879	5.925	0.055	0.055	0.055
Reg1	8.524	8.297	8.411	6.630	6.713	6.672	0.049	0.055	0.052

Reg2	9.093	8.673	8.883	6.698	6.881	6.790	0.082	0.058	0.070
Reg7	5.746	5.938	5.842	5.514	5.131	5.323	0.070	0.086	0.078
Reg8	7.703	7.648	7.676	6.963	6.632	6.798	0.166	0.047	0.107
RR6	8.256	8.099	8.178	7.837	7.831	7.834	0.058	0.102	0.080
RR8	6.990	6.558	6.774	6.235	6.282	6.259	0.054	0.057	0.056
RR9	5.348	5.302	5.325	4.672	4.968	4.820	0.058	0.043	0.051

Appendix 6: Non-F1 hybrids measurements of the pistillate flower, length, and width of bracteoles.

	Length of Pistillate Flower (mm)			Length of Bracteoles (mm)			Width of Bracteoles (mm)		
Sample ID	Sample 1	Sample 2	Average	Sample 1	Sample 2	Average	Sample 1	Sample 2	Average
AVR13	6.009	6.439	6.224	5.580	5.728	5.330	0.056	0.093	0.075
BRDC3	6.176	6.287	6.232	5.640	5.278	5.269	0.105	0.092	0.099
HEAW2	6.291	6.347	6.319	5.797	5.716	5.412	0.052	0.114	0.083
HSCAA18	5.533	5.964	5.749	5.423	5.182	4.975	0.128	0.088	0.108
ML14	4.909	6.622	5.766	4.376	3.876	4.591	0.101	0.087	0.094
ML5	5.731	6.049	5.890	6.118	5.552	5.223	0.151	0.097	0.124
LL5	8.282	8.127	8.205	6.589	6.960	6.694	0.079	0.087	0.083
HOR2-2	5.411	6.007	5.709	5.309	5.269	4.951	0.054	0.069	0.062
HOR2-4	7.925	8.289	8.107	5.517	5.491	6.222	0.075	0.072	0.074
HOR2-5	8.397	8.393	8.395	7.788	7.843	7.136	0.059	0.112	0.086
KEN1	7.817	7.945	7.881	7.130	6.823	6.599	0.070	0.070	0.070
CC-602	1.092	1.375	1.234	1.190	1.098	1.498	0.124	0.107	0.116
OSM-601	3.322	3.324	3.323	2.472	2.382	2.971	0.199	0.093	0.146
OSM-701	2.687	3.086	2.887	2.146	2.196	2.667	0.080	0.061	0.071
OSM-703	3.175	2.732	2.954	2.634	2.462	2.826	0.082	0.105	0.094
PLS-103	7.782	8.063	7.923	7.263	7.275	6.718	0.080	0.099	0.090
PLS-403	7.464	6.842	7.153	6.433	6.635	6.088	0.127	0.074	0.101
PLI-101	2.473	2.072	2.273	1.290	1.507	2.102	0.054	0.054	0.054

PLI-502	4.649	4.724	4.687	5.318	6.477	4.642	0.183	0.121	0.152
RLW-101	2.703	2.716	2.710	1.597	1.846	2.429	0.074	0.084	0.079

Appendix 7: Supplementary methods for bracteole measurements.

A 0.1mm calibration slide (SANON) picture was taken at all magnifications (1x, 2x, 3x, and 4x) on a dissecting microscope (NIKON-SZ800). The software ImageJ/Fiji (Rasband, 1997–2008) was downloaded in Windows 64-bit. Images taken with the 1x objective lens had the 1x calibration slide photo uploaded alongside. In ImageJ, the File→Open option was used to navigate to the file location and to open the image(s). The line tool in the toolbar was selected, and the measurement from each 0.1mm line to the following 0.1mm line on the scale (from the outside of one line to the inside of the line on the calibration slide picture) on the calibration picture was drawn. The measurement (Ctrl + M) was then recorded. This was repeated ten times at various lines on the calibration slide. The measurement average was then taken in the “Results” table, and then in that menu bar: Results→Summarize provides the mean, SD, Min and Max. Since the average distance is desired, the mean was recorded, representing the average distance of all ten measurements in pixels. Under “Analyze” in the menu bar, “Set Scale” was selected. The mean length in pixels from the ten measurements from the results table was imputed into the “Set Scale” table as the “Distance in pixels.” The known distance was set as “0.1”, and the unit of length was set to “mm.” The global checkbox was selected. This applies the average distance in pixels from the ten measurements of the 0.1mm calibration scale to other photos taken under the same conditions. The following mean measurements for the 0.1mm calibration slide from 1x to 4x were 181.33, 374.25, 554.67, and 757.83 pixels/mm, respectively.

Appendix 8: Hex codes, RGB values and perceived lightness values of 2 bracteoles per sample in 25 *T. angustifolia*, 25 F1 *T. x glauca* and 22 non-F1s.

Sample ID	Taxon ID	Hex code	R	G	B	Lightness
7LBA1.1	A	9d755a	157	117	90	52.54089
7LBA1.2	A	a37c72	163	124	114	55.49903
7LBA2.1	A	c3925a	195	146	90	64.07828
7LBA2.2	A	b47f6d	180	127	109	58.02392

7LBA3.1	A	a27159	162	113	89	52.07708
7LBA3.2	A	bd8759	189	135	89	60.66498
7LBA9.1	A	ae7d52	174	125	82	56.36218
7LBA9.2	A	a4776a	164	119	106	54.16125
Craic4.1	A	b47f51	180	127	81	57.53641
Craic4.2	A	b98868	185	136	104	60.7128
FBA3.1	A	996f5a	153	111	90	50.54577
FBA3.2	A	8d716c	141	113	108	50.16327
FBA4.1	A	a37550	163	117	80	53.04599
FBA4.2	A	b17f4f	177	127	79	57.16336
FBA5.1	A	99766f	153	118	111	52.81447
FBA5.2	A	a5714b	165	113	75	52.1944
FBA6.1	A	a57950	165	121	80	54.2947
FBA6.2	A	9e714f	158	113	79	51.43215
FBA7.1	A	8e6e63	142	110	99	49.2569
FBA7.2	A	a07053	160	112	83	51.48085
FBA8.1	A	a07554	160	117	84	52.76982
FBA8.2	A	ac7e55	172	126	85	56.43479
Red3-1.1	A	bd8346	189	131	70	59.42846
Red3-1.2	A	b2815e	178	129	94	58.01202
Red3-2.1	A	b1876f	177	135	111	59.72827
Red3-2.2	A	94746a	148	116	106	51.64138
Red3-3.1	A	a37851	163	120	81	53.82862
Red3-3.2	A	80675c	128	103	92	45.71954
Red3-4.1	A	a47d5b	164	125	91	55.39541

Red3-4.2	A	946e4f	148	110	79	49.51099
Red3-5.1	A	b37c3c	179	124	60	56.41198
Red3-5.2	A	b08054	176	128	84	57.37482
Red3-6.1	A	aa816b	170	129	107	57.37439
Red3-6.2	A	aa8971	170	137	113	59.55264
Red3-7.1	A	a17c5d	161	124	93	54.84779
Red3-7.2	A	a87f60	168	127	96	56.43465
Reg3.1	A	a87b55	168	123	85	55.22305
Reg3.2	A	b48064	180	128	100	58.09561
Reg4.1	A	9f775a	159	119	90	53.27927
Reg4.2	A	b48257	180	130	87	58.37407
Reg5.1	A	b88153	184	129	83	58.52306
Reg5.2	A	9f6f4d	159	111	77	51.00813
Reg6.1	A	c2937c	194	147	124	64.79714
Reg6.2	A	c49885	196	152	133	66.43249
TVRA1.1	A	aa7754	170	119	84	54.42767
TVRA1.2	A	a67a6d	166	122	109	55.20618
TVRA3.1	A	8e6f5a	142	111	90	49.32324
TVRA3.2	A	8f6f57	143	111	87	49.3705
TVRA4.1	A	7c695a	124	105	90	45.80687
TVRA4.2	A	82685a	130	104	90	46.15658
Dav1.1	F1	d7b79d	215	183	157	76.52633
Dav1.2	F1	cda78e	205	167	142	71.26184
Dav2.1	F1	dfbfa1	223	191	161	79.34145
Dav2.2	F1	cfab91	207	171	145	72.51866

Dav3.1	F1	ddbb9c	221	187	156	78.05949
Dav3.2	F1	c79c78	199	156	120	67.48292
Dav4.1	F1	ca9c8a	202	156	138	68.1446
Dav4.2	F1	c39780	195	151	128	65.97679
Kin2-1.1	F1	dab498	218	180	152	75.95553
Kin2-1.2	F1	d9b796	217	183	150	76.57027
Kin2-2.1	F1	d6ac84	214	172	132	73.19842
Kin2-2.2	F1	d0ad92	208	172	146	72.88549
Kin2-3.1	F1	d9b79a	217	183	154	76.64979
Kin2-3.2	F1	ceac92	206	172	146	72.69536
Kin2-4.1	F1	c9976b	201	151	107	66.22259
Kin2-4.2	F1	cea98c	206	169	140	71.81968
Kin2-5.1	F1	d2a587	210	165	135	71.11922
Kin2-5.2	F1	cf9f84	207	159	132	69.27508
MJ2-1.1	F1	d4b29e	212	178	158	75.01781
MJ2-1.2	F1	d8baa8	216	186	168	77.60493
MJ2-2.1	F1	dec1a5	222	193	165	79.83308
MJ2-2.2	F1	e1bfa3	225	191	163	79.56456
MJ2-3.1	F1	dcbda9	220	189	169	78.73813
MJ2-3.2	F1	d8b390	216	179	144	75.35963
MJ2-4.1	F1	e3c9af	227	201	175	82.48578
MJ2-4.2	F1	d0a783	208	167	131	71.34135
Red4-1.1	F1	d7baa4	215	186	169	77.53693
Red4-1.2	F1	c4958a	196	149	138	65.79973
Red4-2.1	F1	cb9a86	203	154	134	67.67199

Red4-2.2	F1	ca9b84	202	155	132	67.77312
Red4-3.1	F1	d9c2b4	217	194	180	79.97136
Red4-3.2	F1	e7d6c3	231	214	195	86.51504
Red4-4.1	F1	d0ab92	208	171	146	72.63504
Red4-4.2	F1	d7b7a2	215	183	162	76.63215
Red4-5.1	F1	ccaa98	204	170	152	72.13068
Red4-5.2	F1	d1b2a1	209	178	161	74.80557
Reg1.1	F1	dbba9b	219	186	155	77.60526
Reg1.2	F1	dbbea7	219	190	167	78.85485
Reg2.1	F1	d6bba9	214	187	169	77.69835
Reg2.2	F1	dcc0a9	220	192	169	79.48816
Reg7.1	F1	d6b49d	214	180	157	75.68278
Reg7.2	F1	d6baa2	214	186	162	77.29434
Reg8.1	F1	d3b29c	211	178	156	74.88164
Reg8.2	F1	d0a797	208	167	151	71.74575
RR6.1	F1	ebcaac	235	202	172	83.38014
RR6.1.2	F1	d2a891	210	168	145	72.06191
RR8.1	F1	dbb393	219	179	147	75.70374
RR8.2	F1	e1c1ad	225	193	173	80.27209
RR9.1	F1	cea694	206	166	148	71.23642
RR9.2	F1	bc989a	188	152	154	66.1214
AVR13.1	Non-F1	b58556	181	133	86	59.22406
AVR13.2	Non-F1	9f7349	159	115	73	51.96522
BRDC3.1	Non-F1	a87e58	168	126	88	56.03882
BRDC3.2	Non-F1	a58269	165	130	105	57.06033

HEAW2.1	Non-F1	9f7452	159	116	82	52.3655
HEAW2.2	Non-F1	a27e63	162	126	99	55.58767
HSCAA18.1	Non-F1	a68165	166	129	101	56.82817
HSCAA18.2	Non-F1	aa7e5d	170	126	93	56.34526
ML14.1	Non-F1	816352	129	99	82	44.51841
ML14.2	Non-F1	ad816a	173	129	106	57.67972
ML5.1	Non-F1	9b734f	155	115	79	51.60412
ML5.2	Non-F1	a17756	161	119	86	53.43053
ML9.1	Non-F1	a3795a	163	121	90	54.238
ML9.2	Non-F1	98734a	152	115	74	51.18801
LL5.1	Non-F1	d5a578	213	165	120	71.15437
LL5.2	Non-F1	d4b4a5	212	180	165	75.67238
HOR2-2.1	Non-F1	d1af9a	209	175	154	73.89994
HOR2-2.2	Non-F1	c8a48b	200	164	139	69.96125
HOR2-4.1	Non-F1	c39470	195	148	112	64.92427
HOR2-4.2	Non-F1	cea990	206	169	144	71.90068
HOR2-5.1	Non-F1	d4a98d	212	169	141	72.42595
HOR2-5.2	Non-F1	e8d1b4	232	209	180	85.02295
KEN1.1	Non-F1	ce9f7e	206	159	126	69.05761
KEN1.2	Non-F1	cf9f84	207	159	132	69.27508
CC-602.1	Non-F1	ac7f5a	172	127	90	56.76975
CC-602.2	Non-F1	aa8479	170	132	121	58.44208
OSM-601.1	Non-F1	a37955	163	121	85	54.15108
OSM-601.2	Non-F1	ac7839	172	120	57	54.5525
OSM-701.1	Non-F1	986c4f	152	108	79	49.45367

OSM-701.2	Non-F1	a67748	166	119	72	53.78054
OSM-703.1	Non-F1	9f706c	159	112	108	51.88464
OSM-703.2	Non-F1	8b6760	139	103	96	47.01905
PLS-103.1	Non-F1	d7bba6	215	187	166	77.72177
PLS-103.2	Non-F1	d6ac87	214	172	135	73.25288
PLS-403.1	Non-F1	e0bfa1	224	191	161	79.43255
PLS-403.2	Non-F1	e1c7ad	225	199	173	81.76831
PLI-101.1	Non-F1	dbc4ad	219	196	173	80.4919
PLI-101.2	Non-F1	d8c2af	216	194	175	79.77158
PLI-502.1	Non-F1	b2804e	178	128	78	57.51452
PLI-502.2	Non-F1	a57652	165	118	82	53.56281
RLW-101.1	Non-F1	d8b9a6	216	185	166	77.31074
RLW-101.2	Non-F1	ddbfad	221	191	173	79.41471

Appendix 9: Supplementary methods for quantifying the colour of bracteoles.

Pictures of individual Calibrite ColorChecker Classic Mini (X-Rite) colour grid patches were taken under 3x magnification on a dissecting microscope. A Nikon D5000 DSLR camera was used for image acquisition. Images were captured using the following settings: shutter speed of 1/60s, aperture F13, ISO 200, and the colour profile was set to Adobe RGB**.** A Fiber-Lite Mi-150 Illuminator controlled image brightness. White balance was set to 'Fluorescent 3' to ensure accurate colour representation under fluorescent lighting conditions. Images were captured in RAW format (.NEF) to preserve maximum image data for post-processing. The resolution was set to 2848 x 4288 pixels. These settings were chosen to minimize motion blur and maintain optimal image quality in lighting conditions.

RAW images of the individual patches were converted to 8-bit TIFF images, and then all images were uploaded to Adobe Photoshop 25.2.0 (Adobe Inc., San Jose, CA).

The canvas size was set to a width of 13 inches and a height of 9 inches, the background colour was set to white, the resolution was set to 300pixels/ich, the Colour Mode was set to RGB Color, and the Color profile was set to Adobe RGB (1998). Individual patches were uploaded into the canvas by going into File → Scripts → Upload files to scripts. Individual patches were then reconstructed together (transformed into 2x2 inches) in the same placements as on the colour grid. Then, the images were saved as an 8-bit TIFF file.

Colour calibration was used to ensure consistency and accuracy in the representation of colours across devices and programs. The reconstructed ColorChecker was then uploaded into ImageJ. The calibration was performed using the IJP-Color plugin (https://github.com/ij-plugins/ijp-color) for free software ImageJ/FIJI (Schindelin et al., 2012). The ROI tool was used to mark the four corners of the reconstructed ColorChecker. Next, under Plugins→Color→IJP Color Calibrator was selected. The calibration was performed using reference colour values for the X-Rite Passport. The following settings were used for calibration: Reference was set to “XYZ.” Reference XYZ is better suited for camera raw images, and the raw CMOS sensor response is close to being linear to CIE XYZ colour. The mapping method was set to “Linear No-Intercept Cross-band” to avoid overfitting and using the lowest possible degrees of freedom. Each of the 24 colours was measured under the microscope using the same conditions for the samples. Test shots were taken before the primary data collection to confirm proper lighting conditions and exposure.

All sample photos were taken under the same conditions as the ColorChecker images. Sample photos were then calibrated in the Color Calibrator dialogue box, and under the “Apply to Another Image” batch apply, the folder where the samples were saved was selected. Another folder was created to output the calibrated sample photos.

In Adobe Photoshop, calibrated sample photos were uploaded. The lasso tool was used to outline the bracteole tip, and then Filter -> Blur -> Average was selected to get the average colour in the menu bar. Next, the eyedropper tool was selected to obtain the colour of that area. The hex code was then recorded. This was done on two bracteoles per sample on 25 *T. angustifolia*, F1 *T.* × *glauca* and 22 non-F1 hybrids.

Appendix 10: R-scripts for perceived lightness (Myndex, 2024).

```
#Perceived Brightness
df <- Colour[, -c(1, 2, 3)]
# Function to convert sRGB to linear RGB
sRGB_to_linear <- function(rgb) {
  linear_rgb <- rgb / 255
  linear_rgb <- ifelse(linear_rgb <= 0.04045, linear_rgb / 12.92, ((linear_rgb + 0.055) /
1.055)^2.4)
  return(linear_rgb)
}
# Function to calculate perceived lightness from linear RGB
rgb_to_lightness <- function(rgb) {
  Y <- 0.2126 * rgb[1] + 0.7152 * rgb[2] + 0.0722 * rgb[3]
  L <- ifelse(Y <= 0.008856, 903.3 * Y, 116 * Y^(1/3) - 16)
  return(L)
}
# Function to apply the transformation to each row of the dataframe
transform_df <- function(df) {
  df$Lightness <- apply(df[, c("R", "G", "B")], 1, function(rgb) {
    rgb_linear <- sRGB_to_linear(rgb)
    lightness <- rgb_to_lightness(rgb_linear)
    return(lightness)
  })
  return(df)
}
# Apply transformation
df_transformed <- transform_df(df)
print(df_transformed)
```