

Hybridization between Invasive Populations of Dalmatian Toadflax (*Linaria dalmatica*) and Yellow Toadflax (*Linaria vulgaris*)

Sarah M. Ward, Caren E. Fleischmann, Marie F. Turner, and Sharlene E. Sing*

Although there is evidence that interspecific hybridization can initiate invasion by nonnative plants, there are few documented examples of novel hybridization events between introduced plant species already exhibiting invasive behavior. We conducted morphometric and molecular analyses of toadflax plants with intermediate morphology found at two sites in Montana, which were co-invaded by yellow toadflax and Dalmatian toadflax. Field-collected putative hybrid plants had intermediate morphometric scores (mean 0.47, on a scale of 0.0 = indistinguishable from Dalmatian toadflax to 1.0 = indistinguishable from yellow toadflax) for a suite of phenotypic traits that differentiate the parent species (leaf length : width ratio, growth form, seed morphology, inflorescence type, and ventral petal shape). Inter-simple sequence repeat (ISSR) analysis of a subset of these putative hybrids revealed combinations of species-diagnostic bands, confirming the presence of DNA from both parent species. Controlled interspecific hand-pollinations generated viable first generation (F_1) hybrid plants that also had intermediate morphometric scores (mean 0.46) and a mix of species-diagnostic ISSR bands from both parents. The hand-generated F_1 hybrids crossed readily with both parent species to produce viable first generation backcrossed (BC_1) plants. Our results confirm that hybridization is occurring between invasive populations of yellow toadflax and Dalmatian toadflax, and that the hybrid progeny are viable and fertile. This example of hybridization between alien congeners is of concern as the parent taxa are already known to be highly invasive. Further research is needed to assess the invasive potential of hybrid toadflax populations, and the likelihood of introgressive trait transfer between the parent species.

Nomenclature: Dalmatian toadflax, *Linaria dalmatica* (L.) P. Mill., LINDA; yellow toadflax, *Linaria vulgaris* P. Mill. LINVU.

Key words: Hybridization, invasive plant, ISSR, morphometric analysis.

The recorded study of plant hybrids (progeny from naturally occurring sexual reproduction between differentiated plant taxa) dates back to observations of spontaneously hybridizing squash and gourd plants in the early 1700s (Zirkle 1935). Because interspecific plant hybrids frequently exhibit reduced fitness, this phenomenon was dismissed for many years as being evolutionarily unimportant (Burke and Arnold 2001). However, there is now growing recognition of the role that interspecific hybridization and subsequent gene flow has played in the evolution of many plant species (Rieseberg 1997; Rieseberg and Carney 1998), and that it can initiate or accelerate

invasion of a novel range by nonnative plants as first suggested by Abbott (1992). In a seminal review, Ellstrand and Schierenbeck (2000) proposed that hybridization can lead to the evolution of invasiveness, and in a recent revision of the topic, these authors cite 35 examples where range expansion by plants was apparently preceded by hybridization between taxa (Schierenbeck and Ellstrand 2009). The underlying genetic mechanisms proposed for increased invasiveness following hybridization include restoration of selectable genetic variation in colonizing species suffering from founder effect, introgression of advantageous locally adapted alleles through hybridization and backcrossing between related nonnative and native taxa, fixable heterosis in early-generation hybrid progeny, and reduced genetic load following exchange of deleterious alleles for neutral or advantageous equivalents (Ellstrand and Schierenbeck 2000; Lee 2002; Rieseberg et al. 2007; Sakai et al. 2001).

A number of studies have investigated hybridization between an introduced species and a native congener as a

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*First, second, and third authors: Associate Professor, Undergraduate Student, and Graduate Student, Department of Soil and Crop Sciences, Colorado State University, Fort Collins, CO 80523; fourth author: Research Entomologist, USDA-USFS, Rocky Mountain Research Station, Bozeman, MT 59717. Corresponding author's E-mail: sarah.ward@colostate.edu

Interpretive Summary

Interspecific hybridization (i.e., cross-pollination between different but related species to produce viable progeny) has been implicated as a causal factor in several major plant invasions. In addition to generating hybrid offspring that could be more invasive in a novel range than either parent species, cross-pollination between different species can lead to the transfer of potentially advantageous traits from one species to another. Yellow toadflax and Dalmatian toadflax are not reported to hybridize within their native European ranges; however, invasive populations of these species in the Rocky Mountains are growing in sufficient proximity at some locations to allow cross-pollination between them. We collected and analyzed plants of intermediate phenotypic appearance from two such sites in Montana, comparing their morphology and DNA with that of unambiguous yellow toadflax and Dalmatian toadflax plants from isolated populations. Molecular marker analysis of these intermediate plants confirmed that they are hybrids containing DNA from both yellow and Dalmatian toadflax. We also generated known hybrid plants by controlled cross-pollination of yellow and Dalmatian toadflax in the greenhouse; significant numbers of these hybrids were viable, fertile, and crossed readily with each other and with the parent species.

Our results confirm that yellow toadflax and Dalmatian toadflax spontaneously hybridize, and that hybrid toadflax populations are developing at sites co-invaded by these introduced species. These hybrid populations may be able to expand into currently noninvaded areas, and could also be less responsive to chemical and biological control than their parents. Further research is needed to determine whether hybrid toadflax will require more aggressive management than current toadflax invasions.

possible stimulus for invasion (Ellstrand and Schierenbeck 2000). Novel hybridization between nonnative plant species within an invaded range has been described in fewer genera, including *Rhododendron* (Milne and Abbott 2000), *Tamarix* (Gaskin and Schall 2002), *Fallopia* (Gammon et al. 2007; Tiebre et al. 2007), and *Wisteria* (Trusty et al. 2007). The results described here provide evidence that spontaneous hybridization is occurring in the U.S. Intermountain West between yellow toadflax (*Linaria vulgaris* P. Mill.) and Dalmatian toadflax [*Linaria dalmatica* (L.) P. Mill.], two weeds already listed as noxious or considered invasive in multiple states. This example of hybridization between alien congeners is of particular concern as the parent taxa are already known to be highly invasive, and hybridization between North American field populations of these toadflax species appears to be a recent phenomenon.

Yellow toadflax is an herbaceous perennial native to north and central Europe, where it has a long history of use as a medicinal and ornamental plant (Fernald 1905). Early settlers brought yellow toadflax to the eastern United States sometime before 1672, and its proliferation in mid-Atlantic coastal settlements by the mid-1700s represents the earliest recorded invasion by a nonnative plant in North America

(Mack 2003). Yellow toadflax has since spread throughout the continent (Lajeunesse 1999). Although Sutton et al. (2007) reported yellow toadflax invading intact native plant communities at high elevations in the northern and central Rocky Mountains, it is more typically found in areas subject to grazing, cultivation, or other forms of disturbance (Clapham et al. 1957). Yellow toadflax has complete and perfect flowers, but is an obligate outcrosser due to a single locus four S-allele gametophytic self-incompatibility system (Docherty 1982). Plants are pollinated by a number of different insects attracted to the clusters of flowers on shoots that are 30 to 100 cm tall (Arnold 1982); it can reproduce both sexually by seed and vegetatively via adventitious shoots from rhizomes (Bakshi and Coupland 1960; Nadeau et al. 1992). The invasiveness of yellow toadflax in the Intermountain West may be due at least in part to its perennial habit, capacity for vegetative reproduction, and production of large numbers of genetically variable seeds (Lajeunesse 1999; Parker and Peabody 1983). A recent study found high levels of intraspecific diversity in yellow toadflax, consistent with its outcrossing breeding system and a probable history of multiple human introductions (Ward et al. 2008).

Dalmatian toadflax is a tall (50–120 cm) perennial herb thought to have originated in the Mediterranean region, with a native range extending from Yugoslavia to northern Iran (Alex 1962). Vujnovic and Wein (1997) suggest that Dalmatian toadflax was first imported to the United States as an ornamental in 1894, although the earliest known authenticated specimen was collected in San Gabriel, CA, in 1920. A putative subspecies, narrow-leaved Dalmatian toadflax, *Linaria dalmatica* (L.) P. Mill. ssp. *macedonica* (Griseb.) D. A. Sutton, distinguished from broad-leaved Dalmatian toadflax primarily by differences in leaf morphology, has been reported, but the taxonomic status of this morphotype remains uncertain, and it is possible that some narrow-leaved Dalmatian toadflax populations actually contain hybrids (Wilson et al. 2005). Like yellow toadflax, Dalmatian toadflax has complete perfect insect-pollinated flowers and is obligately outcrossing (Bruun 1937); however, there are no published reports describing the genetic basis of self-incompatibility in Dalmatian toadflax. Seeds produced from late June to December can germinate both in fall and spring, or remain dormant but viable in the surrounding soil for up to 10 yr (Robocker 1970). Seedlings develop a deep taproot within 8 wk and in the initial growing season can produce 2 to 5 vertical flowering stems; beginning in the second growing season, a single plant can produce up to 25 floral stems and up to 500,000 seeds annually. Dalmatian toadflax also reproduces vegetatively via stems that develop from adventitious buds on both primary and creeping lateral roots (Robocker 1970, 1974).

Spontaneous hybridization between *Linaria* species within the native Eurasian range of the genus has been reported previously but little studied. It appears that despite marked morphological differentiation, species divergence within *Linaria* is relatively recent and reproductive isolation has not yet fully evolved. With the exception of one tetraploid, Jersey toadflax *Linaria pelisseriana* (L.) Mill.) ($2n = 4x = 48$), all *Linaria* species are diploids with the same chromosome number ($2n = 2x = 24$) (Moore 1982), so ploidy levels do not present barriers to hybridization within the genus. Hybrids resulting from cross-pollination between yellow toadflax and creeping toadflax [*Linaria repens* (L.) Mill.] are common in the United Kingdom, where both are native (Stace 1975). Dilleman (1948) reported that specimens of purple toadflax [*Linaria purpurea* (L.) Mill.] cultivated at the Paris Botanical Gardens spontaneously hybridized with both native yellow toadflax and introduced Dalmatian toadflax growing in proximity, and commented that the whole genus appeared to be “promiscuous.” Bruun (1937) described obtaining seed from a hand-pollination of Dalmatian toadflax by yellow toadflax, but provided no further details. We have found no other published reports confirming hybridization between these two toadflax species.

Yellow toadflax is typically found in moister soils such as gully bottoms and riparian zones, while Dalmatian toadflax prefers drier, sunny areas such as well-drained upper slopes. However, common strong-flying insect pollinators of toadflax such as bumble bees (DeClerck-Floate and Richards 1997) can travel over 1.5 km between pollen sources (Osborne et al. 2008) and the two toadflax species can co-invade sites where the microtopography is sufficiently variable to accommodate both their habitat preferences. In 2005, we observed plants with intermediate leaf and flower morphology at two such locations within the Beaverhead-Deerlodge National Forest in Montana. As hundreds of thousands of dollars are spent annually in attempts to control yellow toadflax and Dalmatian toadflax on public lands in the western United States, the possibility of hybridization between these highly invasive species raised concerns among weed managers that hybrid toadflax populations could present even greater management challenges than the parent species (Hal Pearce, U.S. Forest Service, personal communication). The objectives of the research described here were (1) to ascertain the taxonomic status of morphologically intermediate toadflax plants observed at sites co-invaded by yellow and Dalmatian toadflax, (2) to determine the feasibility of hybridization between yellow and Dalmatian toadflax, and (3) to characterize the viability and fertility of hybrid toadflax progeny. Intermediate morphological or other phenotypic characteristics are not always reliable indicators of interspecific hybridization (Rieseberg and Ellstrand

1993). Therefore, in addition to controlled interspecific cross-pollinations to generate known toadflax hybrids, we combined morphometric with molecular analyses in our investigation of putative hybrid plants collected from the field.

Materials and Methods

Plant Materials, Cross-Pollination, and Morphological Characterization. We collected a total of 74 plants with intermediate leaf and floral morphology from two sites in the Beaverhead-Deerlodge National Forest, MT, between 2005 and 2007. Both sites have a history of multiple disturbances due to fire, logging, mining, and livestock grazing, and have actively invading populations of yellow and Dalmatian toadflax growing within a few meters of each other on steeply sloping terrain. To represent the parent species while ensuring that no hybrid or introgressed individuals were included in our experimental stocks, 24 yellow toadflax plants and 27 Dalmatian toadflax plants were collected separately from isolated and taxonomically unambiguous Dalmatian and yellow toadflax populations in Colorado and Montana. All plants were transplanted into commercial potting compost¹ in 30-cm plastic pots and maintained in a greenhouse at Colorado State University. Irrigation was provided via an automated drip system, and plants were fertilized monthly with Miracle-Gro commercial fertilizer (20–20–20).² Greenhouse temperatures were maintained at 20/15 C day/night (corresponding to photoperiod), with supplemental lighting provided during the winter months to maintain a minimum photoperiod of 10 hr daily, increasing to a maximum of 14 hr daily in summer.

We hand-pollinated greenhouse-grown plants by first removing the undehiscent anthers from the flowers of plants designated as female parents. We then detached an entire flower with dehiscing anthers from the designated pollen donor, folded back the petals, and gently rubbed the anthers on the stigma of the pollen recipient. Although both yellow toadflax and Dalmatian toadflax are self-incompatible, emasculating the female parent reduced pollen competition on the stigma and increased seed set. We labeled hand-pollinated flowers and enclosed them in glassine pollination bags³ for 5 to 7 d, then visually examined them for withering of the stigmas and expansion of the ovaries, indicating seed set. We removed the pollination bags and began harvesting seed when the capsules started to dry and turn brown, generally after another 35 to 40 d. We estimated the feasibility of each class of controlled cross as the percentage of individual pollinations resulting in a mature seed-bearing capsule. Details of crosses made to generate F_1 and BC_1 plants are summarized in Table 1. We obtained seed from 51 of the 74 field-collected putative hybrids by hand-pollination

Table 1. Controlled pollinations carried out and resulting seed set.

Cross	Number of pollinations	Percentage seed set
Yellow ^a × Dalmatian ^a	117	49.1
Dalmatian × yellow	109	10.9
F ₁ ^a hybrid (yellow × Dalmatian) × yellow	24	41.6
F ₁ hybrid (Dalmatian × yellow) × yellow	29	22.1
Yellow × F ₁ hybrid (yellow × Dalmatian)	23	52.2
Yellow × F ₁ hybrid (Dalmatian × yellow)	31	35.5
F ₁ hybrid (yellow × Dalmatian) × Dalmatian	31	67.7
F ₁ hybrid (Dalmatian × yellow) × Dalmatian	43	79.1
Dalmatian × F ₁ hybrid (yellow × Dalmatian)	18	27.8
Dalmatian × F ₁ hybrid (Dalmatian × yellow)	29	34.5
F ₁ hybrid (Dalmatian × yellow) × F ₁ hybrid (yellow × Dalmatian)	8	25.0
F ₁ hybrid (yellow × Dalmatian) × F ₁ hybrid (yellow × Dalmatian)	7	57.1

^a Abbreviations: Yellow, yellow toadflax; Dalmatian, Dalmatian toadflax.

with either yellow or Dalmatian toadflax pollen. Plants that failed to set seed were not included in the morphometric analysis.

Morphometric index scores were assigned to 20 Dalmatian toadflax, 20 yellow toadflax, and 51 field-collected putative hybrid plants maintained in the greenhouse, based on the following species-diagnostic characters: leaf length : width ratio, shape of ventral petal, inflorescence type, growth form, and seed type. To reduce the effect of environmental variation on flower and leaf morphology and growth form, plants were scored only after at least 6 mo of growth in a common greenhouse environment and at the same time of year (late April through June). We assigned a sub-score of 0.0 to each feature characteristic of Dalmatian toadflax (pointed ventral petal, branched inflorescence, rosette growth form, pyramid seed), and a sub-score of 1.0 to each feature characteristic of yellow toadflax (round ventral petal, non-branched inflorescence, spreading mat-like growth form, and winged seed). Intermediate phenotypes for these features were scored as 0.25 (more like but not identical

to Dalmatian toadflax), 0.5 (intermediate between the two species), or 0.75 (more like but not identical to yellow toadflax). Leaf length : width ratios were standardized as $(X - X_{dal}) / (X_{yt} - X_{dal})$ where X = mean of leaf length : width ratios for five fully-developed leaves from an individual plant, X_{dal} = mean leaf length : width ratio for 20 Dalmatian toadflax plants sampled, and X_{yt} = mean leaf length : width ratio for 20 yellow toadflax plants sampled. The final morphological hybrid index score for each plant was calculated as $(\Sigma \text{subscores}) / 5$.

Generation and Characterization of F₁ Interspecific Hybrids. To confirm that interspecific hybridization between yellow toadflax and Dalmatian toadflax produced viable offspring, and to generate known F₁ interspecific hybrids for further analysis and comparison with putative field-collected hybrids, we germinated hybrid seed produced by the controlled hand-pollinations described above. Seed dormancy has been reported in yellow toadflax, with germination requiring at least 8 wk of wet stratification at 5 C (Nadeau and King 1991). Germination of Dalmatian toadflax seeds requires prechilling for 2 wk at 3 C according to Andersen (1968). Our F₁ and BC₁ hybrid seed showed stronger dormancy than either of these previous reports suggest: fresh seed failed to germinate either if planted immediately, or if wet stratified for 8 wk at 4 C following Nadeau and King's 1991 protocol (data not shown). We optimized germination by placing freshly collected hybrid seed in paper envelopes sealed in plastic bags in a freezer for a cold stratification period of 20 wk at -20 C. At the end of this period, seed was surface-sterilized in 20% bleach solution for 5 min, rinsed three times in distilled water, placed on wet filter paper in a sealed petri dish, and cold stratified for an additional 6 wk at 4 C. The petri dishes were then placed in a growth chamber at 20 C under continuous light and inspected daily. Germination rates were recorded by counting the number of seeds with a visible emerging radicle at least equal in length to the diameter of the seed. Once recorded, each germinating seed was transferred from the petri dish to a 5-cm square peat pot filled with commercial seed starter compost,⁴ and returned to the growth chamber. We recorded seedling survival as the number of seedlings that developed four to six normal true leaves. At this stage, we transplanted seedlings into 10-cm plastic pots filled with commercial potting compost and transferred them to the greenhouse, where they were maintained as described above. Hybrid seedlings were transplanted into 30-cm pots when they reached sufficient size.

After a minimum of 6 mo of growth in the greenhouse, we assigned morphological index scores (as described above) to 36 F₁ hybrid plants. To test for self-compatibility, we enclosed individual flowering shoots on 15 F₁ plants in glassine pollination bags throughout

Table 2. Inter-simple sequence repeat (ISSR) primer sequences and species-diagnostic amplification products.

ISSR primers	Bands diagnostic for yellow toadflax	Bands diagnostic for Dalmatian toadflax
5'-ACACACACACACACACC-3'	390 bp ^a	700 bp
5'-GAGAGAGAGAGAGAYC-3'	60 bp, 180 bp, 225 bp	75 bp, 250 bp, 275 bp
5'-GTGTGTGTGTGTGTGYC-3'	None	200 bp
5'-TGTGTGTGTGTGTGTGRA-3'	850 bp	None

^aAbbreviation: bp = base pairs.

anthesis and then visually examined them for seed set. To determine hybrid fertility, we made a total of 228 backcrosses between F₁ hybrid plants and the two parent species, following the controlled pollination protocol described above. We also made F₁ × F₁ crosses. Details of all hand-pollinated crosses and resulting seed set can be found in Table 1.

Molecular Confirmation of Interspecific Hybridization.

As noted in the Introduction, intermediate phenotypes are not always a reliable indicator of hybridization. To confirm that morphologically intermediate plants were indeed interspecific hybrids, ISSR markers (Gupta et al. 1994) were used to characterize the genotypic composition of a subset of morphologically intermediate plants. ISSR markers were chosen for this because they require no previous sequence information and are robust and repeatable. The ISSR technique also samples multiple loci throughout the genome simultaneously, increasing the likelihood of identifying species-diagnostic DNA polymorphisms that can be used to confirm the taxonomic status of putative hybrid individuals. DNA for molecular analysis was extracted from approximately 100 mg of young leaf tissue collected from each plant, using the Qiagen DNeasy Plant Mini extraction kit,⁵ which produced final yields of 15 to 20 µg DNA/µL. Each PCR (polymerase chain reaction) tube contained 12.5 µl of Go-Taq Green Master Mix⁶ (equivalent to approximately one unit of Taq polymerase), 2 µl of genomic DNA template solution, 0.5 µl of 25 µM primer solution, and 10 µl nuclease-free sterile water, for a total reaction volume of 25 µl. Amplifications were performed in a MyCycler thermocycler⁷ with a 5-min initial denaturation step at 95 C, followed by 35 cycles of 94 C for 40 s, 47 C for 45 s, and 72 C for 90 s. Amplification products were separated on 1.5% agarose gels and stained with ethidium bromide, and digital images of the gels were generated under UV light using an Alpha-Innotech imager.⁸ Image files were imported into iPhoto,⁹ allowing bands to be visually identified and scored as present or absent using a superimposed grid.

After screening 21 ISSR primers supplied by the University of British Columbia, we selected 4 that generated a total of 10 species-diagnostic bands (i.e.,

distinct and repeatable DNA bands found in more than 90% of individuals in one parent species but fewer than 10% of individuals in the other, based on screening 20 reference plants of each species). To ensure their taxonomic identity, reference plants were collected from allopatric Dalmatian toadflax and yellow toadflax populations that contained no morphologically intermediate individuals. As we did not find any species-specific ISSR amplification products (i.e., bands that are always present in individuals of one species and always absent in individuals of the other species), we selected the 90% threshold for identifying a band as species-diagnostic based on Buerkle (2005). ISSR primer sequences and diagnostic amplification products are listed in Table 2. The selected diagnostic primers were used to amplify DNA from eight putative hybrids from the Montana field sites described previously, and from four known F₁ hybrids produced by hand-pollination. We also carried out ISSR marker analysis on six plants from a population in Durango, CO, that had previously been identified as narrow-leaved Dalmatian toadflax (Rod Cook, La Plata County Weed Office, personal communication).

Results and Discussion

Cross-Pollinations and Hybrid Generation. Table 1 summarizes the numbers of hand-pollinations carried out for the different classes of controlled hybridization, and the percentage seed set obtained for each class. F₁ seed set from interspecific crosses was higher with yellow toadflax as the female parent than with Dalmatian toadflax (49.1 vs. 10.9%), possibly due to some degree of nuclear-cytoplasmic incompatibility. Despite this lower initial seed set when Dalmatian toadflax was the female parent, the 61 F₁ plants and 66 BC₁ plants that we have grown to flowering include vigorous and fertile individuals from this as well as from the reciprocal cross. However, we did observe differences in germination and seedling survival rates among F₁ families that were each derived from a different cross. These rates ranged from 4.0% germination and 0% seedling survival for the least successful F₁ family, to 71.4% germination and 34.3% seedling survival for the most successful. Germination and seedling survival also varied among the five BC₁ families we have raised to date, from 24.7% germination and 7.5% seedling survival for the least

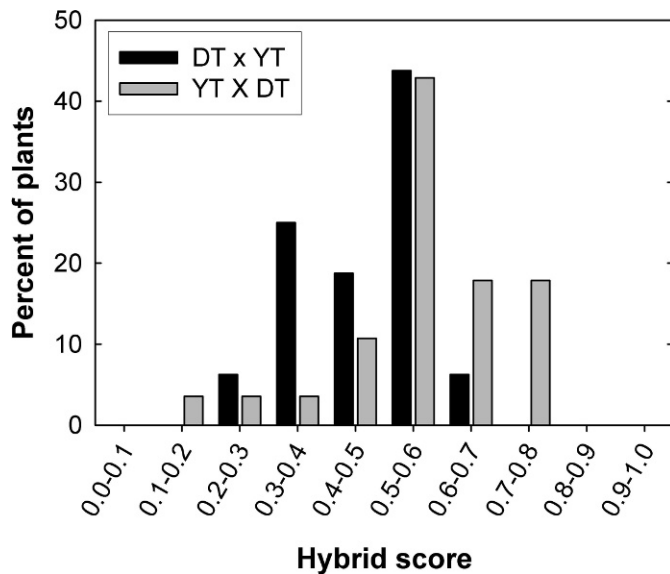


Figure 1. Morphometric hybrid scores for 36 first generation F₁ hybrid plants from controlled pollinations of yellow toadflax by Dalmatian toadflax (YT × DT) and Dalmatian toadflax by yellow toadflax (DT × YT). The scale is from 0.0 (indistinguishable from Dalmatian toadflax) to 1.0 (indistinguishable from yellow toadflax).

successful family to 72.6% germination and 50.0% seedling survival for the most successful. These differences suggest that not all combinations of parental genotypes are equally viable, and even among parental genotypic combinations that do yield F₁ and BC₁ progeny, differences in specific combining ability result in variable vigor among families. Given that high levels of intraspecific genetic diversity have been reported in yellow toadflax (Ward et al. 2008) and may also exist in Dalmatian toadflax, this is not surprising. Nevertheless, our results show that if a sufficiently genetically diverse array of yellow toadflax and Dalmatian toadflax individuals are involved in interspecific pollinations, compatible genotypic combinations will occur in F₁ and BC₁ generations, resulting in viable and vigorous hybrid progeny.

The self-incompatibility of both parent species persisted in their hybrid offspring, with no seed obtained from any of the 15 self-pollinated F₁ plants. However, in all hybridization classes involving F₁ plants as male or female parents, seed was obtained from at least 22% of pollinations, with seed set exceeding 79% in one backcross class (Table 1). This indicates that early-generation hybrids between yellow and Dalmatian toadflax are both viable and fertile. These hybrids, therefore, can form potentially invasive populations containing novel recombinant genotypes. Toadflax hybrids also provide a potential route for introgression of genetic material from one parent species to the other via repeated backcrossing. The implications of this are discussed further below.

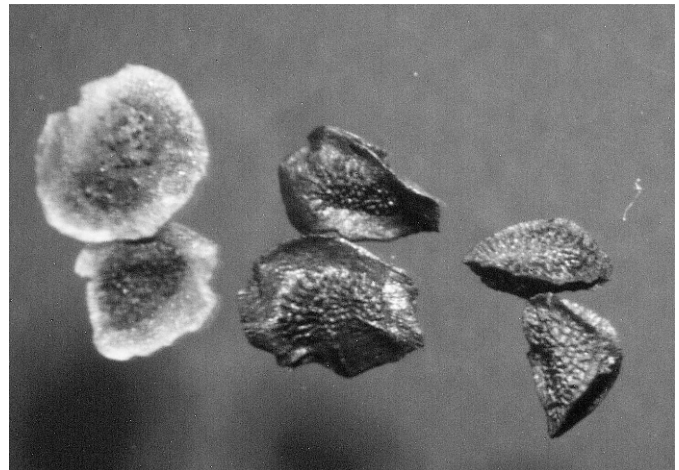


Figure 2. Seeds of yellow toadflax (left), Dalmatian toadflax (right), and a yellow × Dalmatian first generation F₁ hybrid plant (center).

Morphological Characterization of Hybrids. Index scores for hand-generated F₁ hybrids ranged from 0.19 to 0.72, with a mean of 0.46 (Figure 1). These plants were typically intermediate in leaf length : width ratio compared to the parents, with different combinations of parental floral characteristics. Seeds from most F₁ plants were intermediate in appearance, combining the pyramid shape of Dalmatian toadflax seed with the flat winged extension typical of yellow toadflax seed (Figure 2). However, the growth form (yellow-type mat vs. Dalmatian-type rosette) typically resembled that of the female parent. Figure 1 shows that the highest percentage of F₁ plants from both yellow × Dalmatian and Dalmatian × yellow crosses were intermediate in appearance, with morphometric index scores between 0.5 and 0.6. However, there was a considerable range of morphological variation. This lack of uniformity in the F₁ plants is not surprising: both parent species are obligate outcrossers, so high levels of heterozygosity would be expected across the loci contributing to the morphological traits that we scored.

Morphometric index scores for the 51 field-collected hybrids ranged from 0.09 to 0.87, with a mean of 0.47 (Figure 3). This sample is almost certainly biased towards individuals with intermediate phenotype, because we collected putative hybrids in the field based on visual assessment of atypical leaf and flower morphology. Nevertheless, these plants exhibit a range of morphological profiles. Our data on F₁ and BC₁ hybrid progeny produced by hand-pollination suggest that early-generation hybrids can not only survive and persist, but can also cross-pollinate each other and backcross with the parent species to produce a range of hybrid genotypes within a single population. As both yellow toadflax and Dalmatian toadflax are perennial species capable of clonal growth, it is likely that naturally occurring hybrid zones contain a mixture of early and

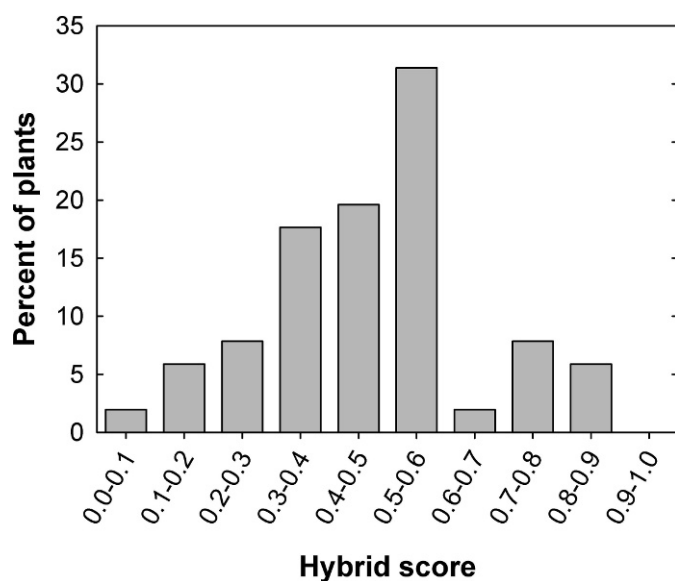


Figure 3. Morphometric hybrid scores for 51 field-collected putative hybrid toadflax plants, on a scale from 0.0 (indistinguishable from Dalmatian toadflax) to 1.0 (indistinguishable from yellow toadflax).

late-generation hybrids, including backcrosses. This is reflected in the varied combinations of parental characteristics we found in our field-collected plants.

Molecular Analysis. The ISSR profiles of all putative field-collected hybrids screened, and the known F_1 individuals, contained at least one diagnostic band from yellow toadflax and at least one from Dalmatian toadflax, although different combinations of the various bands were present in different individuals (see Figure 4 for a representative gel image). Because we were working with relatively few species-diagnostic markers, and we only ran ISSR profiles on a small subset of our field-collected putative hybrid plants, we did not attempt to develop a more comprehensive marker-based numerical hybrid index. Based on our criteria for selecting species-diagnostic markers, the probability is 81% or better that any plant with an ISSR profile containing at least one diagnostic band from each parent species is an interspecific hybrid. For plants with two or more diagnostic bands from each parent species, this probability increases to greater than 99%; this was the case for all four of the known F_1 hybrid individuals screened, and for six of the eight field-collected putative hybrids. Of the other two field-collected plants screened, one had a single diagnostic ISSR band from each parent species (81% hybrid probability), and the other had two bands diagnostic of yellow toadflax and one diagnostic of Dalmatian toadflax (89% hybrid probability). More extensive molecular analyses of hybrid toadflax populations could usefully identify the most prevalent genotypes in a hybrid swarm at an invasion site, and provide information

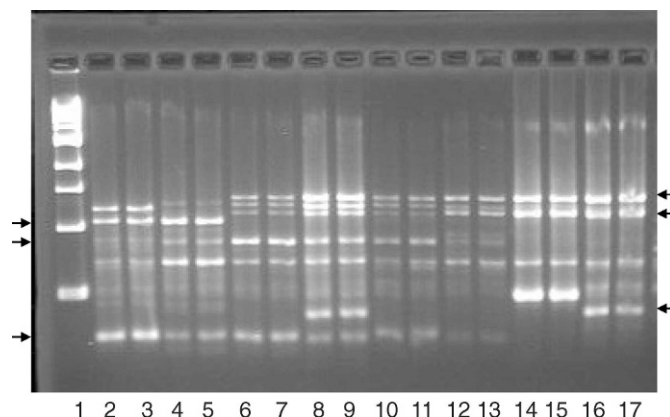


Figure 4. Amplification of total genomic DNA with inter-simple sequence repeat (ISSR) primer 5'-GAGAGAGAGAGAGAGAYC-3'. Each individual genotype is represented by two replicated amplifications. Lane 1: 1 kb ladder; lanes 2 to 3 and 4 to 5: yellow toadflax (two individuals); lanes 6 to 7, 8 to 9, 10 to 11, 12 to 13: putative field-collected hybrids (four individuals); lanes 14 to 15 and 16 to 17: Dalmatian toadflax (two individuals). Arrows indicate species-diagnostic bands.

on the persistence and relative fitness of different hybrid genotypes. Development of microsatellite loci for *Linaria* species would be especially useful in this context: dominant markers such as ISSRs can confirm hybrid genotypes, but do not distinguish effectively between early and later generation hybrids (Buerkle 2005; Minder et al. 2007). However, more detailed marker-based analysis of the composition of naturally occurring *Linaria* hybrid populations was beyond the scope of this study.

ISSR profiles for the plants from Durango, CO, previously identified as narrow-leaved Dalmatian toadflax, all contained diagnostic bands for Dalmatian toadflax, but none for yellow toadflax. It appears, therefore, that this population is indeed a narrow-leaved morphotype of *L. dalmatica*. However, given the variable leaf morphology that we have observed in a range of known hybrid plants, it is possible that some populations thought to be narrow-leaved Dalmatian toadflax do in fact contain hybrid individuals. The extent of hybridization between Dalmatian toadflax and yellow toadflax is currently unknown, but may well be more widespread than previously realized.

Potential Impact of Toadflax Hybrids. Our results confirm that cross-pollination between yellow toadflax and Dalmatian toadflax produces viable and fertile hybrid progeny, and that such hybridization is occurring spontaneously at sites co-invaded by these species. The hybrid swarms that we have sampled to date are relatively small, but without information on the demographics of such populations, we do not know if this is because they tend to be transitory or because gene flow between the parent species in the invaded range is a novel event. Hybridization

has long been recognized as an important mechanism for increasing genetic variation and producing novel gene combinations on which natural selection can act (Lewontin & Birch 1966; Rieseberg and Carney 1998; Stebbins 1959). Gene flow between differentiated plant taxa has several potential outcomes, some of which could be of concern in relation to toadflax. One possibility is that heterosis (“hybrid vigor”) could result in interspecific hybrids that are more widely adapted and aggressively invasive than the parent species; this phenomenon has been reported previously for *Tamarix* in North America (Gaskin and Schaal 2002) and for *Fallopia* in Europe (Tiebre et al. 2007). Heterosis could result in hybrid toadflax displacing one or both parent species, or moving into new habitats not currently invaded by *Linaria*. Continued genetic recombination means that heterosis is transitory and will be most apparent in early-generation hybrid progeny, especially the F₁. However, all the hybrid toadflax plants that we have examined possess the perennial habit and capacity for rhizomatous vegetative reproduction found in both parent species, providing a mechanism by which fixed heterotic F₁ genotypes could potentially persist and spread. It is worth noting that documented examples of aggressive invasion apparently stimulated by stabilized heterosis following interspecific hybridization include *Tamarix* (Gaskin and Schaal 2002) and *Myriophyllum* species where heterosis appears to be maintained by vegetative propagation (Moody and Les 2002, 2007).

An alternative scenario is that early-generation toadflax hybrids do not exhibit heterosis and become invasive, but are sufficiently viable and persistent to allow repeated backcrossing to at least one of the parent populations. Results from our experimental greenhouse pollinations show that F₁ hybrids not only cross-pollinate each other, but also readily backcross to both parent species. This provides a route for introgression of genetic material, with hybrid progeny acting as a bridge to facilitate the transfer of potentially advantageous traits from one species to the other. Hybridization leading to introgression and trait transfer among invasive congeners has been documented in other genera. Whitney et al. (2006) describe how hybridization followed by backcrossing transferred increased herbivore resistance from cucumberleaf sunflower (*Helianthus debilis* Nutt.) into populations of the annual sunflower (*Helianthus annuus* L.) that are now expanding into new areas of central and southern Texas. Milne & Abbott (2000) describe how rhododendron populations were able to expand into Britain’s coldest regions following introgression of genetic material from the cold-tolerant North American species Catawba rosebay (*Rhododendron catawbiense* Michx.) into invasive common rhododendron (*Rhododendron ponticum* L.), a Mediterranean native previously confined to warmer parts of its invaded U.K. range. Based on these examples, even if hybrid toadflax populations are themselves noninvasive and ephemeral, the possibility of

introgression leading to advantageous trait transfer between the two invasive parent species would remain a concern.

The presence of hybrid toadflax populations may also complicate the use of classical biological control in managing toadflax invasions. The two exotic insects most widely released as biocontrol agents on toadflax are *Mecinus janthinus* Germar, a stem-mining weevil that establishes most readily on Dalmatian toadflax (Breiter and Seastedt 2007; McClay and Hughes 2007), and *Brachypterolus pulicarius* L., a seed-feeding beetle that has been reported on both yellow and Dalmatian toadflax, but favors yellow toadflax as a host (Hering 2002; MacKinnon et al. 2005). Whether these insects will be effective on hybrid toadflax plants containing genetic material from both host species is not currently known, but is under investigation. Examples of biocontrol programs where agents have performed poorly on hybrid forms of their target weed include *Lantana* (Zalucki et al. 2007), *Myriophyllum* (Moody and Les 2007), and *Melaleuca* (Dray et al. 2004). Optimizing biocontrol efficacy for managing toadflax invasion is desirable as other options are limited, especially for yellow toadflax, which shows limited and variable response to herbicides (Sebastian and Beck 1998, 1999).

Our data confirm that cross-pollination between yellow toadflax and Dalmatian toadflax produces viable and fertile F₁ progeny that backcross readily to each of the parent species, and that these toadflax species are hybridizing spontaneously at sites in Montana where they co-occur. Toadflax hybrids inherit the outcrossing breeding system, perennial habit, and capacity for clonal growth common to both parent species. Consequently, hybrid populations are likely to consist of multiple genotypes representing both early- and late-generation hybrids, and any hybrid genotype with superior fitness can be rapidly fixed and propagated. As both yellow and Dalmatian toadflax are already known to be highly invasive, hybridization between them is of concern because it generates novel genetic combinations on which selection can act, potentially expanding the range of invasive toadflax populations and making them even more difficult to manage using currently available chemical and biological control options.

Sources of Materials

¹ Potting compost, Fafard Custom Mix, Conrad Fafard Inc., Agawam, MA.

² Miracle-Gro Plant Food, Scotts Miracle-Gro Company, Marysville, OH.

³ Glassine pollination bags, Uline, Chicago, IL.

⁴ Scotts seed starter mix, Scotts Miracle-Gro Company, Marysville, OH.

⁵ DNAeasy extraction kits, Qiagen, Valencia, CA.

⁶ GoTaq green Master Mix, Promega, Madison, WI.

⁷ MyCycler thermocycler, BioRad Laboratories, Hercules, CA.

⁸ AlphaImager HP, Alpha Innotech, San Leandro, CA.

⁹ iPhoto imaging software, Apple Computer Inc., Cupertino, CA.

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