THESIS

DEVELOPMENT OF PCR-RFLP AND DNA BARCODING PLASTID MARKERS FOR YELLOW TOADFLAX AND DALMATIAN TOADFLAX

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ABSTRACT

DEVELOPMENT OF PCR-RFLP AND DNA BARCODING PLASTID MARKERS FOR YELLOW TOADFLAX AND DALMATIAN TOADFLAX

Yellow toadflax and Dalmatian toadflax are problematic invasive plant species in North America. Yellow toadflax was introduced multiple times to the United States from Europe, beginning in the late 1600s. Dalmatian toadflax has similarly been repeatedly introduced to the United States, starting in 1874. Both species are known to inhabit disturbed areas, competing for limiting resources with native plant species. Both are obligate outcrossed species, which allows them to maintain a high level of genetic diversity. Both species are known to inhabit a wide range of ecosystems.

Yellow toadflax and Dalmatian toadflax are difficult species to control with herbicide. The most effective herbicides currently available have a 61% - 95% control rate for Dalmatian toadflax and only a 35% - 69% control rate for yellow toadflax. Herbicides that can not achieve 100% control in a population may select for resistant individuals. Biocontrol agents, specifically *Mecinus janthinus,* have proven to be effective for controlling invasive toadflax, to some extent.

Hybridization between these two outcrossed species has occurred spontaneously under North American field conditions, and the resulting fertile hybrid progeny exhibit heterosis. Neither herbicide nor biocontrol agent effectiveness has been determined for these toadflax hybrids. Gene flow between these two species could cause introgression of advantageous traits, thus making either of these problematic species even more difficult to control. Plastid DNA PCR-RFLP and DNA barcoding markers were therefore developed to track this gene flow.

One PCR-RFLP marker (*trnT/D* digested with *Alu1*) and two DNA barcoding regions (*matK* and *trnL-F*) were discovered to distinguish between cpDNA haplotypes for yellow toadflax and Dalmatian toadflax. Testing on individual plants collected from multiple U.S. field hybridization sites has revealed that yellow toadflax chloroplast DNA occurs more frequently in hybrids than Dalmatian toadflax cytoplasm. These results indicate that gene flow is asymmetric in persistent *L. vulgaris* x *L. dalmatica* populations.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE

Yellow toadflax history and biology

Yellow toadflax (*Linaria vulgaris* Mill.) (ITIS 2013) is known by the common names "common snapdragon", "Jacob's Ladder", and "butter and eggs" (Saner et al. 1995, Lajeunesse 1999). Originally members of the Scrophulariaceae family, all *Linaria* species, including yellow toadflax, were moved with the rest of the tribe Antirrhineae to the Plantaginaceae family (The Angiosperm Phylogeny Group 2003, Fernández-Mazuecos et al. 2013).

Yellow toadflax is an herbaceous creeping perennial forb (Saner et al. 1995) originally introduced to the United States from the United Kingdom in the late 1600s as an ornamental and medicinal plant (Mitich 1993). Up to the early 1990s yellow toadflax could still be purchased under the name "butter and eggs" or "Jacob's ladder" at select nurseries, until it became listed as a noxious weed (Mitich 1993). Horticultural escapes have allowed yellow toadflax to spread and thrive throughout North America.

In the Middle Ages yellow toadflax was believed to have many medicinal properties. It was thought to cure throat ailments, cleanse the liver and spleen, and relieve swelling associated with hot buboes; more recently, yellow toadflax has been used as an astringent, detergent, and dye (Mitich 1993). In the United States during the 1600s, yellow toadflax was mixed with milk to poison flies (Haughton 1978).

Yellow toadflax leaves are 2.4-5.5 cm long, linear to narrow lanceolate, usually alternate, but can seem opposite due to crowding (Saner et al. 1995). Leaves are usually

pointed at both ends and blue to pale green (Lajeunesse 1999). Older leaves have a single vein visible on the underside (Wilson et al. 2005). Stems are usually 0.3-1.0 m high, pale green with tinge of silver, woody near the base, and somewhat branched towards the ends (Lajeunesse 1999). The chromosome number in yellow toadflax is reported as n =12 (Darlington and Wylie 1955, Tandon and Bali 1957).

Yellow toadflax can form creeping colonies via adventitious buds on horizontal roots (Charlton 1966). Vegetative reproduction can begin as early as 2-3 weeks after germination (Zilke 1954). Up to 90-100 secondary roots can be produced in the first year (Zilke 1954). Adventitious buds begin to emerge in March (Saner et al. 1995, Beck 2010) and are well protected from wildfires which allows for rapid post-fire regeneration (Wilson et al. 2005).

Yellow toadflax flowers form a terminal raceme. The flowers are yellow to orange tipped, complete, and resemble snapdragon flowers (Mitich 1993). Flowers are densely packed, with five sepals, corollas 2-3cm long with five petals, the upper portion two lobed, and lower portion 3 lobed (Saner et al. 1995). Flowers usually number between 6 and 30 blossoms per raceme (Wilson et al. 2005). Yellow toadflax is an insect pollinated obligate outcrosser (Arnold 1982, Saner et al. 1995, Stout et al. 2000) and is known to be self-incompatible due to a single locus causing gametophytic self-incompatibility (Docherty 1982). Yellow toadflax has on average 150-250 ovules per capsule, but only 0-47 seeds successfully develop from each capsule (Arnold 1982). Yellow toadflax plants at full reproductive potential can produce up to 20,000 seeds annually per plant (Wilson et al. 2005). Seeds are brown to black, reticulate and winged, which would suggest these seeds are wind dispersed; however, most yellow toadflax seeds are actually gravity dispersed (Nadeau et al. 1991, Saner et al. 1995). Seeds germinate early to mid-May

(Beck 2010). A study by Andersen (1968) found that less than 50% of seeds germinated, which could be due to high seed dormancy or low seed viability.

Yellow toadflax is thought to have originated from the steppes of south-eastern Europe to north-western Asia (Meusel et al. 1978, USDA PLANTS Database 2013). Since its introduction to the U.S. yellow toadflax has spread across the whole of North America except Nunavut (USDA PLANTS Database 2013). Yellow toadflax is listed as a noxious weed in Idaho, Montana, Nevada, New Mexico, Oregon, South Dakota, Washington, and Wyoming (USDA PLANTS Database 2013).

Yellow toadflax prefers sites with well-drained, disturbed, coarse soil in open areas (Wilson et al. 2005). A study by Sutton et al. (2007) found an increased probability for yellow toadflax invasion in the Colorado Flat Tops Wilderness Area in locations that had greater species richness, were close to trails, and were near current infestations of yellow toadflax. Yellow toadflax plants can tolerate a wide range of soil types from coarse gravels to sandy loams, and they have increased competitive ability in areas where the summers tend to be dry (Lajeunesse 1999). Wet and dark conditions are a limiting factor for growth (Zilke 1954). In North America, yellow toadflax normally occurs in gravelly to sandy roadsides, railroad yards, dry fields, gardens, pastures, cultivated fields, and waste areas (Saner et al. 1995).

Dalmatian toadflax history and biology

Dalmatian toadflax, *Linaria dalmatica* (L.) Mill. (ITIS 2013), is commonly known as "wild snapdragon", "broad leaved toadflax", and "Dalmatian toadflax" (Alex 1962, Vujnovic and Wein 1996). Dalmatian toadflax is a member of the Antirrhineae tribe in the Plantaginaceae family (The Angiosperm Phylogeny Group 2003); it is a robust, glaucous, perennial forb that spreads via horizontal rhizomes (Vujnovic and Wein 1996). Dalmatian toadflax has been under

cultivation in Europe since the mid to late 1600s as an ornamental, the earliest documented example of cultivation occurring in Italy in 1592 (Alex 1962). The native range of Dalmatian toadflax extends from northern Croatia and Serbia to southern Iran (Alex 1962). In its native range, Dalmatian toadflax existed in areas that were moderately grazed by multiple ungulate species (Lajeunesse 1999).

Dalmatian toadflax was introduced to the United States as an ornamental plant in 1874 (Alex 1962). Since its introduction, Dalmatian toadflax has spread across the continent, with infestations heaviest in the western United States and Canada (USDA PLANTS Database 2013).

There are two distinct subspecies of Dalmatian toadflax, *Linaria dalmatica* ssp. *dalmatica* (L.) Mill., and *Linaria dalmatica* ssp. *macedonica* (Griseb.) D.A. Sutton (ITIS 2013). The subspecies are primarily distinguished by leaf morphology. Many members of the genus *Linaria* can hybridize with one another (Bruun 1936), so it is unknown which *L. dalmatica* subspecies is invasive in North America (Wilson et al. 2005, Sing and Peterson 2011).The Dalmatian toadflax subspecies evaluated in this study is thought to be *Linaria dalmatica* ssp. *dalmatica.*

Dalmatian toadflax stems are annual, semi-woody, increasing in woodiness approaching the base; there are usually 1-20 stems per crown (De Clerck-Floate and Harris 2002) that are 40-100 cm tall, becoming increasingly branched with height (Vujnovic and Wein 1996). Leaves are 1.5-4.7 cm in length and 0.2-1.1 cm in width (Alex 1962), alternating, cordate in shape, and lacking a peduncle (Alex 1962, Vujnovic and Wein 1996). Lower leaves are lanceolate to ovate, while the upper leaves are linear lanceolate to broad lanceolate.

Dalmatian toadflax is known to spread via horizontal roots, which can penetrate up to 1.8 m vertically and spread to 3.6 m (De Clerck-Floate and Harris 2002). The roots are semi-woody, and produce adventitious buds (De Clerck-Floate and Harris 2002). Root-generated fertile

offshoots are usually produced in the autumn and number from 0-40 (Alex 1962). Adventitious buds can be produced from rhizomes as early as 9 weeks after germination, which give rise to independent daughter shoots (Lajeunesse 1999).

Flowers for Dalmatian toadflax are assembled in simple erect racemes, which are 15-55 cm long (Vujnovic and Wein 1996). Flowers are complete, yellow to orange, insect-pollinated, and look similar to snapdragon flowers (Alex 1962, Lajeunesse 1999). Unlike yellow toadflax flowers, Dalmatian toadflax flowers have a yellow throat (Wilson et al. 2005). Flowering usually begins in June and continues into September or October (Lajeunesse 1999). Dalmatian toadflax is known to be an obligate outcrosser (Bruun 1936). Dalmatian toadflax can produce up to 500,000 viable seeds annually per mature plant (Robocker 1974). Seeds usually germinate in the following spring, but can still be viable after a decade (Robocker 1970). Seeds are gravity dispersed (Alex 1962, Mitich 1993, Lajeunesse 1999).

The worst infestations of Dalmatian toadflax occur in the northwestern United States, Alberta, and British Columbia (Lajeunesse 1999). Broad-leaved Dalmatian toadflax is the more widely distributed of the two subspecies, but narrow-leaved Dalmatian toadflax has also spread to northwestern United States and British Columbia (Lajeunesse 1999). Dalmatian toadflax is classified as a noxious weed in Arizona, California, Colorado, Idaho, Montana, Nevada, New Mexico, North Dakota, Oregon, South Dakota, Washington, and Wyoming (USDA PLANTS Database 2013).

Dalmatian toadflax is known to tolerate a broad range of environmental conditions, which is part of the reason for its invasive success. In North America it occurs at latitudes between 33° to 55° N, and between 35° and 47° latitude in its Old World native range (Vujnovic and Wein 1996). It grows in sunny open rocky areas and up to 2800 m altitude. The species can invade a

variety of soils from sandy loam to coarse gravel (Alex 1962, Robocker 1970). Dalmatian toadflax is usually found in regions with well-drained soils and dry summers (Lajeunesse 1999).

Toadflax invasion and ecological impact

In a study by Nadeau et al. (1991), a 10 cm piece of yellow toadflax was able to increase to a patch 2 m in diameter in one growing season. Yellow toadflax can also effectively spread by seeds: a study by Nadeau et al. (1991) showed that up to 92% of seeds fall within 50 cm of the parent plant. These two studies illustrate how easily yellow toadflax density can increase in invaded areas through sexual and vegetative propagation. In most cases, Dalmatian toadflax spreads via sexual reproduction although the majority of the seeds fall near the parent plant (Jacobs and Sing 2006). Once established, yellow toadflax and Dalmatian toadflax can use vegetative expansion to overrun competitive native foliage and create a monoculture (Lajeunesse 1999). Rhizomes allow these two species to survive in harsh environments and cope with abiotic stresses such as herbicides and fire. Being obligate outcrossers, both species are genetically diverse, which helps them adapt to the variety of habitats they inhabitant (Vujnovic and Wein 1996). In early spring, adventitious buds begin to regenerate (Alex 1962, Vujnovic and Wein 1996), which are not dependent on soil moisture, unlike many native plants (Colorado Department of Agriculture 2008, Sing and Peterson 2011). Yellow toadflax and Dalmatian toadflax are known to invade perennial forage crops, annual crops, and summer fallow areas (Saner et al. 1995). Several studies have shown that Dalmatian toadflax contains iridoid glycosides and alkaloids (Handjieva et al. 1993, Ilieva et al. 1993, Jamieson and Bowers 2010). These iridoid glycosides are known to be mildly toxic to cattle, compounding problems associated with toadflax infestation (Mitich 1999). These secondary metabolites might not be lethal, but they can act as a grazing deterrent for livestock (Lajeunesse 1993). A risk assessment

conducted by Sing and Peterson (2011) reported large areas of uncertainty about the effects of Dalmatian toadflax and yellow toadflax on wildfire, erosion, human and animal toxicity. Further research is needed to fully grasp the total effect of these species on invaded ecosystems.

Toadflax Hybrids

Hybridization between two species has been suggested as a way to increase invasive adaptations that allow a species to successfully invade new regions (Ellstrand and Schierenbeck 2000). In plants, hybridization plays a major role in evolution, which potentially results in the development of new taxa (Grant 1981). Yellow toadflax and Dalmatian toadflax are obligate outcrossers, thus maintaining a high level of genetic diversity (Mitich 1993, Saner et al. 1995, Stout et al. 2000, Ward et al. 2008). In a study by Ward et al. (2008), results for individuals sampled from five different states and genotyped using ISSRs (inter simple sequence repeats) demonstrated that yellow toadflax populations sampled within the United States maintain a very high degree of genetic variation. It is likely that Dalmatian toadflax has a high degree of genetic variation as well. Genetic transfer of beneficial traits between these two species could increase their adaptability to new environments and expedite their range increase. Yellow toadflax and Dalmatian toadflax are already successful invaders, and hybrids between these two species could have greater adaptive capacity than either of the parents.

There are multiple examples of invasive species hybridizing and creating new taxa or biotypes that are an even greater threat than either of the invasive parents. The *Tamarix* genus is known to have 54 species that are capable of hybridizing (Ellstrand and Schierenbeck 2000, Whitcraft et al. 2007). *Tamarix* hybrids (*Tamarix chinensis* x *T. ramosissima)* occupy 470,000-600,000 ha in the United States, mostly in riparian areas. These hybrids are depleting water sources, altering hydrologic patterns and hindering use of riparian recreation areas (Zavaleta

2000, Gaskin and Kazmer 2009). *Spartina alterniflora* x *S. foliosa* hybrids have been an invasive problem in estuaries in California, specifically the San Francisco Bay (Ayres et al., 1999). *S. alterniflora* was introduced in the mid 1970s and is native to eastern USA. *S. alterniflora* x *S. foliosa* hybrids have a more aggressive growth and sexual reproduction pattern than either of the parents (Callaway and Josselyn 1992). If allowed to form dense enough patches these hybrids can alter the hydrologic tables, impede ship traffic, and trap silt which will increase the elevation of infested areas (Ayres et al., 1999). *S. alterniflora* hybridizing with native *Spartina* species created an invasive problem in mud-flats across the UK (Hubbard 1957, Goodman et al. 1969).

Plants with intermediate morphologies between yellow toadflax and Dalmatian toadflax have been characterized by Ward et al. (2009) in a study that determined these plants are hybrids between yellow toadflax and Dalmatian toadflax occurring spontaneously in the field. This study also reported that controlled greenhouse crosses with yellow toadflax as the female parent had a higher seed set (49.1%) than those crosses that used Dalmatian toadflax as the female (10.1%). These results suggest that hybrids with a yellow toadflax maternal parent may be more numerous in the field. If there is a higher percentage seed set of hybrids with yellow toadflax as the maternal parent, later generations could have an increased accumulation of yellow toadflax traits. Backcrossing into yellow toadflax populations could lead to introgression of Dalmatian toadflax traits. These results from Ward et al. (2009) may initially suggest that yellow toadflax maternal haplotype is favored over Dalmatian toadflax maternal haplotype, but these results do not show what could happen in later backcrossing generations. More research is needed to determine what hybrid maternal haplotypes predominate in the field.

In the field yellow toadflax and Dalmatian toadflax typically occupy two different microhabitats. Yellow toadflax prefers moist soils such as riparian areas, while Dalmatian

toadflax prefers sunny and drier areas (De Clerck-Floate and Richards 1997). Since both species reproduce via outcrossing and are insect pollinated, pollen could be transferred between species by strong flying pollinators, usually bumblebees. Some bumblebees have a nectar foraging range can be as far as 2 km from their nest (Castro et al. 2008). Since bumblebees are a shared pollinator, this would allow pollen transfer between species even if they inhabit different microhabitats in a given area.

Heterosis, also known as hybrid vigor, is defined as "superiority of the F1 generation over the parental generation for a specific trait" (Stuber et al. 1992). There are two major models used to describe heterosis. One is the dominance model, in which deleterious recessive alleles are suppressed by dominant alleles at a specific locus in the hybrid. The second model of heterosis is the overdominance model which can be expressed in two forms: true overdominance (hybrid vigor caused by interaction of different dominant alleles at the same locus) and pseudo-overdominance, interaction between recessive alleles that are in close association, but on different homologs (Stuber et al. 1992, Birchler et al. 2010). The increased growth rate and fecundity seen in the F1 hybrids between yellow toadflax and Dalmatian toadflax tested in common garden trials indicate heterosis (Turner et al. 2011) which could cause displacement of parental populations by the hybrids.

In many cases, hybridization between two species results in asymmetric gene flow where one species is more commonly the maternal parent (Tiffin et al. 2001). There are multiple pre- and post-zygotic factors that cause gene flow to be asymmetrical. Differences in flowering time and divergence of floral characters can limit when and if pollen of another species can attach to and germinate on the stigma (Levin 1971. Some pollen tubes may be unable to penetrate the ovule wall, which would inhibit zygote formation (Snow 1994). In previous studies, pDNA has

been used to track gene flow in interspecific hybridization (Cruzan et al. 1993, Wang and Schmidt 1994). In a study examining gene flow between hybridizing *Eucalyptus* species, *Eucalyptus aggregata* had a higher genetic contribution to a hybrid population than *E. rubida* (Field et al. 2011), which indicated asymmetric gene flow. In another study by Bacilieri et al. (1996), in a sympatric population of *Q. petraea* and *Q. robur* ovules of *Q. petraea* trees were fertilized by 'extreme' *Q. petraea* genotypes, indicating that asymmetric backcrosses were occurring.

Management of toadflax invasion

Yellow toadflax and Dalmatian toadflax have deep and expansive root systems allowing these species to withstand many different control methods (Saner et al. 1995). Each plant can produce up to 100 secondary roots per year, which makes many cultural methods of control ineffective (Zilke 1954).

Tillage can reduce toadflax growth, but due to their creeping horizontal roots, tillage would increase the spread of either species having an overall negative effect on their management (Nadeau et al. 1991). Physically removing the plant through root pulling can be effective if done on a regular basis for 5-6 years (Lajeunesse 1999), but given the tendency in yellow toadflax for creeping mat production (Saner et al. 1995), root pulling for yellow toadflax might be an ineffective means of control. Considering the time and monitoring that would be required, physical means of control may not be the preferred method.

For herbicide control of yellow toadflax and Dalmatian toadflax, chlorsulfuron (an acetolactate synthase inhibitor), picloram (an auxinic herbicide) or a mixture of the two is recommended (Colorado Dept. of Agriculture, 2008). 2,4-D + Dicamba is suggested for Dalmatian toadflax control applied during the pre-bloom period (Colorado Department of

Agriculture 2008). Chlorsulfuron is suggested for use on yellow toadflax in protected areas where collateral damage should be limited (Krick 2011). Spraying in the fall or during flowering gives the most consistent results and reapplications should be made as needed (Beck 2010). However this spraying regime only provides 61%-95% control for Dalmatian toadflax and 35%-69% control for yellow toadflax even if followed correctly. This study demonstrated that there is a high degree of variation in control between sites, and yellow toadflax was more difficult to control of the two species. If 100% control of these species using herbicides is not achieved, selection could occur for individuals with some level of resistance (Mortensen et al. 2012).

Biological control is controlling a pest species, in this case a weed, using a natural enemy such as an insect or pathogen that reduces target weed fitness through predation (for weeds, herbivory), parasitism, or disease. Plant herbivory can alter a plant on many levels from decreased weight, branching, and growth to reproductive losses in seed size, count and viability (Crawley 1989a, Crawley 1989b, Schat et al. 2011). *Brachypterolus pulicarius, Calophasia lunula, Eteobalea intermediella, Rhinusa antirrhini, Rhinusa neta, Rhinusa linariae* are all potential insect biocontrols for yellow and Dalmatian toadflax (Wilson et al. 2005). *Mecinus janthinus* is an elongate, ovular, dark metallic blue-black weevil (snout beetle) (Wilson et al. 2005). This stem mining weevil originates from central to southern Europe (Jeanneret and Schroeder 1992). Overwintered adults emerge in March to early April, feed on toadflax leaves and shoot meristems for 2-6 weeks, then begin mating and ovipositing into host stems (Schat et al. 2011). From May to June females chew small holes in host stems where they then deposit individual eggs (Wilson et al. 2005, Schat et al. 2011). Intra-stem mining or boring by feeding larvae results in stem wilting and flower suppression proportional to the intensity of *M. janthinus* infestation (Jeanneret and Schroeder 1992).

Mecinus janthinus has been identified as one of the most effective biocontrol agents for yellow and Dalmatian toadflax (Sing et al. 2005, Jacobs and Sing 2006). Van Hezewijk et al. (2010) found that Dalmatian toadflax stem height and quantity decreased significantly within treatment areas in the seven years following the release of *M. janthinus*. A shift in Dalmatian toadflax dominance was also observed in the study area with large monocultures of Dalmatian toadflax reduced over the study period to patchy clumps (Van Hezewijk et al. 2010).

Two biotypes of *M. janthinus* have recently been discovered, one associated with Dalmatian toadflax and the other with yellow toadflax (Toševski et al. 2011, Sing 2011). Production of a signature secondary chemical profile frequently determines how biocontrol agents recognize or accept host plants (Rosenthal and Janzen 1979). The effect of hybridization can produce secondary metabolites that closely resemble one of the parent taxa, are intermediate between those of the parent taxa, are over or under-expressed, absent, or represent a completely novel expression of phytochemical compounds (Cheng et al. 2011). This change in secondary compounds caused by hybridization could confuse species-specific feeders, which means biocontrol agents may not readily establish on toadflax hybrids. For *M. janthinus* to be an effective biocontrol agent, the genetic background of toadflax in a given population should be determined, so a land manager can decide which weevil biotype should be released. At the present moment there is no easy way to track the genetic background of either *Mecinus* biotype.

Using PCR-RFLP markers and pDNA barcoding to identify plastid haplotypes

Plastids are semi-autonomous organelles thought to be derived from free living organisms (Sagan 1967). Plastid DNA (pDNA) is known to divide separately and independently from nucleus DNA (ncDNA) via binary fission (Mereschkowsky 1905). Plastid genomes have gone

through genomic reduction DNA insertions into nuclear genomic DNA (Brennicke et al. 1993, Martin and Herrmann 1998, Race et al. 1999).

Plastid DNA is highly conserved and has a minimal size variation between angiosperms, from 120 kb to 160 kb (Palmer 1985). Initial research into plastid genome structure revealed that pDNA might be circular (Kolodner and Tewari 1975, Herrmann et al. 1975). But more recent research by Oldenburg and Bendich (2004) reveals that pDNA may be linear and branched in structure. More research needs to be performed to determine the structure of plastid DNA.

Most of the plastid genome is comprised of three major types of DNA sequence. First, the conserved open reading frame and genes involved in photosynthetic processes (Palmer 1985, Ravi et al. 2008). Second, the genes that encode the photosynthetic machinery (photosystem 1, photosystem 2, cytochrome b, etc.) (Palmer 1985, Ravi et al. 2008). Last are the genes that encode the RNA machinery: RNA polymerase, ribosomal RNA, and transfer RNA (Palmer 1985, Ravi et al. 2007). pDNA is known to insert itself into nuclear DNA, which can make inferring the origin of plastid related pseudogenes difficult (Arthofer et al. 2010).

In most angiosperms the pDNA is maternally inherited (Neale and Sederoff 1989, Szmidt et al. 1987). In gymnosperms the plastids are usually paternally inherited, except for Gnetophyta, Cycadophyta , and Ginkgophyta which are all thought to be maternally inherited (Chamberlain 1935, Swamy 1948, Moussel 1978, Neale and Sederoff 1989). Some angiosperms are known to have bipaternal (e.g. *Epilobium hirsutum, Medicago sativa* and *Nepeta cataria),* or paternal inheritance (e.g. *Passiflora edulis,* and *Plumbago auriculata)* (Corriveau and Coleman 1988, Reboud and Zeyl 1994). For this study, it was assumed that yellow toadflax and Dalmatian toadflax cytoplasm is maternally inherited since there are no studies that show the contrary. In the cases of maternal inheritance, plastids are removed from the male gametes during either male

gamete development or fertilization (Sears 1980). The combination of maternal inheritance and highly conserved DNA makes pDNA a better marker system to study hybridization gene flow in the field than ncDNA (Ravi et al. 2007).

Plastid DNA is highly conserved for two reasons. The first is its mode of inheritance, which allows the cytoplasm to be inherited in a uniparental fashion. The second reason is that a large percentage of any plastid genome is made up of coding DNA which has a high degree of conservation due to the need to retain its cellular functionality (Palmer 1985, Ravi et al. 2007). However, there are still tandem repeat regions, single nucleotide polymorphism, and other pDNA regions that have increased mutation rates. These high mutation regions can be utilized as species diagnostic markers by monitoring species-specific polymorphisms. There are several known types of plastid markers: SSRs (Panaud et al. 1996), ISSRs (Inter Simple Sequence Repeat) (Mahmudul and Bian 2010), AFLPs (Elderkin et al. 2004), SNPs (Martínez-Arias et al. 2001, Thorisson et al. 2005), PCR-RFLPs (Restriction Fragment Length Polymorphism) (Dahouk et al. 2005) and DNA barcoding (Kress et al. 2005).

The plastid genome was chosen as the target for marker development for this study rather than the mitochondrial genome (mtDNA) or nuclear genome (ncDNA) for several reasons. First, mtDNA has a very low rate of point mutations, thus it has a low rate of base substitution accumulation, like pDNA, but mtDNA has a high rate of internal rearrangements, such as inversions (Palmer and Herbon 1988). Second, pDNA has a uniparental maternal mode of inheritance. Because of its uniparental inheritance, pDNA can be used to track plastid inheritance. Nuclear DNA is known to undergo recombination and is biparentally inherited in sexually reproducing outcrossing plants, which causes a decrease in the estimates for population differentiation and subdivision as compared to mtDNA and pDNA (Corriveau and Coleman

1988, Birky et al. 1989, Birky 1995, Petit et al. 2005). Both Dalmatian and yellow toadflax are obligate outcrossers, so these species maintain a high degree of genetic diversity. Since yellow toadflax is known to have a high degree of genetic variation (Ward et al. 2008), using ncDNA as a marker system would make tracking hybrid haplotypes very difficult. But pDNA can bypass this problem since it is inherited maternally. The slower rate of mutation and uniparental inheritance in pDNA make this marker system an excellent choice to tease apart interspecific variation, but the pDNA genome mutates too slowly to work well in to helping determine intraspecifc variation.

PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) utilizes conventional PCR techniques to amplify a large amount of specific DNA sequence to be digested with restriction endonucleases into smaller polymorphic fragments (Zhu et al. 2011). The subsequent digested fragments are visualized on agarose by gel electrophoresis making distinct DNA polymorphisms more apparent (Dahouk et al. 2005). These distinct DNA polymorphisms have arisen by spontaneous mutations, which are created due to the inherent error rates of DNA replication (Lewin 2006). Point mutations and indels (insertions and deletions) can occur naturally through mistakes in the DNA replication process (Lewin 2006). PCR-RFLP can take advantage of these DNA polymorphism by visualizing them as markers for differentiation between different genotypes. PCR-RFLP markers are able to not only distinguish between two closely related species (Poczai et al. 2011, Strydom et al. 2011), but also to determine plastid inheritance in hybrids between two closely related species (Arnold et al. 1991, Hollingsworth et al. 1998).

DNA barcoding also utilizes conventional PCR techniques and sequencing to visualize nucleotide polymorphisms (SNPs and indels) (Newmaster and Ragupathy 2009). In one of the

first studies using DNA barcoding, variation in the *cox1* mitochondrial gene was used to identify species of Lepidoptera (Brown et al. 1999). DNA barcoding determines nucleotide polymorphisms by DNA sequence alone, not by restriction digest patterns. This is one of the major advantages for DNA barcoding over PCR-RFLP. By using the DNA sequence itself as the comparison, rather than a restriction cutting pattern, one may have a higher opportunity to find a larger number of unique species-specific identifiers, since one would be comparing polymorphisms in an entire DNA sequence rather than a short 4-20 bp restriction site. DNA barcodes can be scored based upon species-specific conserved nucleotide regions rather than restriction cutting pattern. The use of DNA barcoding as species diagnostic markers has been demonstrated in multiple studies (Kress et al. 2005, Kress and Erickson 2007, Van De Wiel et al. 2009, Bruni et al. 2010). In a study by Van De Wiel et al. (2009), *trnH/psbA* was used as a DNA barcode region to delineate *Hydrocotyle ranunculoides* from closely related congeners. In a study by Newmaster and Ragupathy (2009), *rbcL*, *matK* and *trnH-psbA* DNA barcoding regions were used to distinguish between the genera *Vachellia* and *Acacia*. With DNA sequencing becoming more accessible and affordable each day, DNA barcodes could be more useful to the scientific community than PCR-RFLPs. DNA barcoding has multiple uses such as identifying contaminants in food products or determining if products are from endangered species (Hebert et al. 2003, Hollingsworth et al. 2011). However both PCR-RFLP and DNA barcoding can yield similar results (Bertin et al. 2010).

DNA barcodes can be used in two very different ways. One method is to use these character-based barcodes as a species diagnostic tool (Kress et al. 2005, Hollingsworth et al. 2011), which has been demonstrated to be an effective method for species identification and discrimination (Kelly et al. 2007, Rach et al. 2008, Bergmann et al. 2009). In a study by Bruni et

al. (2010), 50 different land plant were identified using two nuclear regions (*sqd1* and *At103*) and three plastid regions (*matK , trnH/psbA*, and *rpoB*) by character-based methods. DNA barcodes can also be used with distance-based methodologies to delineate phylogeny and make species discovery (Chase et al. 2005, Taylor and Harris 2012). In a study by Ren et al. (2010), 23 out of the 26 known *Alnus* species were identified using *rbcL*, *matK*, *trnH*/*psbA* and internal transcribed spacer (ITS) sequences by distance-based methods. However, a problem with distance-based studies is that they assume that intraspecfic variation is less than interspecific variation, which may not always be the case (Meyer and Paulay 2005). For this study, DNA barcoding was used as a character-based method to distinguish between two closely related species by using multiple pDNA regions.

DNA barcoding has many more issues as a marker system in plants than in animals. In animals, a universal primer has been established that amplifies the mitochondrial gene *cox1* (Hebert et al. 2003, Smith et al. 2005). However, the plant community has not established a specific primer region that can be used across all taxa, a problem that must be resolved to increase the utility of DNA barcoding to plant researchers (Chase et al. 2005). There is also a bias in the DNA barcoding community towards animals, specifically invertebrates (Taylor and Harris 2012). In a recent review Taylor and Harris (2012) noted that 41 different loci have been investigated in attempts to identify a universal plant based marker. In a study by Hollingsworth et al. (2009), *rbcL* and *matK* were suggested as core regions for DNA barcoding in plants. However, these regions are still under review (Hollingsworth et al. 2011). DNA barcoding is becoming a global standard in species identification (Chase et al. 2005, Hollingsworth et al. 2011) and is widely used due to its accuracy and precision in species identification (Botti and Giuffra 2010). However, DNA barcoding relies on the purity of DNA molecules and the

availability of sequencing facilities. In many cases DNA is collected from fresh tissues and preserved, so DNA quality usually is not an issue with using DNA barcodes. Another issue is a lack of universal standardized workflow and analysis, making repetition of experiments difficult (Chase et al. 2005, Hollingsworth et al. 2011). These major problems need to be addressed by the DNA barcoding community for this technique to reach its full potential.

SNPs (Single Nucleotide Polymorphisms) are one of the many polymorphic features that form DNA barcodes. SNPs consist of single base changes in a DNA sequence. SNPs given their nature occur rather frequently, in the human genome, there is an SNP on average every 100-300 bps (Thorisson et al. 2005). SNP sites are usually diallelic if not triallelic, so these SNPs will usually have two preferred nucleic forms (Martínez-Arias et al. 2001).

For this study DNA barcoding and PCR-RFLP markers were chosen to track the inheritance of pDNA in yellow toadflax and Dalmatian toadflax hybrids. AFLP is an extremely sensitive technique for evaluating polymorphism at the genomic level (Meudt and Clarke 2007), but for this study pDNA was the target for the marker system not nuclear DNA. RAPDs amplify random fragments of DNA, which might or might not include plastid DNA (William et al. 1990). ISSR amplifies regions using SSRs as primers, which means no prior DNA sequence knowledge of the species is required (Mahmudul and Bian 2010). ISSRs and AFLPs work best with large variable lengths of DNA (Elderkin et al. 2004), but since this study worked with pDNA which is highly conserved and short these marker systems are not the best choice. SSR primers anneal to regions flanking short sequence repeats so the SSR itself is amplified. Plastid SSRs have been used in previous studies as species diagnostic markers in plants (Grassi et al. 2003, Zeinalabedini et al. 2008). SSRs could have been used as diagnostic system for this study, but no previous sequencing has been performed on *Linaria* so potential primer sequences were unavailable.

Utilizing nucleotide polymorphisms, PCR-RFLP pDNA markers can be used to identify pDNA lineage within a hybridizing population. Due to maternal inheritance, plastids will be transferred through each subsequent generation from the maternal parent (Szmidt et al. 1987, Neale and Sederoff 1989). However it is important to note that pDNA markers can only track the plastid lineage. These markers cannot be used to infer any ncDNA inheritance patterns, since they cannot determine the paternal parent in a cross. The goal of this research was to develop species-specific pDNA markers for yellow toadflax and Dalmatian toadflax, then to test toadflax hybrids in the field to determine the gene flow for pDNA of hybridizing toadflax populations.

CHAPTER 2: DEVELOPMENT OF PLASTID PCR-RFLP MARKERS TO DISTINGUISH BETWEEN YELLOW TOADFLAX AND DALMATIAN TOADFLAX pDNA HAPLOTYPES

Introduction

For this study a PCR-RFLP (polymerase chain reaction restriction fragment length polymorphism) marker system was developed to distinguish between Dalmatian toadflax and yellow toadflax pDNA. PCR-RFLP uses the polymerase chain reaction to amplify selected DNA regions, which are then digested by restriction enzymes and visualized on an agarose gel with a DNA staining agent (Neale and Sederoff 1989, Szmidt et al. 1987). PCR-RFLP markers for different pDNA regions have been shown in multiple studies to be a reliable method for identification of plant species (Parani et al. 2000, Pharmawati et al. 2004). It is a repeatable method that is not as costly as DNA sequencing (Haider et al. 2012). The materials and equipment that are required for PCR-RFLP are in most biotechnology or molecular biology laboratories. Previous studies have shown that PCR-RFLP can be used to determine the maternal parents of hybrids in interspecific crosses between *Fallopia japonica* and *F. sacchalinensis,* and between *Iris fulva, I. hexagona,* and *I. nelsonii* (Arnold et. al. 1991, Hollingsworth et al. 1998). The objective of this study was to create species-diagnostic PCR-RFLP based markers that can distinguish between yellow toadflax pDNA haplotypes and Dalmatian toadflax pDNA haplotypes.

For this study, candidate pDNA marker regions were selected based on their PIC (polymorphic information content), which are the total number of nucleotide substitutions, indels, and inversions compared between two ingroup species and an outgroup species, scored according to Shaw et al. (2005). Only pDNA regions with a high PIC score were chosen for further study: *trnL c-d, trnT/D, rpS16, trnsugA-trnfmcaU,* and *59rpS12-rpL20.* These chosen

regions were sequenced in Dalmatian toadflax and yellow toadflax individuals sampled from taxonomically unambiguous populations where no hybridization could occur due to geographic isolation from the other species.

Methods and Materials

DNA was extracted from 217 individuals (Tables 2 and 3) using a DNeasy DNA mini prep kit from Qiagen. Extracted DNA was amplified using PCR. Primer sequences are given in Table 1. The reaction reagents were as follows: 10 ul Promega Gotaq Reaction Buffer, 0.5 mM dNTP's, 0.4 uM each of upstream and downstream primers, and 0.25 Promega Gotaq DNA Polymerase. PCR was performed in a Bio-Rad C1000 thermocycler. PCR parameters for the different amplified regions were as follows:

trnL c-d: 95ºC for 10 min., then 30 cycles of 95ºC for 1 min., 53ºC for 30 sec., then 72ºC for 45 sec. followed by a final annealing step at 72ºC for 10 min.

trnT/D: 80ºC for 5 min., then 30 cycles of 94ºC for 45 sec., then 52-58ºC for 30 sec., and 72ºC for 1 min., followed by a final annealing step at 72ºC for 5 min.

rpS16: 80ºC for 5 min., then 35 cycles of 94ºC for 30 sec., then 50–55ºC for 30 sec., and 72ºC for 1 min., followed by a final annealing step at 72ºC for 5 min.

trnsugA-trnfmcaU: 80ºC 5 min., then 30 cycles of 94ºC for 30 sec., then 55ºC for 30 sec., and 72ºC for 2 min., followed by a final annealing step at 72ºC for 5 min.

rpL20: 96ºC for 5 min., then 35 cycles of 96ºC for 1 min., then 50–55ºC for 1 min., and 72ºC for 1 min., followed by a final annealing step at 72ºC for 5 min.

Amplified DNA was run on a 1% agarose gel and visualized using ethidium bromide (EtBr) to determine quality of amplification. Amplified regions were then short run sequenced

using an ABI 3130xL Genetic Analyzer with BigDye® Terminator v3.1. Serial Cloner 2.1 software, which compares the desired sequence against an extensive list of restriction enzymes, was used to analyze DNA sequences for possible unique restriction sites. Separate restriction digests using the enzymes Alu1, Fok1, and Sau1, were performed for 3 hours at 37ºC. Digested DNA fragments were separated and visualized using EtBr on a 3% agarose gel to determine restriction pattern. Pearson's chi-squared goodness of fit test, $\chi^2 = (O-E)^2/E$, was used to test deviation from a 1:1 ratio for yellow toadflax to Dalmatian toadflax pDNA within hybrid populations (Fisher and Yates 1963).

Table 1. List of Primers.

PLASTID REGION AMPLIFIED	PRIMER SEQUENCE	PRIMER SOURCE
trnL c	*CGA AAT CGG TAG ACG CTA CG*	*Taberlet et al. 1991*
trnL d	*GCG GAT AGA GGG ACT TGA AC*	*Taberlet et al. 1991*
trnD	*ACC AAT TGA ACT ACA ATC CC*	*Shaw et al. 2005*
trnT	*CTA CCA CTG AGT TAA AAG GG*	*Shaw et al. 2005*
rpS16F	*AAA CGA TGT GGT ARA AAG CAA C*	*Shaw et al. 2005*
rpS16R	*AAC ATC WAT TGC AAS GAT TCG ATA*	*Shaw et al. 2005*
trnsugA	*GAG AGA GAG GGA TTC GAA CC*	*Shaw et al. 2005*
trnfmcaU	*CAT AAC CTT GAG GTC ACG GG*	*Shaw et al. 2005*
59rpS12	*ATT AGA AAN RCA AGA CAG CCA AT*	*Shaw et al. 2005*
rpL20	*CGY YAY CGA GCT ATA TAT CC*	*Shaw et al. 2005*

Table 2. Location of sampled toadflax populations.

Accession	Location	Latitude	Longitude
DT/ YT HT	Horsetooth Reservoir Fort Collins CO	40.5	-105.2
YT FT	Flat Tops Wilderness Area, CO	39.55005	-105.782
YT AK	Fairbanks, AK	64.8396	-147.788
YT PC	Pine Creek, MT	45.06431	-110.581
DT LSP	Lory State Park, CO	40.5755	-105.189
B or LB	Boulder, MT	46.23	-112.12
R	Radersburg, MT	46.19576	-111.631
DT MT	Missoula, MT	46.85456	-113.953
YT MT	Ovando, MT	47.02193	-113.13
DT Helena	Helena, MT	46.59733	-112.022
DT LM	Lee Martinez Park, Fort Collins, CO	40.59629	-105.082
DT EH	Elkhorn Mountains, MT	46.27389	-111.94
YT Alta WY	Alta, WY	43.75374	-111.037
YT SD	Leola, SD	45.67613	-99.1429
YT ND	Burlington, ND	48.293	-101.501
ID or P	Palisades, ID	43.4005	-111.209
DT Cherry	Fort Collins, CO	40.5	-105
Abbey	Red Feather, CO	40.802	-105.58

Results

The *trnT/D* plastid region was the only DNA region out of the five tested that was informative in identifying species-diagnostic plastid haplotypes. Plastid regions *trnL c-d, rpS16, trnsugA-trnfmcaU,* and *59rpS12-rpL20* were not good candidates for developing species-diagnostic PCR-RFLP markers. In order for a region to be considered informative it needed to give a consistent RFLP banding pattern for the same species, and the banding pattern had to be easily distinguishable between yellow toadflax and Dalmatian toadflax, i.e. the resulting DNA fragments of yellow toadflax and Dalmatian toadflax had to be visually unique when compared to one another. The region to be amplified needed to be consistent, meaning that only one specific genomic region of the plastid is amplified, not multiple regions that could confound results by giving false positives. After sequencing and analyzing these regions for possible unique restriction banding patterns, four out of five regions were found to not possess the DNA polymorphisms required as informative species-diagnostic markers. The *trnL c-d* region remained undigested with Fok1 and Sau1, so this region and enzyme combinations were not useful markers. After sequencing and analysis, *rpS16, trnsugA-trnfmcaU,*and *59rpS12-rpL20,* contained too little interspecific variation to be useful as species-specific markers. The variation displayed between *rpS16, trnsugA-trnfmcaU,* and *59rpS12-rpL20* was insufficient to accurately distinguish between the yellow toadflax and Dalmatian toadflax haplotypes.

The sequenced *trnT/D* region was ~865 bp for the yellow toadflax individual and ~884 bp for the Dalmatian toadflax. After an initial screening of the *trnT/D* regions with Serial Cloner 2.1, four possible candidate endonuclease restriction enzymes, Alu1, Acc1, Sal1 and Fok1, were tested on amplified pDNA from 39 yellow toadflax and 33 Dalmatian toadflax plants considered taxonomically unambiguous based upon morphology and their location in discrete populations

that were geographically isolated from the other species. After performing restriction digests and agarose gel analysis, only *trnT/D* region digested with Alu1 met all the species-diagnostic criteria listed above. Acc1 and Fok1 did not give a unique digestion pattern for the *trnT/D* regions. After Sal1 failed to digest the *trnT/D* region, the regions were resequenced and the Sal1 digestion sites were found to be no longer present on *in silico* digestion. After digestion with Alu1, the *trn*T/D region in yellow toadflax had two bands: 748 bp and 117 bp (see Figures 5 and 6). The *trn*T/D region in Dalmatian toadflax had 4 restriction bands after digestion with Alu1: 608, 137,137, and 2 bp (see Figures 4 and 7).

A total of 217 individuals were tested with the Alu1 marker (see Table 3). Of the 217 total individuals, the *trnT/D* pDNA region failed to amplify in 13 individuals, most likely due to poor quality of extracted DNA. Of the 204 individuals that amplified, 39 were considered unambiguous yellow toadflax and 33 were considered unambiguous Dalmatian toadflax, based on their source populations as described above. The remaining 132 individuals were either hybrids generated by controlled greenhouse crosses or plants collected from hybrid sites in the field, which were confirmed as hybrids using ISSRs as described in Ward et al. (2009). Plastid DNA from all of the 39 unambiguous yellow toadflax plants gave the expected yellow toadflax Alu1 restriction banding pattern (haplotype) with fragments of 748 bp and 117 bp. The 12 Dalmatian toadflax plants that were collected from Lory State Park, CO and Cherry Street, Fort Collins, CO displayed the yellow toadflax pDNA haplotype and the other 21 Dalmatian toadflax individuals displayed the Dalmatian toadflax pDNA haplotype of 608 bp, 137 bp, 137 bp, and 2 bp (see Table 1 and Figures 8-11). All unambiguous Dalmatian toadflax accessions that were sampled from Helena MT, Elkhorn Mountains, MT, Missoula, MT and Lee Martinez Park Fort Collins, CO displayed the Dalmatian toadflax pDNA haplotype. The known 20 F1 yellow by

Dalmatian toadflax hybrids gave the yellow toadflax pDNA haplotype. Nine out of the ten of the Dalmatian by yellow toadflax F1 hybrids created by greenhouse crosses gave a yellow pDNA haplotype. All the field collected hybrids from Palisades, ID displayed the yellow toadflax pDNA haplotype. The Radersburg, MT, Abbey, CO and Boulder, MT field collected hybrid populations included in this study contained both yellow toadflax and Dalmatian toadflax haplotypes. Results are summarized in Table 3.

Discussion

The *trnT/D* region digested with the Alu1 marker was found to be a reliable species diagnostic marker for both yellow toadflax and Dalmatian toadflax individuals. The yellow toadflax pDNA haplotype was detected in 100% of the 39 yellow toadflax individuals tested that were considered taxonomically unambiguous based on morphology and location. However, the Dalmatian toadflax haplotype was detected in only 20 of the 33 unambiguous Dalmatian toadflax individuals tested. Dalmatian toadflax plants collected from Lee Martinez Park, CO, and across Montana gave a consistent Dalmatian pDNA haplotype. The two Dalmatian toadflax plants collected at Cherry St. Fort Collins, CO displayed a yellow toadflax pDNA haplotype, which means these are most likely hybrids. All samples collected from a presumed Dalmatian toadflax population in Lory State Park, CO displayed yellow toadflax pDNA. Hybridization between species has occurred within this location, although no known yellow toadflax currently inhabits Lory State Park. Most likely all the individuals occurring within Lory State Park originated from backcrossed individuals. If the Cherry St., Fort Collins and the Lory State Park individuals are excluded from the determination of marker accuracy, the *trnT/D* region is 100% accurate as a pDNA diagnostic marker.

Previous studies by Ward et al. (2009) found that interspecific hybridization occurred at a higher rate with a yellow toadflax maternal parent. This is partially confirmed by hybrids collected and analyzed from the Palisades, ID site. All 25 hybrids tested from this site had identical yellow toadflax pDNA haplotypes ($p = 0.0004$). This means that either hybrids with yellow toadflax pDNA are favored in this area or there were no Dalmatian maternal parents. It could also mean that the entire Palisades population was derived from a very small amount of introduced hybrid seed, or even a single hybridization event. Upon further inspection of this site, no yellow toadflax or Dalmatian toadflax plants could be found (personal observation by Sing). This could mean that the hybrids at this site have displaced the parental species.

Only 2 (DY F1 15 and DY F1 07) of the 10 Dalmatian by yellow toadflax hand-crossed hybrids had the expected Dalmatian toadflax pDNA. DY 4 and DY 6 were offspring from crosses made using the same Dalmatian toadflax female parent from Lory State Park. DY 13 and DY 14 were crosses made with another Dalmatian toadflax female parent that also originated from Lory State Park. The same is true for DY 09, but crossed with a different yellow toadflax male parent. As described earlier, all presumed Dalmatian toadflax individuals tested from Lory State Park displayed yellow toadflax pDNA which was passed on to their progeny when these plants were used as female parents. The Dalmatian toadflax at Lory State Park may in fact be a hybrid population since it possesses yellow toadflax pDNA, but morphologically resembles Dalmatian toadflax. DY 1, DY 5, and DY 11 all had the same presumed Dalmatian toadflax female parent that came from a meadow at Cherokee Park Wildlife Area near Livermore, CO. This parent was observed to be unusually large and vigorous looking plant, and it was also the only toadflax plant in the area (Barry Ogg, personal communication). There was no tissue left from this parent to test to determine its cytoplasm. However, the observation that this parent

looked so vigorous leads one to believe it could have been a hybrid. In all of the above sampled plants, the presence of yellow toadflax pDNA is explained by their Dalmatian toadflax lineage.

Four out of 15 of the Boulder, MT hybrid accessions possessed Dalmatian toadflax pDNA ($p=0.2012$); this is not a significant difference between yellow toadflax and Dalmatian toadflax pDNA. This hybridization zone has a higher percentage of individuals with yellow toadflax cytoplasm, but a lower percentage of yellow toadflax pDNA haplotypes than the Palisades, ID site. This could be due to a higher percentage of Dalmatian individuals occurring at these sites to be maternal parents than in Palisades (personal observation by Ward and Sing). However, it is worth noting that at no one hybrid site tested was there a higher occurrence of Dalmatian pDNA haplotype than yellow pDNA haplotype. However, one of the two hybrid individuals collected from the Abbey, CO site had Dalmatian maternal pDNA. This shows that both the yellow toadflax and the Dalmatian toadflax haplotypes occur at the Abbey site.

Thirteen of the twenty collected individuals from Radersburg, MT, site displayed a yellow toadflax maternal background ($p = 0.3428$); this is not a significant difference between yellow toadflax and Dalmatian toadflax pDNA. It was noted from observations in the field, that there were a larger proportion of Dalmatian toadflax plants occurring at this site than yellow toadflax. This partially confirms findings from Ward et al. (2009) that Dalmatian toadflax can act as the maternal parent in hybrid crosses, but at a much lower frequency than yellow toadflax. Results from this location did not show a lower proportion of hybrids with Dalmatian toadflax plasmid DNA, but the sample size used in this experiment could have been too small to determine if the difference in number of yellow toadflax and Dalmatian toadflax is statistically significant.

At the Radersburg site, yellow toadflax occurs in a gulley that has no standing water, but excess water would drain through this gulley during times of precipitation. Dalmatian toadflax occurs strictly at the rocky slopes overlooking this gulley. The hybrids occur in the intermediate zone between the yellow toadflax and Dalmatian toadflax (see Figures 1 and 2). Yellow toadflax and Dalmatian toadflax seed can be moved a small distance by wind, but they are mostly gravity dispersed (Alex 1962, Saner et al. 1995, Lajeunesse 1999). The floor of this site is partially covered in low lying grass and forbs, so there is little to no wind at ground level to move these seeds into the hybrid zone. Radersburg is highly disturbed site due to over a century of mining activity (Davidson 2011). Many of the hybrids in the intermediate zone have a yellow pDNA haplotype, which means this seed is being moved uphill to reach the hybrid zone. This uphill seed movement would be difficult, since most toadflax seed falls in close proximity to the parental plant (Nadeau et al. 1991). It seems that this hybrid seed is being transported by some means other than wind dispersal. Further study needs to be done on yellow toadflax and Dalmatian toadflax to determine if mammals or birds could be possible vectors for seed dispersal.

For the five hybrid sites tested, only one, Palisades, ID ($p = 0.0004$), displayed a significant difference in the proportion of hybrids with yellow toadflax pDNA being the more commonly occurring haplotype. This suggests that there is asymmetric pDNA gene flow occurring in the field, but this asymmetry is site specific. Not every site will display the same asymmetry that occurred at Palisades, ID due to environmental variation from site to site. Since only four hybrid populations were sampled in this study it is difficult to make an all-encompassing conclusion about pDNA gene flow occurring in the field, especially due to the wide range of habitats in which these two species and their hybrids can occur.

Figure 1. Image of Radersburg, MT site.
Photograph taken by Ward and Sing 2008.

The Boulder, MT site landscape is very similar to that of Radersburg, MT. It is a highly disturbed site due to small claims mining, livestock and wildlife grazing, and continuous maintenance of a U.S. Forest Service road (personal observation by Ward). The site is a sloped hill, which starts at a rocky ridge and leads down into a ditch (see Figure 3). Dalmatian toadflax plants are located on the ridge and the yellow toadflax plants are located around the ditch. The hybrids predominantly occur in a zone between the two parent areas. The relative proportions of yellow toadflax and Dalmatian toadflax haplotypes for both the Radersburg and the Boulder sites were similar. At both sites there was a higher proportion of yellow toadflax cytoplasm in the hybrids, although this difference was not statistically significant.

Figure 2. Image of Radersburg, MT site, looking at intermediate hybrid zone. Photograph taken by Sharlene Sing 2008.

```
<Serial Cloner V2.1> -- <Tue, Mar 01, 2011   12:38 PM>

Restriction analysis of 01,07,2010DTm7trnT.xdna [Linear]
Incubated with AluI

4 fragments generated.

   1:      608 bp  -  From AluI[139]     To  AluI[747]

   2:      137 bp  -  From AluI[747]     To  ---

   3:      137 bp  -  From AluI[2]       To  AluI[139]

   4:        2 bp  -  From ---           To  AluI[2]
```

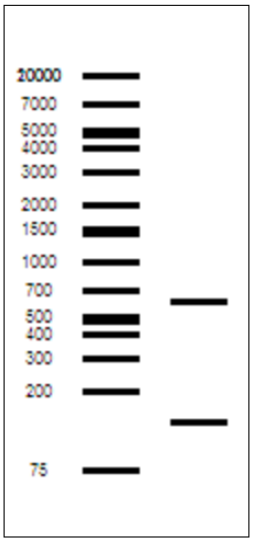

Figure 3. in silico Alu1 digestion of the trnT/D plastid region of Dalmatian toadflax using Serial Cloner 2.0.

```
<Serial Cloner V2.1> -- <Tue, Mar 01, 2011   12:40 PM>

Restriction analysis of 01,07,2010YT01trnT.xdna [Linear]
Incubated with AluI

2 fragments generated.

   1:      748 bp  -  From ---           To  AluI[748]

   2:      117 bp  -  From AluI[748]     To  ---
```

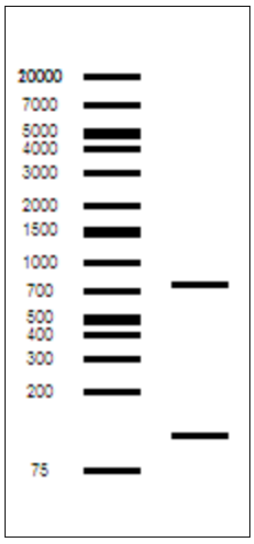

Figure 4. in silico Alu1 digestion of the trnT/D plastid region of yellow toadflax using Serial Cloner 2.0.

TCATTAGTCGGACAAGTTCTATGTTGAGAAATACCTACATGCATATATTATATATAT
GCAGTAGACTCATAGCGACTAGTGGCTTATTTTTATTTTTGAATAATCAAATGAATTT
TCCTAGCACCTCTAGGGTAAAATAAACTGTGGACCTAATTTGAAATGATCCTTATCA
AAACAAAATGGACTTGATTGATACATAACATACATGTACAATTACTAATTTGACATA
AAAGAAATTTCAAGATATTTGATCAAATCATGGGATGAAGGGATTTTACTTGTACCT
TTGAACGAAATTCGATTTTTAGAGAAAGTTTTGAATAACTTCTTGATCCCGCTTACTA
AATTTAGTTTAGTATATTTCTATAGAGAATGTCGATTCTAATGAATCGATTCATGACT
ATGGGTGACGAATCAAAAAATTATATCTAATTCTGAAAAACGGAAAGATACCTCGG
ATCTAATCATACCATTCCATTATATTGACAATTTCAAAAAATGATCATACTATGATC
ATAGTATGAGGGCGGTTGGGTAAGTCGGCCCCCATCGTCTAGTGGTTTAGGACATCT
CTCTTTCAAGGAGGCAGCGGGGATTCGAATTCCCCTGGGGGTAGGGTACTACGAAA
GGAAATTGATCATGGATTAACAATAAGCCTAAAATTGATTCTTCCTGGGTCGATGCC
CGAGCGGTTAATGGGGACGGACTGTAAATTCGTTGGCAATATGTCTACGCTGGTTCA
AATCCAGCTTATAACCCCCTTTGTGTTGCAGAAATCCTGCATACGAAGAGAAAAAAG
AATAAAATTTTCTGCTAGATCCCTTATTTCCTGGG

Figure 5. The Yellow toadflax trnT/D plastid region with the position of the Alu1 restriction site *(AGCT) is highlighted in red.

CAGCTTAGTGACAAGTTCTATGTTGAGAAATACCTACATGCATATATTATATATATG
CAGTAGACTCATAGCGACTAGTGGCTTATTTTTATTTTTGAATAATCAAATGAATTTT
CCTAGCACCTCTAGGGTAAAATAAGCTGTGGACCTAATTTGAAATGATCCTTATCAA
AACAAAATGGACTTGATTGATACATAACATACATGTACAATTACTAATTTGACATAA
AAGAAATTTCAAGATATTTGATCAAATCATGGGATGAAGGGATTTTACTTGTACCTT
TGAACGAAATTCGATTTTTAGAGAAAGTTTTGAATAACTTCTTGATCCCGCTTACTA
AATTTAGTTTAGTATATTTCTATAGAGAATGTCGATTCTAATGAATCGATTCATGACT
ATGGGTGACGAATCAAAAAATTATATCTAATTCTGAAAAACGGAAAGATACCTCGG
ATCTAATCATACCATTCCATTATATTGACAATTTCAAAAAATGATCATACTATGATC
ATAGTATGAGGGTGGTTGGGTAAGTCGGCCCCCATCGTCTAGTGGTTTAGGACATCT
CTCTTTCAAGGAGGCAGCGGGGATTCGAATTCCCCTGGGGGTAGGGTACTACGAAA
GGAAATTGATCATGGATTAACAATAAGCCTAAAATTGATTCTTCCTGGGTCGATGCC
CGAGCGGTTAATGGGGACGGACTGTAAATTCGTTGGCAATATGTCTACGCTGGTTCA
AATCCAGCTCGACCCAAGAAATTTGCCAATATACCATGATATAACCCCCTTTGTGTT
GCAGAAATCCTGCATACGAAGAGAAAAAAGAATAAAATTTTCTGCTAGATCCCTTA
TTTCCCTGGGATTGAGTTTTAAATTGGATA

Figure 6. The Dalmatian toadflax trnT/D plastid region positions of the Alu1 restriction sites *(AGCT) is highlighted in red.

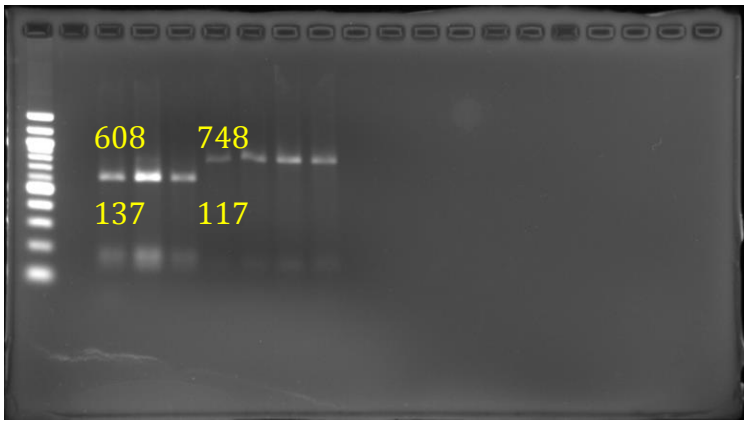

Figure 7. Alu1 digestion of the trnT/D plastid region in unambiguous yellow toadflax and Dalmatian toadflax.

*Approximate sizes of bands are in yellow
Lane in order of appearance
1- Ladder
2-blank
3- DT-MT-M3 09
4- DT-MT-M9 09
5- DT-Helena
6- YT-AK-1
7- YT-AK-4
8- YT-MT-02-09
9- YT-MT-07-09

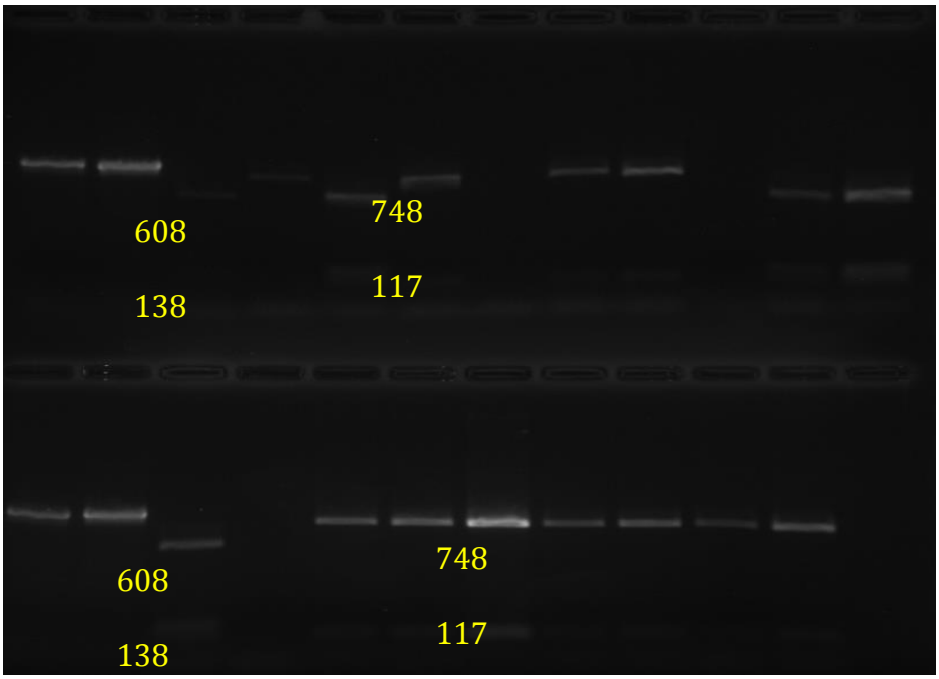

Figure 8. Alu1 digestion of the trnT/D plastid region in handcrossed individuals.
ºApproximate sizes of bands are in yellow
1- Uncut TrnT/D amplicon
2- Uncut TrnT/D amplicon
3- LB 07 PC 01
4- BC-HH-01
5- B05-1-7
6- BC-CC-01
7- BC-W-01
8- BC-DD-01
9- BC-X-01
10- BC-BB-01
11- DYF-F1-15
12- RO5-2-01
1- Uncut TrnT/D amplicon
2- Uncut TrnT/D amplicon
3- BO2-2
4- R05-2-7
5- BCW-01
6- BC-GG-01
7- BC-AA-01
8- BC-D-03
9- BC-EE-01
10- BC-A-01
11- BC-R-01
12- blank

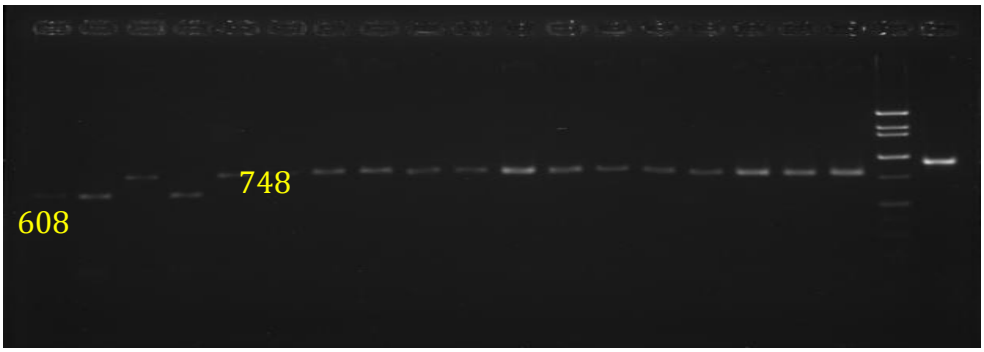

Figure 9. Alu1 digestion of the trnT/D plastid region of Palisades ID, site.

ºApproximate sizes of bands are in yellow
1-DT Alu1 positive control
2- DT Alu1 positive control
3- YT Alu1 positive control
4-DT Alu1 positive control
5-YT Alu1 positive control
6-YT Alu1 positive control
7-Palisade-1
8-Palisade-2
9-Palisade-3
10-Palisade-4
11-Palisade-5
12-Palisade-6
13-Palisade-7
14-Palisade-8
15-Palisade-9
16-Palisade-10
17-Palisade-11
18-Palisade-12
19-Ladder
20-Uncut TrnT/D amplicon

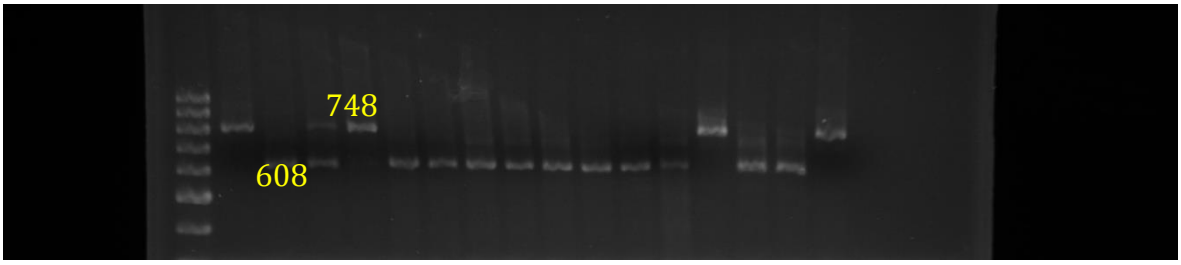

Figure 10. Alu1 digestion of the trnT/D plastid region of Radersburg, MT and Boulder MT, sites.

ºApproximate sizes of bands are in yellow
1-Ladder
2-Abbey 1
3-R-06-15
4-B-06-14
5-R-05-2-4
6-B0-1-7
7-R-06-07
8-R-06-08
9-R-06-04
10-R-06-06
11-R-05-2-1
12-B-06-07
13-B-06-03
14-B-06-11
15-R-05-2-9
16-B-06-15
17-Blank
18-Blank
19-Blank
20-Blank

Table 3. Plastid haplotypes of 204 toadflax plants based on restriction of the trnT/trnD region with Alu 1.

Accession Name	Source	Assigned taxon (based on morphology)	pDNA haplotype (based on Alu1 marker)
Abbey 1	Red Feather, CO	Hybrid	DT
Abbey 2	Red Feather, CO	Hybrid	YT
ALTA, WY 12T 0497380 4844876	Alta, WY	YT	YT
ALTA, WY 12T 0497389 4844873	Alta, WY	YT	YT
ALTA, WY 12T 0497390 4844872	Alta, WY	YT	YT
ALTA, WY 12T 0497390 4844873	Alta, WY	YT	YT
ALTA, WY 12T 0497391 4844866	Alta, WY	YT	YT
B0-1-7	Boulder, MT	Hybrid	YT
B-02-02	Boulder, MT	Hybrid	YT
B02-2	Boulder MT	Hybrid	DT
B-03-4	Boulder, MT	Hybrid	DT
B05-2-1	Boulder, MT	Hybrid	DT
B-06-02	Boulder, MT	Hybrid	YT
B-06-03	Boulder, MT	Hybrid	YT
B-06-07	Boulder, MT	Hybrid	YT
B-06-09	Boulder, MT	Hybrid	YT
B-06-10	Boulder, MT	Hybrid	YT
B-06-11	Boulder, MT	Hybrid	DT
B-06-14	Boulder, MT	Hybrid	YT
B-06-15	Boulder, MT	Hybrid	YT
BO5-1-7	Boulder, MT	Hybrid	DT
BC-A-01	Handcrossed	Hybrid	YT
BC-A-06	Handcrossed	Hybrid	YT
BC-AA-01	Handcrossed	Hybrid	YT
BC-B-01	Handcrossed	Hybrid	YT
BC-BB-01	Handcrossed	Hybrid	YT
BC-C-01	Handcrossed	Hybrid	YT
BC-C-02	Handcrossed	Hybrid	YT
BC-C-06	Handcrossed	Hybrid	YT

BC-CC-01	Handcrossed	Hybrid	YT
BC-D-01	Handcrossed	Hybrid	YT
BC-D-02	Handcrossed	Hybrid	YT
BC-D-03	Handcrossed	Hybrid	YT
BC-D-04	Handcrossed	Hybrid	YT
BC-D-05	Handcrossed	Hybrid	DT
BC-DD-01	Handcrossed	Hybrid	YT
BC-E-02	Handcrossed	Hybrid	YT
BC-E-04	Handcrossed	Hybrid	YT
BC-E-09	Handcrossed	Hybrid	DT
BC-EE-01	Handcrossed	Hybrid	YT
BC-F-01	Handcrossed	Hybrid	YT
BC-F-05	Handcrossed	Hybrid	YT
BC-G-02	Handcrossed	Hybrid	YT
BC-GG-01	Handcrossed	Hybrid	YT
BC-H-02	Handcrossed	Hybrid	YT
BC-H-05	Handcrossed	Hybrid	YT
BC-HH-01	Handcrossed	Hybrid	YT
BC-I-07	Handcrossed	Hybrid	YT
BC-I-11	Handcrossed	Hybrid	YT
BC-R-01	Handcrossed	Hybrid	YT
BC-R-02	Handcrossed	Parent	DT
BCW-01	Handcrossed	Hybrid	YT
BC-W-01	Handcrossed	Hybrid	YT
BC-X-01	Handcrossed	Hybrid	YT
D-R06-1	Handcrossed	Hybrid	DT
DT Cherry 1	Fort Collins, CO	DT	YT
DT Cherry 2	Fort Collins, CO	DT	YT
DT EH	Elkhorns, MT	DT	DT
DT EH P1	Elkhorns, MT	DT	DT
DT HELENA	Helena, MT	DT	DT
DT HT 1	Horsetooth Reservoir, CO	Hybrid	YT
DT HT 1 08	Horsetooth Reservoir, CO	Hybrid	YT
DT HT 2	Horsetooth Reservoir, CO	Hybrid	YT
DT LSP 10	Lory State Park, CO	DT	YT
DT LSP 11	Lory State Park, CO	DT	YT

DT LSP 12	Lory State Park, CO	DT	YT
DT LSP 13	Lory State Park, CO	DT	YT
DT LSP 14	Lory State Park, CO	DT	YT
DT LSP 15	Lory State Park, CO	DT	YT
DT LSP 17	Lory State Park, CO	DT	YT
DT LSP 18	Lory State Park, CO	DT	YT
DT LSP 19	Lory State Park, CO	DT	YT
DT LSP 20	Lory State Park, CO	DT	YT
DT MT	Missoula, MT	DT	DT
DT MT 02	Missoula, MT	DT	DT
DT MT 09	Missoula, MT	DT	DT
DT MT 2	Missoula, MT	DT	DT
DT MT M1	Missoula, MT	DT	DT
DT MT M2	Missoula, MT	DT	DT
DT MT M3 09	Missoula, MT	DT	DT
DT MT M6 09	Missoula, MT	DT	DT
DT MT M8 09	Missoula, MT	DT	DT
DT MT M9 09	Missoula, MT	DT	DT
DT-Elk horn	Missoula, MT	DT	DT
DT-LM-1	Lee Martinez Park, CO	DT	DT
DT-LM-2	Lee Martinez Park, CO	DT	DT
DT-LM-5	Lee Martinez Park, CO	DT	DT
DT-LM-6	Lee Martinez Park, CO	DT	DT
DT-LM-7	Lee Martinez Park, CO	DT	DT
DT-LSP-16	Lory State Park, CO	DT	YT
DT-Missoula	Missoula, MT	DT	DT
DY 01	Handcrossed	Hybrid	YT
DY 04	Handcrossed	Hybrid	YT
DY 09	Handcrossed	Hybrid	YT
DY 13	Handcrossed	Hybrid	YT
DY 14	Handcrossed	Hybrid	YT
DY F1 05	Handcrossed	Hybrid	YT
DY f1 06	Handcrossed	Hybrid	YT
DY F1 07	Handcrossed	Hybrid	DT

DY F1 11	Handcrossed	Hybrid	YT
DY F1 15	Handcrossed	Hybrid	DT
ID DL HY 01	Palisades, ID	Hybrid	YT
ID-L1-09	Palisades, ID	Hybrid	YT
ID-L2-09	Palisades, ID	Hybrid	YT
ID-L3-09	Palisades, ID	Hybrid	YT
ID-L5-09	Palisades, ID	Hybrid	YT
ID-M5-09	Palisades, ID	Hybrid	YT
ID-U10-09	Palisades, ID	Hybrid	YT
ID-U1-09	Palisades, ID	Hybrid	YT
ID-U12-09	Palisades, ID	Hybrid	YT
ID-U2-09	Palisades, ID	Hybrid	YT
ID-U3-09	Palisades, ID	Hybrid	YT
ID-U4-09	Palisades, ID	Hybrid	YT
ID-U9-09	Palisades, ID	Hybrid	YT
LB07 P1 E1	Boulder, MT	Hybrid	YT
LB07 P2 C1	Boulder, MT	Hybrid	YT
ND 14U 0314499 5351890	Burlington, ND	YT	YT
ND 14U 0314503 5351862	Burlington, ND	YT	YT
ND 14U 0314504 5351862	Burlington, ND	YT	YT
P1	Palisades, ID	Hybrid	YT
P10	Palisades, ID	Hybrid	YT
P11	Palisades, ID	Hybrid	YT
P12	Palisades, ID	Hybrid	YT
P2	Palisades, ID	Hybrid	YT
P3	Palisades, ID	Hybrid	YT
P4	Palisades, ID	Hybrid	YT
P5	Palisades, ID	Hybrid	YT
P6	Palisades, ID	Hybrid	YT
P7	Palisades, ID	Hybrid	YT
P8	Palisades, ID	Hybrid	YT
P9	Palisades, ID	Hybrid	YT
R-05-2-1	Radersburg, MT	Hybrid	YT
R05-2-2	Radersburg, MT	Hybrid	YT
R-05-2-4	Radersburg, MT	Hybrid	DT
R-05-2-9	Radersburg, MT	Hybrid	YT

R-06-01	Radersburg, MT	Hybrid	YT
R-06-03	Radersburg, MT	Hybrid	DT
R-06-04	Radersburg, MT	Hybrid	YT
R06-06	Radersburg, MT	Hybrid	DT
R-06-06	Radersburg, MT	Hybrid	YT
R-06-07	Radersburg, MT	Hybrid	YT
R-06-08	Radersburg, MT	Hybrid	YT
R-06-11	Radersburg, MT	Hybrid	YT
R-06-12	Radersburg, MT	Hybrid	YT
R-06-15	Radersburg, MT	Hybrid	YT
R-06-16	Radersburg, MT	Hybrid	YT
R-06-18	Radersburg, MT	Hybrid	YT
R-DT-4	Radersburg, MT	Hybrid	DT
RO2-2	Radersburg, MT	Hybrid	DT
RO5-2-01	Radersburg, MT	Hybrid	DT
RO5-2-7	Radersburg, MT	Hybrid	DT
SD 14T 0488874 505 875	Leola, SD	YT	YT
SD 14T 0488874 5058074	Leola, SD	YT	YT
SD 14T 0488875 5058077	Leola, SD	YT	YT
SD 14T 0488877 5058067	Leola, SD	YT	YT
SD 14T 0488879 5058069	Leola, SD	YT	YT
YD 18	Handcrossed	Hybrid	YT
YD 20	Handcrossed	Hybrid	YT
YD 23	Handcrossed	Hybrid	YT
YD 25	Handcrossed	Hybrid	YT
YD 26	Handcrossed	Hybrid	YT
YD 27	Handcrossed	Hybrid	YT
YD 29	Handcrossed	Hybrid	YT
YD 31	Handcrossed	Hybrid	YT
YD 32	Handcrossed	Hybrid	YT
YD 33	Handcrossed	Hybrid	YT
YD 34	Handcrossed	Hybrid	YT
YD 35	Handcrossed	Hybrid	YT
YD 39	Handcrossed	Hybrid	YT

YD 44	Handcrossed	Hybrid	YT
YD 45	Handcrossed	Hybrid	YT
YD F1 17	Handcrossed	Hybrid	YT
YD F1 18	Handcrossed	Hybrid	YT
YD f1 24	Handcrossed	Hybrid	YT
YD F1 38	Handcrossed	Hybrid	YT
YD F1 46	Handcrossed	Hybrid	YT
YT AK 08-1	Fairbanks, AK	YT	YT
YT AK 08-2	Fairbanks, AK	YT	YT
YT AK 08-3	Fairbanks, AK	YT	YT
YT AK 08-4	Fairbanks, AK	YT	YT
YT AK 08-5	Fairbanks, AK	YT	YT
YT AK 08-6	Fairbanks, AK	YT	YT
YT AK 4	Fairbanks, AK	YT	YT
YT FT BM-1	Flat Tops, CO	YT	YT
YT FT BM-2	Flat Tops, CO	YT	YT
YT FT DSP 2 08	Flat Tops, CO	YT	YT
YT FT EP 5	Flat Tops, CO	YT	YT
YT FT FS 2	Flat Tops, CO	YT	YT
YT FT OS P1	Flat Tops, CO	YT	YT
YT FT OS P2	Flat Tops, CO	YT	YT
YT FT OS P4	Flat Tops, CO	YT	YT
YT FT PS 2	Flat Tops, CO	YT	YT
YT HT 4 08	Horsetooth Reservoir, CO	YT	YT
YT MT 01	Ovando, MT	YT	YT
YT MT 02 09	Ovando, MT	YT	YT
YT MT 05 09	Ovando, MT	YT	YT
YT MT 06 09	Ovando, MT	YT	YT
YT MT 07 09	Ovando, MT	YT	YT
YT MT 09	Ovando, MT	YT	YT
YT PC-7	Pine Creek, MT	YT	YT
YT PC8	Pine Creek, MT	YT	YT
YT-Fort Ellis	Fort Ellis, MT	YT	YT

CHAPTER 3: THE DEVELOPMENT OF PLASTID DNA BARCODING MARKERS TO DISTINGUISH BETWEEN YELLOW TOADFLAX AND DALMATIAN TOADFLAX PDNA HAPLOTYPES

Introduction

DNA barcoding is a technique used to identify species by short run sequencing of a specific genomic (nuclear or plastid) location (Arif et al. 2010). Coupling individual DNA barcodes with BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990) to compare marker regions with a reference region allows for successful species identification in plants using the *matK* (99%), *trnH/psbA* (95%) and *rbclA* (75%) regions (Arif et al. 2010). The application of DNA barcoding as species diagnostic markers has been demonstrated in multiple studies (Kress et al. 2005, Kress and Erickson 2007, Van De Wiel et al. 2009, Bruni et al. 2010). DNA barcoding is used as diagnostic method based on the presence of one or more unique locus nucleotide polymorphisms that can be used as a species identification tool. The main purpose of DNA barcoding is to provide unambiguous identification of an unknown species (Kress and Erickson 2007).

DNA barcoding regions must exhibit enough variation to demonstrate interspecific variation, but not so much as to have a high degree of intraspecific variation, which is one of the reasons why mtDNA and pDNA regions are utilized instead of ncDNA (Lahaye et al. 2008). In animals, a universal primer has been established, which amplifies the mitochondrial gene *cox1* (Hebert et al. 2003, Smith et al. 2005). However, the plant community has not established a specific primer region that can be used across all taxa. To increase DNA barcoding diagnostic power, multiple diagnostic regions should be used identify an individual (Hollingsworth et al. 2011). The Consortium for the Barcode for Life recently released a study stating that a universal primer region needs to be determined for plant use (Hollingsworth et al. 2011). This study

suggested that it would be useful to consider a core set of pDNA regions, specifically *rbcla* and *matK,* for barcoding in plant systematic and taxonomic studies (Hollingsworth et al. 2011). The barcoding regions *trnH/psbA* and *trnL-F* have also been shown to be useful (Chase et al. 2005, Kress and Erickson 2007, Van De Wiel et al. 2009, Newmaster and Ragupathy 2009, Hollingsworth et al. 2011, De Groot et al. 2011). Since no part of the *Linaria* genome has been sequenced, all four of the above regions were tested in this study in case any one of them proved to have little or no diagnostic power.

The *matK* gene, which is ~1500 bp, is a quickly evolving region of the plastid genome that has high discriminatory power (Hilu and Liang 1997, Lahaye et al. 2008, Bruni et al. 2010, Liu et al. 2011). The *rbcL* (ribulose-1,5-biphosphate carboxylase) plastid gene, which is roughly 500bp (Manen and Natali 1995, Lahaye et al. 2008), has proven to be useful in taxonomic studies (Van De Wiel et al. 2009, De Groot et al. 2011). Both *trnH*/*psbA* and *trnL-F* are variable intron/intergenic spacer regions that are known to have high species resolution power (Kress and Erickson 2007, Lahaye et al. 2008 Van De Wiel et al. 2009, Bruni et al. 2010, Liu et al. 2011).

For this study the plastid regions used were *trnH*/*psbA, matK, rbcL,* and *trnL-F*. These plastid primer combinations sequence 2 conserved gene regions (*matK* and *rbcla*) and 2 variable intron/intergenic spacer regions (*trnH*/p*sbA, trnL-F).* Testing both gene and intergenic/intronic regions should increase the chances of identifying species-specific diagnostic markers for both yellow toadflax and Dalmatian toadflax, since there is no sequence data for either species. Without sequence data it is hard to know if it would be better to use highly conserved (gene) or highly variable (intergenic/intronic) pDNA regions. The purpose of this study was to create species-diagnostic DNA barcodes that can distinguish between yellow toadflax pDNA haplotypes and Dalmatian toadflax pDNA haplotypes.

Methods and Materials

DNA was extracted from 63 individuals using a DNeasy DNA mini prep kit from Qiagen. These 63 individuals were a subset of the 204 individuals described in Chapter Two that were sampled for PCR-RFLP markers (see Table 5) and were selected to provide a representative sample of each of the different populations that were tested for the presence of both yellow and Dalmatian toadflax haplotypes (see Table 7). Extracted DNA was amplified by PCR performed in a C1000 thermocycler, using Promega GoTaq DNA polymerase. The reaction components were as follows: 10 ul Gotaq Reaction Buffer, 0.5mM dNTPs, 0.4 uM upstream and downstream primer (Table 4), and 0.25 Gotaq DNA polymerase for a final reaction volume of 50uL. PCR parameters were as follows:

The middle part of *matK* was amplified for 5 min. at 94°C; 40x (1 min. at 94°C, 30 sec. at 48°C, 1 min. at 72°C), with a 7 min. final extension at 72°C.

trnL-F was amplified for 5 min. at 94°C; 35x (1 min. at 94°C, 1 min. at 50-55°C, 2 min. at 72°C), with a 10 min. final extension at 72°C.

rbcLa was amplified for 5 min. at 95°C; 32 cycles (30 sec. at 95°C, 30 sec. at 53-55°C, 1 min. at 72°C), with a 10 min. final extension at 72°C.

trnH/psbA was amplified with 5 min. at 95°C; 35 cycles (1 min. at 95°C, 45 sec. at 55°C, 2 min. at 72°C), with a 10 min. final extension at 72°C.

Initially TX2 and TX4 were used as *matK* primers (Liu et al. 2011), but a low percentage of individuals tested amplified. The *matK* primers *390F* and *1326R* were used in place of TX2 and TX4. Sequences, sources, and additional information on primers used to generate amplicons are given in Table 4.

Four genotypic classes of individuals were tested: taxonomically unambiguous yellow toadflax and Dalmatian toadflax (based upon morphology and source location), known hybrids generated from controlled greenhouse crosses, and plants collected in the field that were confirmed as hybrids using ISSR analysis (Ward et al. 2009). The taxonomically unambiguous yellow and Dalmatian toadflax was used to create the species diagnostic DNA barcodes. The greenhouse crossed hybrids were used to test the reliability of the DNA barcodes, and then the field collected hybrids were barcoded to determine their pDNA haplotype. The goal of this study was to create species-diagnostic DNA barcodes that can distinguish between yellow toadflax pDNA haplotypes and Dalmatian toadflax pDNA haplotypes.

Amplified DNA regions were run on a 1% agarose gel stained with ethidium bromide (EtBr) and visually examined under UV light to ensure proper amplification. For correct amplification there should only be one band occurring at a size corresponding to that shown in Table 3. Amplified regions were precipitated used Ethanol/EDTA Precipitation following the protocol provided in the BigDye Terminator v3.1 Cycle Sequencing Kit, and were then short run sequenced using an ABI 3130xL Genetic Analyzer with ABI's BigDye® Terminator v3.1. MEGA v5 software (http://www.megasoftware.net) was used to analyze the sequenced regions and ClustalW v1.81 was used for multiple sequence alignment. All multiple sequence alignments were trimmed, meaning the first ~50-70 bp and last 50 to 100 bp were excluded from the results due their low signal strength on the resulting chromatogram generated by the ABI 3130xL Genetic Analyzer.

Results

The four pDNA regions sequenced varied greatly in the number of sequence polymorphisms and their potential as DNA barcodes (see Tables 6-8). Only *trnL-F* and *matK* included enough consistent sequence polymorphisms to be useful as diagnostic markers. The *rbcla* and *trnH/psbA* did not have enough consistent polymorphisms to be used as DNA barcodes. All four of the tested regions had a high PCR amplification success rate (61 out of 63). The two individuals (YT MT 09 and YT PC-7) that failed to amplify did so for all four regions, which was likely due to poor DNA quality. One sample, DT MT M1, had poor sequencing for *trnL-F* which was the result of poor PCR amplification. For *matK* DT LSP 11 and DT Cherry 1 gave poor sequencing results. For *trnH/psbA* poor sequencing was also obtained for DT Cherry 1. *TrnL-F* had poor sequencing results for both ALTA WY 12T 0497389 4844873 and ALTA WY 12T 0497389 4844873. The cause of poor sequencing was most likely reagents from the PCR reaction that had not been removed completely during precipitation. These samples were excluded from the multiple sequence alignment. Samples B-06-14 and DT M2 09 had poor sequencing results for *trnH/psbA* and *rbcLa* respectively. This was determined by the strength of signals these samples produced during sequencing on their chromatograms. They were still included in the multiple sequence alignment, because the poor sequencing only caused some of the sites to have inconsistent polymorphisms; these samples were not included in the final analysis. The *trnH/Psba* and *rbcLa* sites were too inconsistent across species to be used as reliable DNA barcoding regions: fewer than 50% of accessions exhibited polymorphisms and these were not consistent across populations or species. The results of the aligned sequences for *trnL-F* and *matK* are shown in Tables 6 and 7.

The plastid region *trnL-F* had multiple diagnostic regions (SNPs) that distinguished between yellow and Dalmatian toadflax. At sites 284 and 331, 10 out of 16 Dalmatian individuals tested possessed an adenine, while all unambiguous yellow toadflax individuals possessed a guanine. The only Dalmatian samples that did not have an adenine at these sites were: DT LSP 11, DT LSP 13, DT LSP 18, DT Cherry 1, DT Cherry 2, and Abbey 2. Both of these populations possessed a guanine at positions 284 and 331. A presumed yellow toadflax accession, ND 14U 0314499 5351890, displayed polymorphisms consistent with a Dalmatian haplotype at both sites 284 and 331.

Table 4. List of Primers for DNA Barcoding.

Primers name	Primer sequence (5' to3')	Amplified seqeunce length	Primer source
TrnL/TrnF: B49873	GGTTCAAGTCCCTCTATCCC	~360 bp	Kress et al. 2005
TrnL/TrnF: A50272	ATTTGAACTGGTGACACGAG	~360 bp	Kress et al. 2005
matK: 390F	CGATCTATTCATTCAATATTTC	~885	Lahaye, R. 2008
matK: 1326R	TCTAGCACACGAAAGTCGAAGT	~885	Lahaye, R. 2008
TrnH/PsbA	GTTATGCATGAACGTAATGCTC	~450 bp	Sang et al. 1997
TrnH/PsbA	CGCGCATGGTGGATTCACAATCC	~450 bp	Sang et al. 1997
rbclA-F	ATGTCACCACAAACAGAGACTAAAGC	~1430 bp	Kress et al. 2005
rbclA-R	GTAAAATCAAGTCCACCRCG	~1430 bp	Kress et al. 2005

Table 5. Locations of toadflax accessions used for DNA barcoding.

Accession Name	Location	Latitude	Longitude
[1]DT/ [2]YT HT	Horsetooth Reservoir,CO	40.5	-105.2
YT FT	Flat Tops, CO	39.55	-105.78
YT AK	Fairbanks, AK	64.83	-147.78
YT PC	Pine Creek, MT	45.06	-110.58
DT LSP	Lory State Park, CO	40.57	-105.18
B	Boulder, MT	39.9	-105.3
R	Radersburg, MT	46.19	-111.63
DT MT	Missoula, MT	46.85	-113.95
YT MT	Ovando, MT	47.02	-113.12
DT Helena	Helena, MT	46.59	-112.02
DT LM	Lee Martinez Park, CO	40.59	-105.08
DT EH	Elkhorn Mountains, MT	46.27	-111.93
YT Alta WY	Alta, WY	43.75	-111.03
YT SD	Leola, SD	45.67	-99.14
YT ND	Burlington, ND	48.29	-101.50
ID or P	Palisade, ID	43.40	-111.20
DT Cherry	Fort Collins, CO	40.5	-105

[1]DT denotes Dalmatian toadflax.
[2]YT denotes yellow toadflax.

Table 6. Sites of *trnL-F* polymorphisms.

Accession name	Site 284 (Stats at 283)	Site 331 (Starts at 330)
ALTA WY 12T 0497389 4844873	AGACCC	-GAAAT
ALTA WY 12T 0497390 4844873	AGACCC	-GAAAT
ALTA WY 12T 0497391 4844866	AGACCC	-GAAAT
ND 14U 0314503 5351862	AGACCC	-GAAAT
SD 14T 0488874 505 875	AGACCC	-GAAAT
SD 14T 0488875 5058077	AGACCC	-GAAAT
SD 14T 0488877 5058067 3a	AGACCC	-GAAAT
YT MT 05 09	AGACCC	-GAAAT
YT FT OS P1	AGACCC	-GAAAT
YT MT 07 09	AGACCC	-GAAAT
YT PC8	AGACCC	-GAAAT
LB07 P1 E1	AGACCC	-GAAAT
YD	AGACCC	-GAAAT
BC-F-05	AGACCC	-GAAAT
SD 14T 0488875 5058077	AGACCC	-GAAAT
SD 14T 0488874 505 875	AGACCC	-GAAAT
ID-U2-09	AGACCC	-GAAAT
ID-M5-09	AGACCC	-GAAAT
Abbey 2	AGACCC	-GAAAT
DT LSP 11	AGACCC	-GAAAT
DT LSP 18	AGACCC	-GAAAT
DT LSP 13	AGACCC	-GAAAT
DT Cherry 1	AGACCC	-GAAAT
DT Cherry 2	AGACCC	-GAAAT
B-06-07	AGACCC	-GAAAT
B-06-10	AGACCC	-GAAAT
B-06-15	AGACCC	-GAAAT
B-06-03	AGACCC	-GAAAT
R-06-07	AGACCC	-GAAAT
R-O6-15	AGACCC	-GAAAT
R-06-08	AGACCC	-GAAAT
LB07 P2 C1	AGACCC	-GAAAT
4499 5351890	AAACCC	-AAAAT
DY	AAACCC	-AAAAT

BC-E-09	AAACCC	-AAAAT
DT LM 1	AAACCC	-AAAAT
DT LM 2	AAACCC	-AAAAT
R-06-03	AAACCC	-AAAAT
DT HELENA	AAACCC	-AAAAT
DT-M1	AAACCC	-AAAAT
DT MT M9 09	AAACCC	-AAAAT
DT MT M8 09	AAACCC	-AAAAT
DT MT M3 09	AAACCC	-AAAAT
DT MT M2 09	AAACCC	-AAAAT
(2nd try)	AAACCC	-AAAAT
ABBEY 1	AAACCC	-AAAAT
DT EH	AAACCC	-AAAAT
DT EH P1	AAACCC	-AAAAT
B-06-11	AAACCC	-AAAAT

The plastid region *matK* had 8 diagnostic polymorphic sites. Sites 88, 354, 359 and 379 were diagnostic markers for yellow toadflax originating from North Dakota (YT ND) and South Dakota (YT SD) populations. Sites 88 and 354 contained a guanine, instead of a cytosine, for the YT ND and YT SD populations. Site 359 had a thymine, in place of a cytosine, for YT SD and YT ND populations. Site 379 had a cytosine, instead of thymine, for YT SD and YT ND populations. At site 297, yellow toadflax originating from Montana (YT MT) and Alaska (YT AK) populations had a polymorphic marker, which was a thymine in place of a cytosine. At site 456, Dalmatian toadflax originating from a Montana (DT MT) population had a distinct marker, which was a guanine in place of adenine. At site 502, yellow toadflax originating from Alta, WY (YT ALTA), South Dakota (YT SD), North Dakota (YT ND), Pine Creek, MT (YT PC), a hand-crossed hybrid (BC–E- 06), and Dalmatian toadflax from Fort Collins, CO (DT Cherry 2) had a diagnostic marker, which was a cytosine in place of a thymine. At site 713 Dalmatian toadflax from three Montana populations (DT EH, DT MT, DT Helena) had the same polymorphism of a guanine replacing an adenine.

Table 7. Sites of *matK* polymorphisms.

Accession Name	88 (starts at 85)	297 (starts at 296)	354 and359 (starts at 353)	379 (starts at 375)
DT MT M6 09	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT MT M8 09	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT MT M2	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT MT M3 09	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT MT M9 09	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT MT M1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT LM 1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT LM 2	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT Helena	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT EH P1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT EH	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
Cherry 2	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
Cherry 1	------	--------	--------	------
DT LSP 11	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
DT LSP 13	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
YT MT 07 09	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
YT MT 06 09	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
YT MT 05 09	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
YT PC8	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
YT AK 4	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
SD 14T 0488874 505 875	AAAGCC	TGCATTTT	TGCATTTT	CGTCCG
SD 14T 0488875 5058077	AAAGCC	TGCATTTT	TGCATTTT	CGTCCG

SD 14T 0488874 5058074	AAAGCC	TGCATTTT	TGCATTTT	CGTCCG	
SD 14T 0488877 5058067	AAAGCC	TGCATTTT	TGCATTTT	CGTCCG	
ND 14U 0314499 5351890	AAAGCC	TGCATTTT	TGCATTTT	CGTCCG	
ND 14U 0314503 5351862	AAAGCC	TGCATTTT	TGCATTTT	CGTCCG	
ALTA WY 12T 0497390 4844872	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
ALTA WY 12T 0497391 4844866	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
ALTA WY 12T 0497390 4844873	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
ALTA WY 12T 0497389 4844873	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
YT FT FS 2	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
YT FT OS P1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
Abbey 1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
ABBEY 2	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
DY F1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
BC-D-03	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
BC-E-09	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
LB07 P2 C1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
LB07 P1 E1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
R-06-03	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
R-06-08	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
R-06-15	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	
R-06-07	AAACCC	TCCATTCT	TCCATTCT	CGTCTG	

R-06-01	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
B-06-10	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
B-06-11	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
B-06-03	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
B-06-07	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
P2	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
P3	AAACCC	TCCATTCT	TCCATTCT	CGTCTG
ID L1	AAACCC	TCCATTCT	TCCATTCT	CGTCTG

Table 7. Sites of *matK* polymorphisms (continued).

Accession Name	456 (starts at 455)	502 (starts at 500)	706 (starts at 705)
DT MT M6 09	AAGATT	GGTTAT	AATATT
DT MT M8 09	AAGATT	GGTTAT	AATATT
DT MT M2	AAGATT	GGTTAT	AATATT
DT MT M3 09	AAGATT	GGTTAT	AATATT
DT MT M9 09	AAGATT	GGTTAT	AATATT
DT MT M1	AAGATT	GGTTAT	AATATT
DT LM 1	AGGATT	GGTTAT	AGTATT
DT LM 2	AGGATT	GGTTAT	AGTATT
DT Helena	AGGATT	GGTTAT	AATATT
DT EH P1	AGGATT	GGTTAT	AATATT
DT EH	AGGATT	GGTTAT	AATATT
Cherry 2	AGGATT	GGCTAT	AGTATT
Cherry 1	------	------	------
DT LSP 11	AGGATT	GGTTAT	------
DT LSP 13	AGGATT	GGTTAT	AGTATT
YT MT 07 09	AGGATT	GGTTAT	AGTATT
YT MT 06 09	AGGATT	GGTTAT	AGTATT
YT MT 05 09	AGGATT	GGTTAT	AGTATT
YT PC8	AGGATT	GGCTAT	AGTATT
YT AK 4	AGGATT	GGTTAT	AGTATT
SD 14T 0488874 505 875	AGGATT	GGCTAT	AGTATT
SD 14T 0488875 5058077	AGGATT	GGCTAT	AGTATT
SD 14T 0488874 5058074	AGGATT	GGCTAT	AGTATT

SD 14T 0488877 5058067	AGGATT	GGCTAT	AGTATT
ND 14U 0314499 5351890	AGGATT	GGCTAT	AGTATT
ND 14U 0314503 5351862	AGGATT	GGCTAT	AGTATT
ALTA WY 12T 0497390 4844872	AGGATT	GGCTAT	AGTATT
ALTA WY 12T 0497391 4844866	AGGATT	GGCTAT	AGTATT
ALTA WY 12T 0497390 4844873	AGGATT	GGCTAT	AGTATT
ALTA WY 12T 0497389 4844873	AGGATT	GGCTAT	AGTATT
YT FT FS 2	AGGATT	GGTTAT	AGTATT
YT FT OS P1	AGGATT	GGTTAT	AGTATT
Abbey 1	AGGATT	GGTTAT	AGTATT
ABBEY 2	AGGATT	GGTTAT	AGTATT
DY F1	AGGATT	GGTTAT	AGTATT
BC-D-03	AGGATT	GGTTAT	AGTATT
BC-E-09	AGGATT	GGCTAT	AGTATT
LB07 P2 C1	AAGATT	GGTTAT	AGTATT
LB07 P1 E1	AGGATT	GGTTAT	AGTATT
R-06-03	AGGATT	GGTTAT	AATATT
R-06-08	AGGATT	GGTTAT	AGTATT
R-06-15	AGGATT	GGTTAT	AGTATT
R-06-07	AGGATT	GGTTAT	AGTATT
R-06-01	AAGATT	GGTTAT	AGTATT
B-06-10	AGGATT	GGTTAT	AGTATT
B-06-11	AGGATT	GGTTAT	AATATT
B-06-03	AAGATT	GGTTAT	AGTATT
B-06-07	AGGATT	GGTTAT	AGTATT
P2	AGGATT	GGTTAT	AGTATT
P3	AGGATT	GGTTAT	AGTATT
ID L1	AGGATT	GGTTAT	--------

Table 8. Comparison of pDNA haplotypes based on DNA barcode and PCR-RFLP.

Accession Name	Source	Assigned taxon (based on morphology)	DNA Barcoding marker used	pDNA based on DNA Barcode	pDNA haplotype Based on PCR-RFLP
Abbey 1	Red Feather, CO	Hybrid	(trnL-F)	DT	DT
Abbey 2	Red Feather, CO	Hybrid	(trnL-F)	YT	YT
ALTA, WY 12T 0497389 4844873	Alta, WY	YT	(trnL-F)	YT	YT
ALTA, WY 12T 0497390 4844872	Alta, WY	YT	matK	YT	YT
ALTA, WY 12T 0497390 4844873	Alta, WY	YT	(trnL-F)	YT	YT
ALTA, WY 12T 0497391 4844866	Alta, WY	YT	(trnL-F), matK	YT	YT
B-06-03	Boulder MT,	Hybrid	(trnL-F)	YT	YT
B-06-07	Boulder MT,	Hybrid	(trnL-F)	YT	YT
B-06-10	Boulder MT,	Hybrid	(trnL-F)	YT	YT
B-06-11	Boulder MT,	Hybrid	(trnL-F)	DT	DT
B-06-14	Boulder MT,	Hybrid	none		YT
B-06-15	Boulder MT,	Hybrid	(trnL-F)	YT	YT
BC-E-09	Hand crossed	Hybrid	(trnL-F), matK	DT	DT
BC-F-05	Hand crossed	Hybrid	(trnL-F)	YT	YT
DT Cherry 1	Fort Collins CO	DT	(trnL-F)	YT	YT
DT Cherry 2	Fort Collins CO	DT	(trnL-F), matK	YT	YT
DT EH	Elkhorns, MT	DT	(trnL-F), matK	DT	DT
DT EH P1	Elkhorns, MT	DT	(trnL-F), matK	DT	DT
DT HELENA	Helena, MT	DT	(trnL-F), matK	DT	DT

DT-LM-1	Lee Martinez Park, Fort Collins, CO	DT	(trnL-F)	DT	DT
DT-LM-2	Lee Martinez Park, Fort Collins, CO	DT	(trnL-F)	DT	DT
DT LSP 11	Lory State Park, CO	DT	(trnL-F), matK	YT	YT
DT LSP 13	Lory State Park, CO	DT	(trnL-F)	YT	YT
DT LSP 18	Lory State Park, CO	DT	(trnL-F)	YT	YT
DT MT M1	Missoula, MT	DT	(trnL-F)	DT	DT
DT MT M2	Missoula, MT	DT	(trnL-F), matK	DT	DT
DT MT	Missoula, MT	DT	none		DT
DT MT 2	Missoula, MT	DT	none		DT
DT MT M3 09	Missoula, MT	DT	(trnL-F), matK	DT	DT
DT MT M9 09	Missoula, MT	DT	(trnL-F), matK	DT	DT
DT MT M6 09	Missoula, MT	DT	matK	DT	DT
DT MT M8 09	Missoula, MT	DT	(trnL-F), matK	DT	DT
DY F1 15	Hand crossed	Hybrid	(trnL-F)	DT	DT
ID-M5-09	Palisades, ID	Hybrid	(trnL-F)	YT	YT
ID-U2-09	Palisades, ID	Hybrid	(trnL-F)	YT	YT
LB07 P1 E1	Boulder, MT	Hybrid	(trnL-F), matK	YT	YT
LB07 P2 C1	Boulder, MT	Hybrid	(trnL-F)	YT	YT
ND 140 0314499 5351890 1a	Burlington, ND	YT	(trnL-F), matK	DT, YT in matk	YT
ND 14U 0314503 5351862 2a	Burlington, ND	YT	(trnL-F), matK	YT	YT
P2	Palisades, ID	Hybrid	matK	YT	YT
P3	Palisades, ID	Hybrid	matK	YT	YT
R-06-01	Radersburg, MT	Hybrid	matK	YT	YT
R-06-03	Radersburg, MT	Hybrid	(trnL-F), matK	DT	DT

R-06-07	Radersburg, MT	Hybrid	(trnL-F)	YT	YT
R-06-08	Radersburg, MT	Hybrid	(trnL-F)	YT	YT
R-06-15	Radersburg, MT	Hybrid	(trnL-F)	YT	YT
SD 14T 0488874 5058875	Leola, SD	YT	(trnL-F), matK	YT	YT
SD 14T 0488874 5058074	Leola, SD	YT	(trnL-F), matK	YT	YT
SD 14T 0488875 5058077	Leola, SD	YT	(trnL-F), matK	YT	YT
SD 14T 0488877 5058067	Leola, SD	YT	(trnL-F), matK	YT	YT
YD 23	Hand crossed	Hybrid	(trnL-F)	YT	YT
YT AK 4	Fairbanks, AK	YT	matK	YT	YT
YT AK 08-1	Fairbanks, AK	YT	none		YT
YT AK 08-4	Fairbanks, AK	YT	none		YT
YT FT FS 2	Flat Tops, CO	YT	none		YT
YT FT OS P1	Flat Tops, CO	YT	(trnL-F)	YT	YT
YT FT PS 2	Flat Tops, CO	YT	none		YT
YT MT 05 09	Ovando, MT	YT	(trnL-F), matK	YT	YT
YT MT 06 09	Ovando, MT	YT	matK	YT	YT
YT MT 07 09	Ovando, MT	YT	(trnL-F), matK	YT	YT
YT PC8	Pine Creek, MT	YT	(trnL-F)	YT	YT
YT PC-7	Pine Creek, MT	YT	failed	YT	YT
YT MT 09	Ovando, MT	YT	failed	YT	YT

Discussion

The best DNA barcodes should exhibit a high degree of interspecific variation and small degree of intraspecfic variation (Lahaye et al. 2008). Our results indicate that *TrnL-F* was the best candidate pDNA barcoding region for yellow and Dalmatian toadflax. *TrnL-F* had two polymorphic sites that could be used to readily distinguish between these two species. Each of these polymorphic sites was distinct and consistent for each species, for many of the sampled individuals. The only individuals which were not consistent were DT LSP, DT Cherry Street and ND 14U 0314499 5351890. DT LSP and DT Cherry individuals morphologically resembled Dalmatian toadflax, but possessed a yellow toadflax haplotype based on these results and on the PCR-RFLP results (see Chapter 2). These findings suggest that these individuals are most likely backcrossed individuals that have a predominantly Dalmatian toadflax ncDNA. The ND 14U 0314499 5351890 individual expressed a Dalmatian haplotype for the *trnL-F* DNA barcode. This same individual expressed a yellow toadflax haplotype based on the *matK* DNA barcode at 3 different sites and the Alu1 PCR-RFLP marker. The polymorphism that occurred in the *trnL-F* sequence is therefore most likely a sequencing error.

The *matK* plastid region revealed strong population resolution, which works as a reliable species diagnostic for only specific yellow toadflax (YT SD, YT ND, YT AK, YT PC, YT ALTA) and Dalmatian toadflax (DT MT, DT Helena, DT EH) populations. The *matK* region had 8 polymorphic characters: 88, 297, 354, 359, 379, 456, 502, and 706 (see Table 9). Polymorphism at site 88 (G place of C), 354 (G in place of C), 359 (T in place of C) could distinguish YT SD or ND from other yellow toadflax and Dalmatian toadflax individuals. The unique character (T in place of C) at 297 was able to distinguish YT MT and YT AK accessions from other yellow toadflax accessions. The polymorphic characters at 456 (C in place of T) and

706 (G in place of A) could delineate DT MT, DT EH, and DT Helena from other Dalmatian toadflax and yellow toadflax accessions. The polymorphism at 502 (C in place of T) could distinguish YT SD, YT PC, YT SD, YT ALTA, from other yellow toadflax and Dalmatian toadflax accessions. Combined as a barcode, these 8 polymorphic sites could distinguish yellow toadflax pDNA from Dalmatian toadflax pDNA in all accessions except DT LM.

Table 9. pDNA Haplotypes based on *matK* pDNA Barcode.

pDNA Haplotpyes	Site 88, 354,359, 379	Site 297	Site 456	Site 502	Site 706
YT SD	X			X	
YT ND	X			X	
YT WY				X	
YT AK		X			
YT FT					
YT LM					
YT MT		X			
YT PC				X	
DT MT			X		X
DT Helena					X
DT EH					X
DT LSP					
DT Cherry					

Of the four regions used, *matK* and *trnL-F* regions had the best interspecific variation with low intraspecfic variation, with *trnL-F* being the more reliable of the two. The pDNA region *trnL-F* could delineate between yellow toadflax and Dalmatian toadflax The *matK* region had a moderate degree of interspecific variation, but the variation was consistent across populations within each species. This variation could possibly be used to delineate population haplotypes, but more individuals from each population need to be analyzed before *matK* barcode can be used for that purpose. Currently it seems that there are 5 haplotypes that can be determined by *matK*, 3 yellow toadflax haplotypes and 2 Dalmatian toadflax haplotypes. The degree of genetic

variation occurring among haplotypes found in Dalmatian toadflax individuals from Colorado populations at Lory State Park and from the Cherry Street site in Fort Collins suggests that these individuals are not taxonomically unambigious Dalmatian toadflax. More investigation into these individuals should be conducted to determine if this variation is a result of hybridization or because these are another subspecies of Dalmatian toadflax. The *matK* and *trnL-F* regions taken together as a DNA barcode can be used as a tool to help land managers using biocontrol to determine the maternal background of hybrid toadflax plants invading their area. Most importantly, using these DNA barcodes to determine pDNA haplotypes of toadflax hybrids can determine gene flow patterns, which could help researchers determine the genetic makeup of future hybrid populations.

CONCLUSION

The *trnT/D* region digested with Alu1 plastid marker and the *matk* and *trnL-F* DNA barcodes had multiple interesting points of confirmation (see Table 8). Both marker systems identified discrepancies in Dalmatian toadflax from Cherry Street in Fort Collins and from Lory State Park. Both LSP and Cherry Street individuals apparently contained yellow toadflax pDNA, which was detected using both marker systems. These individuals appeared to be Dalmatian toadflax based upon morphology. There could be some form of hybridization occurring at these sites or these individuals could be another biotype of Dalmatian toadflax. But more likely these individuals are the offspring of an earlier hybridization event, since no parental plants are present at this location. The pDNA haplotypes based on the PCR-RFLP Alu1 markers that were observed in plants from Radersburg, Palisades, LB, Boulder, and Abbey sites were all exactly the same haplotypes based on the DNA barcoding results. The number of plants from the Radersburg, Palisades, and Boulder locations that were barcoded was smaller than the number of individuals haplotyped using PCR-RFLP markers. However, those individuals that were tested with DNA barcodes possessed the same pDNA as was determined by the Alu1 marker, except for the ND 140 0314499 5351890 individual, which was probably a sequencing error. In the hand-crossed hybrid individuals tested with both marker systems (F1 (DY F1 15, YD 23) and BC (BC-E-09 and BC-F-05)), there were identical results, which further confirms the reliability of these two markers. That fact that *trnL-F* and *matK* individuals tested displayed the same species identity as the *trnT/D* region digested with Alu1, supports the reliability of these two marker systems as pDNA species diagnostic markers.

These results also suggest that yellow toadflax pDNA is more common than Dalmatian toadflax pDNA in hybrids from field locations. This indicates that there may be some sort of

prezygotic or postzygotic incompatibility causing an increased proportion of hybrids with yellow toadflax pDNA to survive, resulting in asymmetric pDNA gene flow occurring in some hybrid populations in the field. In the future as more hybrid populations are discovered, each population should be tested to determine its pDNA to get a better understanding about pDNA gene flow within hybrid toadflax populations.

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