Evolutionary Applications www.evolutionaryapplications.org

Evolutionary Applications ISSN 1752-4571

ORIGINAL ARTICLE

Hybridization and invasion: an experimental test with diffuse knapweed (*Centaurea diffusa* Lam.)

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Keywords

Centaurea diffusa, *Centaurea stoebe*, common garden experiment, diffuse knapweed, interspecific hybridization, invasion, spotted knapweed.

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Data for this study are available at Dryad: doi:10.5061/dryad.gh21j.

Received: 6 June 2011
Accepted: 25 July 2011

doi:10.1111/j.1752-4571.2011.00203.x

Abstract

A number of studies have suggested a link between hybridization and invasion. In this study, we experimentally test the potential for hybridization to influence invasion through a greenhouse common garden study. Diffuse knapweed (DK) (*Centaurea diffusa* Lam.) was introduced to North America with admixture from spotted knapweed (SK) (*Centaurea stoebe* subsp. *stoebe* L.). Comparisons between North American DK (including hybrid phenotypes) and native (European) DK in a common garden did not reveal enhanced performance or increased phenotypic variance, suggesting that pre-introduction hybridization or, more generally, post-introduction evolutionary change has not significantly contributed to the invasion of DK. In contrast, early generation hybrids [artificially created Backcross 1 (BC1) plants] exhibited increased variance for eight of the examined traits, and greater leaf and reproductive shoot production when compared to North American DK. Individual BC1 lines differed for several traits, suggesting the importance of the cross for drawing conclusions from such comparisons. When compared to the parental species (DK and SK), the BC1 plants were not transgressive for any of the measured traits. Overall, these findings suggest that if diploid SK is introduced to North America, interspecific hybridization has the potential to result in even more aggressive invaders.

Introduction

The role of hybridization in evolution historically split biologists into two groups—those that saw hybridization as a reinforcement to species boundaries because hybrids are less fit than the parental species (e.g., Mayr 1963; Dobzhansky 1970) and others that viewed hybridization as an important creative evolutionary force (e.g., Anderson and Stebbins 1954). This latter perspective has received support from recent research (Arnold et al. 1999; Rieseberg et al. 2003; Seehausen 2004), which raises the question of whether hybridization may contribute to or even be a primary cause of some biological invasions (Ellstrand and Schierenbeck 2000). Studying the role of hybridization in invasive taxa therefore provides opportunities to examine both the evolutionary outcomes of hybridization and deepen our understanding of its importance in invasion.

When species establish in new ranges, they encounter an array of novel abiotic and biotic environmental factors (Blossey and Notzold 1995; Facon et al. 2006; Mitchell et al. 2006). Interspecific hybridization, by increasing variation, may help some species respond to the novel selective challenges and opportunities associated with these new environments. In contrast, in the native range where species are likely to be locally adapted to their environment, interspecific hybridization might result in maladapted individuals that would be quickly selected against. Additionally, hybrids can exhibit transgressive (i.e., extreme) values of traits relative to their two parents. While most novel transgressive phenotypes may result in individuals less fit than the parental species, some might

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be more fit than either parent species, particularly in an introduced species' new environment (Rieseberg et al. 2003). This could potentially provide the necessary fitness boost for a species to become invasive.

One line of evidence supporting the hypothesis that hybridization might facilitate invasion comes from plant species that became invasive only after hybridization occurred (Ellstrand and Schierenbeck 2000). While this suggests that hybridization might influence invasion, experimental studies are necessary to more rigorously examine how hybridization can influence plant traits that are relevant to invasion. We used a common garden experiment to examine whether interspecific hybridization could potentially influence the invasion of diffuse knapweed (DK) (*Centaurea diffusa* Lam.). Blair and Hufbauer (2009a, 2010) recently concluded that this problematic invasive plant was likely introduced to North America with admixture from a close relative, diploid spotted knapweed (SK) (*Centaurea stoebe* subsp. *stoebe* L.). While the more common tetraploid SK [*C. stoebe* subsp. *micranthos* (Gugler) Hayek] is abundant in North America, the diploid has not been found even after extensive sampling (see Study Species below; Moore and Frankton 1954; Müller 1989; Treier et al. 2009; H. Müller-Schärer, personal communication). Given that diploid DK and tetraploid SK are incompatible (personal observation), it is unlikely that North American populations of these species have hybridized as they were introduced approximately 100 years ago. With the tremendous volume of global trade, however, it is plausible that diploid SK will be introduced into North America from the native range, enabling hybridization.

We addressed the following two sets of questions with the DK system. First, how does North American DK, which contains introgression from diploid SK, perform relative to pure DK of European origin and relative to plants from hybrid sites in Europe (i.e., European sites that appear similar in composition to those in North America)? These comparisons help us evaluate the role of evolution in the invasion in general while taking hybridization into account. For example, if North American DK plants outperform all European DK plants, this suggests that adaptive evolution occurred during the invasion. If instead, North American and European hybrids are similar but outperform pure European DK, we can postulate that pre-introduction hybridization may have played a role in the invasion. Second, if diploid SK are introduced to North America, could post-introduction hybridization increase performance and/or phenotypic variance in newly hybridized populations? In other words, could new hybridization events potentially lead to even more aggressive invaders? We considered increased size (e.g., height, leaf number, and biomass) and faster time to germination, bolting, and flowering as enhanced performance because such differences could lead to more aggressive or successful invaders (sensu Blossey and Notzold 1995).

Methods

Study species

Diffuse knapweed (*C. diffusa* Lam.) and SK (*C. stoebe* L.) were accidentally introduced into North America from Eurasia in the late 1800s or early 1900s (Watson and Renney 1974; Roché and Roché 1991); both species were likely introduced several times (Hufbauer and Sforza 2008; Marrs et al. 2008a,b). They have become major threats to rangeland productivity and quality across western North America (Watson and Renney 1974; Roché and Roché 1991; Sheley et al. 1999). These plants can increase soil erosion (Lacey et al. 1989; Sheley et al. 1997), alter plant community composition (Tyser and Key 1988), negatively impact biodiversity (Ortega et al. 2006), and have been thought to have allelopathic effects on other plants (Fletcher and Renney 1963; Callaway and Aschehoug 2000; but see Locken and Kelsey 1987; Blair et al. 2005, 2006, 2009b; Duke et al. 2009).

It has been reported that both *C. diffusa* and *C. stoebe* have diploid ($2n = 18$) and tetraploid ($4n = 36$) cytotypes (Ochsmann 2000). Both cytotypes of DK are referred to simply as *C. diffusa* Lam. The tetraploid has only been reported twice in the literature from one specimen in Bulgaria (Löve 1979) and one in Yugoslavia (Löve 1978). The diploid is more common (Moore and Frankton 1954; Ochsmann 2001a), and a recent study of microsatellite variation suggested that only diploid DK is present in North America (Marrs et al. 2008a). Chromosome counts with flow cytometry conducted for this study also revealed exclusively diploid DK in North America (Appendix 1). The two cytotypes of SK are both under *C. stoebe* L., a name that takes precedence over the commonly used *Centaurea maculosa* (Ochsmann 2000). The monocarpic diploid is designated *C. stoebe* subsp. *stoebe* L, and the polycarpic tetraploid is designated *C. stoebe* subsp. *micranthos* (Gugler) Hayek (for which *Centaurea biebersteinii* DC. is a synonym). Ploidy number is the only way to unambiguously distinguish these subspecies (Ochsmann 2001b). In the literature, all of the North American SK plants that have been assayed for chromosome number are tetraploids (i.e., *C. stoebe* subsp. *micranthos*; Moore and Frankton 1954; Müller 1989; Treier et al. 2009; H. Müller-Schärer, personal communication).

Floral morphology (Blair and Hufbauer 2009a) and molecular evidence (Blair and Hufbauer 2010) indicate that hybrids between diploid *C. diffusa* and diploid *C. stoebe* (*C.* x *psammogena*) are present in North American DK sites. On the contrary, hybrids are not found in

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North American SK sites (Blair and Hufbauer 2009a). In field surveys, Blair and Hufbauer (2009a) detected plants with hybrid morphology in 39 of 40 DK sites surveyed in western North America. The percentage of plants with hybrid morphology ranged from 1% to 95% with most sites containing <20% hybrid-like individuals. Thus, a majority of the plants in these sites have phenotypes resembling pure DK. In molecular assays, Blair and Hufbauer (2010) detected significant introgression from SK in 10 of 36 DK individuals from nine western North American sites that contained apparent hybrids. Interestingly, the plants that exhibited the hybrid morphology were not necessarily the ones with detectable admixture. These findings and several other lines of evidence (e.g., reproductive incompatibility between the tetraploid SK and diploid DK) suggest that diploid hybrids were introduced with diploid DK early in the invasion and not created post-introduction (Blair and Hufbauer 2009a, 2010).

Generation of Backcross 1 (BC1) seed

Comparisons of hybrids and parent species can be sensitive to which hybrid class is used in the investigation (e.g., F1, F2, Backcrosses) (Arnold and Hodges 1995). For example, F1 hybrids have a complete haploid set of genes from each parent, and they are often heterotic (Lexer et al. 2003a). Findings from studies with only F1 hybrids can be misleading, as recombination in later crosses shuffles parental gene combinations, possibly resulting in positive and/or negative transgression. We focused on Backcross 1 (BC1) individuals in this experiment because we wanted to assess a wide range of variation as a result of the recombination of the genomes of hybridizing species (Rieseberg et al. 1996, 2003). All hand pollinations were made with dead bees (Carolina Biological Inc., Burlington, NC, USA) glued to toothpicks. When conducting a pollination, we removed the pollen from the stigmas of all of the florets of a freshly opened inflorescence (i.e., flower head) with one bee (maternal plant). With another bee, we collected all of the pollen from a freshly opened inflorescence from the donor plant (paternal plant). To ensure no pollen contamination, we kept pairs of plants for crosses in separate rooms. SK and DK are self-incompatible (A.C. Blair, personal observation; Harrod and Taylor 1995), so unpollinated flowers could serve as controls. Flowers were generally pollinated during 2–4 weeks, and they were left to set seed for approximately another 4 weeks.

Because spotted × diffuse hybrids result from crosses between diploid SK and DK and the former is not present in North America, we crossed European diploid SK with North American diploid DK to generate F1 hybrids during summer of 2006. This is the relevant cross to examine the potential outcomes if diploid SK is eventually introduced to North America and hybridizes with DK established in the introduced range. Our parents were diploid SK from East Austria (pollen donor) and diploid DK from Vantage, WA. F1 hybrid status was confirmed with microsatellites (A.C. Blair, unpublished data, primers: cm26, 38cm22, cd37, and 21cm36; see Marrs et al. 2006 for details on methods).

By early spring 2007, several of the F1 plants generated from the aforementioned diploid SK × diploid DK cross were flowering. We generated BC1 seed by pollinating three such F1 hybrids with pollen from a paired plant from the Vantage, WA, DK site. Seed from these three backcrosses (A, B, and C) was used in the greenhouse common garden. The individuals within each BC1 line (A,B, or C) were full sibs. BC1 generation was confirmed by running microsatellites on 10% of the plants from each cross [A. C. Blair, unpublished data, primer pairs: BC1 A and B = 42CM27, CM15, and CM17; BC1 C = cm26, 38cm22, cd37, and 21cm36 (Marrs et al. 2006)]. Because all molecular assays confirmed BC1 status, we did not analyze all BC1 plants.

Greenhouse common garden experiment

Backcross 1 individuals from the three separate crosses (A, B, and C described above) were included in the common garden. Additionally, seeds from maternal collections from the following sites were included (Table 1, Figs 1 and 2): seven DK + hybrid sites from North America, eight diploid DK sites from Europe, three diploid SK sites from Europe, and three diploid DK + hybrid sites from Europe (similar to the North American DK sites) (see Appendix 1 for details on ploidy confirmation with flow cytometry).

Seeds were planted in small containers (diameter 3.8 cm, depth 21 cm) in Sunshine Mix #3 (Sun Gro Horticulture Canada Ltd., Vancouver, BC, Canada) on May 28, 2007, in the University Greenhouse at Colorado State University. When enough seed was available, we planted two seeds per container to increase our likelihood of having a plant from each maternal line. When and if the second seed germinated, we noted and removed it. As the environment can be critical to evaluating traits linked to fitness, we also varied competition, which strongly influences *C. diffusa* invasion (Seastedt and Suding 2007). Thus, there were two treatments in this experiment: with and without competition from *Pascopyrum smithii* (western wheatgrass; L&H Seeds Inc., Connell, WA). This North American native grass was chosen as an ecologically relevant competitor because it is a plant that invasive populations of DK encounter in the field. Additionally, western wheatgrass is highly valued for forage production, and it is thus of interest to see how it

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Table 1. Source sites included in the greenhouse common garden experiment.

Site	GPS location
North American diffuse + hybrid	
Douglas County 2, CO	N39°20′23.8″ W104°49′53.3″
Estes Park, CO	N40°22′09.5″ W105°31′54.2″
I-84, Exit 147, OR	N45°47′28.2″ W120°01′51.8″
Mosier, OR	N45°41′01.9″ W121°24′08.3″
Tygh Valley, OR	N45°15′14.9″ W 121°09′05.8″
Vantage, WA	Unknown
Kittitas, WA	Unknown
European diffuse (verified 2n)	
Crimea 20	Unknown
Crimea 21	Unknown
Romania 5	N44°94′34.3″ E28°91′4.9″
Romania 6	N45°11′8.8″ E28°47′8.3″
Russia 1119	N44°3′0.0″ E43°3′36″
Russia 1120	N44°8′24″ E44°1′12″
Russia 1142	N51°22′48″ E56°48′0.0″
Ukraine 9	N47°51′43.2″ E38°27′38.5″
European spotted (verified 2n)	
SUAC (Ukraine) 2nSK	N49°13′13.4″ E24°42.3′17.6″
Ukraine 2 2nSK	N49°55′48.5″ E24°50.1′8.9″
Ukraine 5 2nSK	N49°46″19.2′ E27°17.5′27.6″
European diffuse + hybrid (verified 2n)	
Ukraine 2 DK	N48°38′45.7″ E30°46′30.3″
Ukraine 10 DK	N48°06′02.4″ E37°48′58.0″
Ukraine 11 DK	N48°09′8.4″ E37°50′26.1″
Backcross 1	
F1 × Vantage, WA DK A	N/A
F1 × Vantage, WA DK B	N/A
F1 × Vantage, WA DK C	N/A

responds to competition from the experimental plants. One seed of western wheatgrass was planted in the competition containers at the same time as the knapweed seeds. Eight seeds from each maternal knapweed plant were started from each of the above sites per treatment, in addition to 15, 20, and 65 seeds per treatment from the three BC1 lines (with the uneven numbers from the three BC1 crosses reflecting the variable number of seeds produced). The larger number of backcrosses than parental individuals follows other work on hybridization (e.g., Lexer et al. 2003b; Gross et al. 2004; Ludwig et al. 2004) and reflects the fact that backcrosses are likely to have highly variable phenotypes (e.g., Lexer et al. 2003b), and therefore, more individuals are needed to obtain an accurate assessment of the mean and variance. Furthermore, transgressive variants can be important ecologically and evolutionarily, yet quite rare, and large number of BC1 individuals improved our ability to detect such transgressive variation.

A total of 536 containers were started in this experiment (8 individual × 21 sites × 2 treatments = 336; 100 BC1 individuals × 2 treatments = 200). Ninety-six

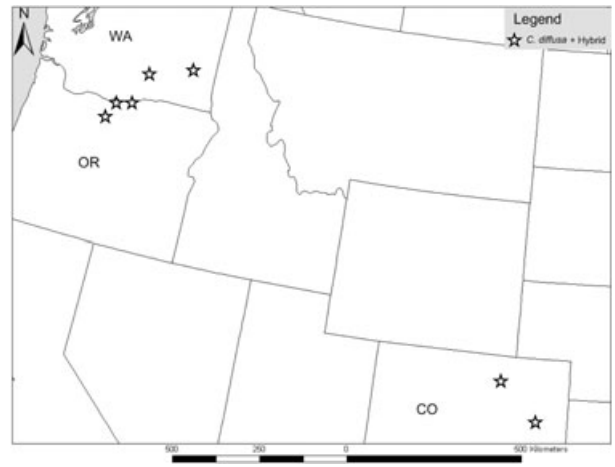

Figure 1 North American sites for *Centaurea diffusa* + hybrids included in the common garden experiment.

Figure 2 European sites for *Centaurea diffusa*, *C. diffusa* + hybrids, and *Centaurea stoebe* subs. *stoebe* included in the common garden experiment.

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Table 2. Traits measured during the greenhouse common garden experiment.

Trait type	Specific traits measured
Life history	Germination rate, time to germination, leaf number at 1 month, leaf number when first plant in the experiment flowered (≈3 months), time to bolt, leaf number at bolt, time to first flower
Morphological	Cotyledon length and width, overall height at first flower, above-ground biomass, below-ground biomass (only plants in the absence of competition)
Floral (first fully opened flower)	Flower color (ranked 1-white to 5-solid purple), bract pigmentation (ranked 0-no pigmentation to 3-deeply pigmented), capitula length and width, spine length (if present), flower diameter

individual western wheatgrass seeds were started in containers to serve as replacements for seeds that did not germinate in the experiment. The remaining plants served as the control to those grown in competition with knapweed. Containers were misted daily, trays randomized weekly, and plants in trays randomized every two weeks. Containers were examined daily, and the number of days to germination was recorded. Additionally, cotyledon length and width were measured on the germination day.

Four weeks after starting the experiment, plants were transplanted into pots (Hummert Custom-tainers, 6.25″ × 6″ × 4.5″) with Sunshine Mix #2 potting soil and fertilized with one teaspoon Osmocote® (The Scotts Company LLC, Marysville, OH, USA)(14-14-14). The number of true leaves was also counted at this time. Throughout the experiment, a number of life history, morphological, and floral traits were recorded (Table 2). The experiment was terminated in April 2008, when a majority of plants had flowered and some were beginning to senesce.

Data analysis

We conducted our statistical analyses with SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Residuals were inspected for normality and heteroscedasticity, and final number of stems + 1 was log-transformed prior to analysis; original values are reported here. All values reported in the text are the LS Mean ± 1SE. To determine whether DK plants of North American origin outperform pure DK plants of European origin, plants from sites of European origin that contain morphological hybrids, or both (Question 1 above), we compared traits among these three plant classes. We analyzed the morphological and life history traits with a mixed model (PROC MIXED) including plant classification (i.e., diffuse North American, diffuse pure European, diffuse hybrid European) as a fixed effect, treatment (i.e., alone or in competition) as a fixed effect, site nested within plant classification as a random effect, and treatment × plant classification as a fixed effect. For below-ground biomass, which was only assayed on the plants in the absence of competition, we used a model with plant classification as a fixed effect and site nested within plant classification as a random effect. We used the same model to compare the above-ground biomass of the grass grown with the three plant classes. We used Levene's test for homogeneity of variance to determine whether plants containing introgression from diploid SK are more variable than pure DK. Because we were interested in a broad comparison, we pooled all of the data from the sites within each plant class. To compare the proportion of seed that germinated between (i) DK of North American origin and pure DK of European origin and (ii) DK of North American origin and DK of European origin that contained plants with hybrid morphology, we analyzed the seed data as x seeds that germinated out of y seeds planted (i.e., events/trial) for each maternal plant. We used mixed models (PROC GLIMMIX with a binomial distribution and a logit link function), including plant classification as a fixed effect and site nested in plant classification as a random effect.

To then determine whether new hybridization events could potentially lead to more aggressive invaders if diploid SK is introduced to North America (Question 2 above), we compared traits between BC1 plants and North American DK. We used the same mixed models as described earlier. Similarly, we used Levene's test for homogeneity of variance to examine whether the newly created BC1 hybrids were more variable than the North American DK. We used the same mixed model as above (PROC GLIMMIX) to compare the proportion of seed that germinated between the two plant classes. Additionally, to examine whether the specific hybrid BC1 lines performed differently from one another, we used one-way ANOVA followed by Tukey–Kramer *post hoc* tests.

Finally, we examined phenotypic transgression by comparing BC1 plants to European spotted and European DK with student's t-tests. A trait was considered transgressive if the mean value for BC1 plants was significantly higher or lower than the mean value for both SK and DK (*sensu* Ludwig et al. 2004).

Results

Greenhouse common garden experiment – question 1 (North American versus European diffuse knapweed)

The three plant classes (North American DK + hybrids, European pure DK, European DK + hybrids) differed

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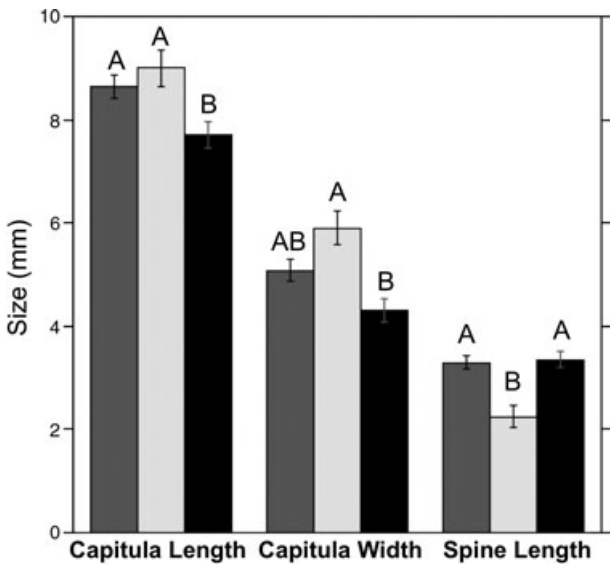

Figure 3 A comparison of floral traits of North American diffuse knapweed (DK) + hybrids (dark gray bars), European DK + hybrids (light gray bars), and pure European DK (black bars). Different letters denote significantly different means (Tukey–Kramer $P < 0.05$). Values represent means ± 1 SE.

significantly in three of the four measured floral traits (Fig. 3) (floral diameter did not differ $P = 0.31$). North American DK and the European DK + hybrids had similar capitula length, while the North American DK was intermediate to the two groups for capitula width. North American DK and pure European DK had similar spine length.

The three plant classes did not differ for any of the measured size or life history traits (Table 3). More North American DK seeds germinated than pure European DK seeds (89.3% vs 63.3%; $F_{1,13} = 7.0$, $P = 0.02$) or European DK + hybrid seeds (89.3% vs 53.1%; $F_{1,8} = 5.6$, $P = 0.04$). Because field-collected seed was used in this experiment, maternal environments could potentially influence traits of offspring used in this experiment (Roach and Wulff 1987). However, this seems unlikely because the plants were very similar at the cotyledon stage, and significant differences were not found for the measured traits (Table 3) (see Parker et al. 2003).

Competition resulted in significantly smaller plants throughout their life cycle (Table 3). The three plant classes did not respond differently to competition (plant class × competition $P > 0.05$). Above-ground biomass of the competitor grass was similar when grown in competition with plants from all three size classes ($P = 0.28$). Based on Levene's test for homogeneity of variance, the three plant classes displayed similar variance for all measured traits (Table 3).

Greenhouse common garden experiment – question 2 (BC1 hybrids versus North American diffuse knapweed)

Of the measured floral traits, none differed between the BC1 plants and the North American DK. BC1 plants had

Table 3. Summary of mixed model nested ANOVA for comparisons among pure European diffuse knapweed (DK), North American DK + hybrids, and European DK + hybrids for size and life history traits. See the text for model details.

	Plant class			Treatment			Population (plant class)	Levene's
Response variable	DF	*F*	*P*	DF	*F*	*P*	*P*	*P*
Size traits								
Cotyledon area	2,15	2.01	0.17	1,217	0.13	0.72	0.38	0.53
Leaf # 1-month	2,15	0.59	0.57	1,218	26.93	**<0.0001**	0.26	0.87
Leaf # 3-month*	2,15	2.4	0.12	1,215	6.08	**0.01**	**<0.01**	0.84
Leaf # - at bolting*	2,15	0.78	0.48	1,181	20.86	**<0.0001**	0.11	0.09
Height at first flower	2,13	0.94	0.41	1,64	0.28	0.60	**<0.001**	0.99
Log final stem # +1	2,15	0.97	0.40	1,180	12.34	**<0.001**	0.11	0.67
Above-ground biomass	2,15	0.17	0.85	1,212	76.87	**<0.0001**	**<0.0001**	0.18
Root biomass	2,15	0.06	0.94	–	–	–	**0.05**	0.61
Above + below biomass	2,15	0.33	0.72	–	–	–	**0.04**	0.96
Life history traits								
Days to germination	2,15	0.94	0.41	1,219	0.09	0.76	**0.03**	0.17
Time to bolt	2,15	0.18	0.84	1,181	0.03	0.87	**<0.0001**	0.12
Time to first flower	2,13	1.77	0.21	1,64	0.05	0.82	0.15	0.16

Results significant at $P < 0.05$ are noted in bold. The findings from Levene's test to compare levels of variance are also shown. Plant class = pure European DK, North American DK + hybrids, and European DK + hybrids; Treatment = competition or no competition.
Plant class × Treatment was never significant, so it was not included in the table.
*Leaf # 3 month was a leaf count taken simultaneously on all plants when the first plant in the experiment flowered, which was at approximately 3 months. Leaf # - at bolting was a leaf count taken on individual plants when we noted each had started to bolt. The time of this count varied per plant.

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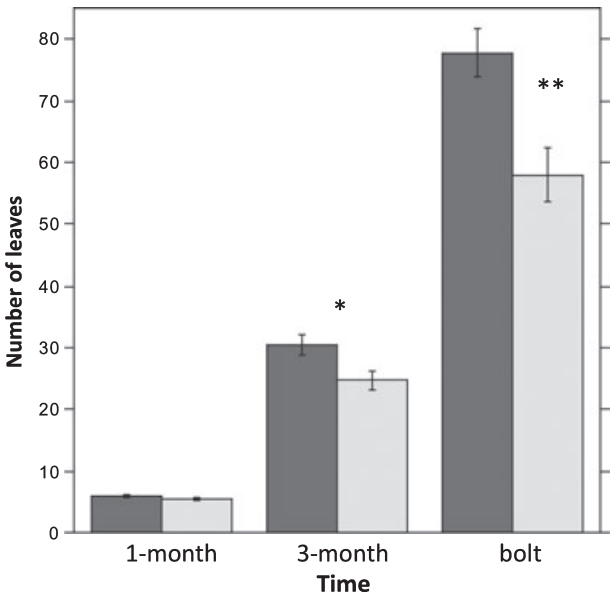

Figure 4 A comparison of leaf number through time of Backcross 1 (dark bars) versus North American diffuse knapweed (light bars). While all plants were assayed at 1 and 3 months, plants were individually counted when they started bolting. Values represent mean ± 1 SE. $*P < 0.05$; $**P = 0.01$.

significantly greater leaf production through time (Fig. 4) and produced more reproductive shoots by the end of the experiment (BC1 = 3.8 ± 0.05, DK = 2.7 ± 0.3; Table 4). On average, BC1 seed took significantly longer to germinate than pure DK (BC1 = 6.2 ± 0.37 days, DK = 4.07 ± 0.42 days; Table 4). None of the other life history or morphological traits significantly differed (Table 4). A similar proportion of BC1 seeds and North American DK seeds germinated (88.5% and 89.3%, respectively; $F_{1,8} = 0.27$, $P = 0.62$). Again, because we used field-collected seed of DK in this experiment, maternal effects could potentially influence the interpretation of trait comparisons. While BC1 seedlings had significantly greater cotyledon area than DK (6.14 ± 0.23 vs 4.71 ± 0.28 cm^2, respectively; Table 4) suggesting differential maternal provisioning, the BC1 cotyledon area was not significantly different than the field-collected SK seedlings (Table 4). This similarity to one of the parents suggests that the increased cotyledon area of the BC1 seedlings is a consequence of hybridization rather than strictly owing to maternal environment. Further, maternal effects typically decrease as a plant ages (Wolfe 1993), but in this study, observed differences in leaf number increased as plants aged (Fig. 3).

Table 4. Summary of mixed model nested anova for comparisons between North American diffuse knapweed (DK) and Backcross 1 (BC1) hybrids for size and life history traits. See the text for model details.

	Plant class			Treatment			Population (plant class)	Levene's test	Transgression*	
Response variable	DF	*F*	*P*	DF	*F*	*P*	*P*	*P*	Comp.	No Comp.
Size traits										
Cotyledon area	1,8	15.8	**<0.01**	1,265	0.00	0.96	0.5	**0.05**, BC > DK	SK	SK
Leaf # 1 month	1,8	2.04	0.19	1,264	13.4	**<0.001**	**<0.0001**	0.39	SK	DK & SK
Leaf # 3 month†	1,8	6.08	**0.04**	1,262	6.7	**0.01**	**0.04**	**<0.001**, BC > DK	SK	SK
Leaf # - at bolting†	1,8	11.3	**<0.01**	1,227	9.9	**<0.01**	0.33	**<0.0001**, BC > DK	SK	SK
Height at first flower	1,8	0.05	0.83	1,90	0.26	0.61	**<0.0001**	**0.04**, BC > DK	DK	DK
Log final stem # +1	1,8	6.6	**0.03**	1,222	20.4	**<0.0001**	0.17	0.6	SK	SK
Above-ground biomass	1,8	0.09	0.77	1,256	104.4	**<0.0001**	**<0.001**	0.22	DK & SK	SK
Root biomass	1,8	4.5	0.07	–	–	–	**<0.01**	**<0.001**, BC > DK	–	SK
Above + below biomass	1,8	1.9	0.20	–	–	–	**0.02**	0.77	–	SK
Life history traits										
Days to germination	1,8	14.7	**<0.01**	1,269	0.49	0.48	0.38	**<0.0001**, BC > DK	DK & SK	DK & SK
Time to bolt	1,8	0.01	0.91	1,227	2.75	0.10	0.24	**0.02**, BC > DK	DK & SK	DK & SK
Time to first flower	1,8	0.60	0.46	1,90	0.02	0.88	**0.04**	**<0.001**, BC > DK	DK & SK	DK & SK

Results significant at $P < 0.05$ are noted in bold. The findings from Levene's test to compare levels of variance are also shown. Plant class = North American DK or BC1; Treatment = competition or no competition. Transgression was compared between the BC1 hybrids and European diploid spotted knapweed (SK) and European diploid DK (i.e., the parental species).

Plant class × Treatment was never significant, so it was not included in the table.

*Transgression of BC1 hybrids was tested by examining whether the trait mean was significantly higher or lower than both SK and DK with student's *t*-tests (*sensu* Ludwig et al. 2004). Comparisons were made among plants grown either in the presence or absence of competition. SK = BC1 plants did not significantly differ from SK; DK = BC1 plants did not significantly differ from DK; DK & SK = BC1 plants did not significantly differ from either SK or DK. None of the measured traits were transgressive (i.e., significantly different from both species).

†Leaf # 3 month was a leaf count taken simultaneously on all plants when the first plant in the experiment flowered, which was at approximately 3 months. Leaf # - at bolting was a leaf count taken on individual plants when we noted each had started to bolt. The time of this count varied per plant.

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As predicted, competition with a native grass reduced plant size (Table 4); plants grown in competition compared to alone had significantly fewer leaves at 1 month (5.4 ± 0.2 vs 5.9 ± 0.2, respectively) and 3 months (25.7 ± 1.4 vs 29.7 ± 1.4, respectively), and when they bolted (59.6 ± 4.0 vs 75.8 ± 3.8, respectively), had fewer stems at the end of the experiment (2.7 ± 0.3 vs 4.4 ± 0.3, respectively) and lower above-ground biomass compared to plants grown in the absence of competition (35.3 ± 3.1 vs 59.8 ± 3.2 g, respectively). North American DK and BC1 plants did not respond differently to competition, as none of the plant class × competition terms were significant. Above-ground biomass of the competitor grass (western wheatgrass) was similar when grown with BC1 or North American DK plants ($P = 0.62$).

Individual BC1 lines differed for several morphological and life history traits (Table 5). Interestingly, BC1-B plants reduced biomass of the competitor grass more than the other BC1 lines and plants from the parental DK site (Vantage, WA) (Fig. 5). BC1-B plants also initially produced more leaves, while BC1-A plants were substantially taller than plants from the other two crosses (Table 5). Thus, the specific backcross performed created plants that differed significantly with respect to potentially important ecological traits.

Based on Levene's test for homogeneity of variance, BC1 plants were more variable than pure European DK for eight size and life history traits (Table 4) including days to germination (1 SD = 5.5 vs 1.6 days, respectively), cotyledon area (1 SD = 3.3 vs 2.2 cm^2), leaf number at approximately 3 months (1 SD = 14.4 vs 9.9 leaves, respectively), leaf number at time of bolting (1 SD = 44.5 vs 27.0 leaves, respectively), time to bolt (1 SD = 71.8 vs 56.4 days), height at first flower (1 SD = 21.0 vs 16.6 cm, respectively), time to first flower (86.3 vs 63.8 days), and root biomass (1 SD = 11.7 vs 8.2 g, respectively).

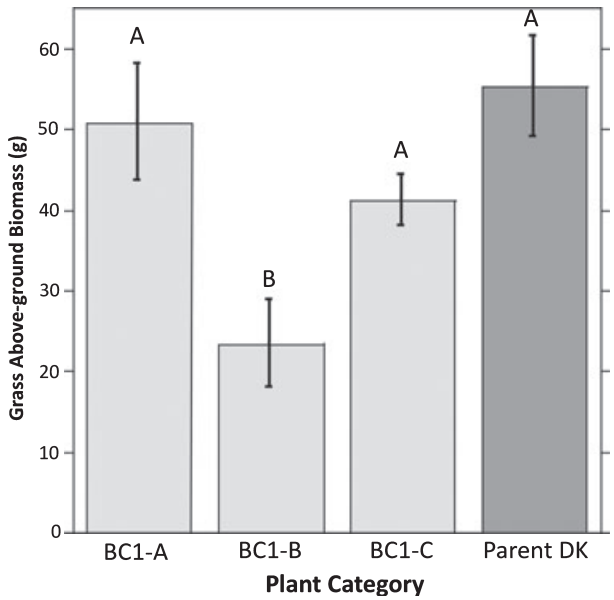

Figure 5 Above-ground biomass of the competitor (western wheatgrass) grown with plants from the three hybrid lines [Backcross 1 (BC1)-A, BC1-B, and BC1-C] and the parental diffuse knapweed population used to generate the hybrid lines (Vantage, WA). Different letters denote significantly different means (Tukey–Kramer $P < 0.05$). Values represent means ± 1 SE.

While the focus of our question was on DK and BC1 plants, we also wanted to examine transgression of the hybrids relative to both parental species. None of the assayed traits were transgressive (Table 4). Interestingly, BC1 plants performed more like SK for a number of traits.

Discussion

Hybridization has been hypothesized as one of many mechanisms that may aid the success of some invasive

Table 5. Results from one-way anova comparing the three Backcross 1 (BC1) hybrid lines (A, B, and C). All traits were analyzed (see Tables 3 and 4), but only those with significant results are shown here. The values for the parental diffuse knapweed population (Vantage, WA) are included for comparison to the BC1 lines.

Trait	*F*-value	*P*-value	BC1-A	BC1-B	BC1-C	Vantage DK
No competition						
Leaf # 1-month	$F_{2,77} = 9.9$	<0.01	4.7 ± 0.4^{A}	6.8 ± 0.3^{B}	6.0 ± 0.2^{C}	**6.0 ± 0.4**
Root biomass (g)	$F_{2,68} = 2.4$	0.10*	10.5 ± 4.7	21.4 ± 2.9	15.8 ± 1.6	**23.3 ± 3.9**
Above + below biomass (g)	$F_{2,66} = 2.7$	0.07*	72.3 ± 8.5	83.7 ± 5.4	69.4 ± 3.0	**94.2 ± 7.8**
Competition†						
Leaf # 1 month	$F_{2,84} = 6.59$	<0.01	$5.6 \pm 0.4^{A,B}$	6.5 ± 0.3^{A}	5.4 ± 0.2^{B}	**5.8 ± 0.3**
Leaf # 3 month	$F_{2,87} = 3.6$	0.03	$31.4 \pm 4.9^{A,B}$	36.0 ± 3.4^{A}	25.7 ± 2.0^{B}	**25.3 ± 3.6**
Height – 1st flower (cm)	$F_{2,27} = 6.7$	<0.01	95.0 ± 13.9^{A}	$68.2 \pm 8.0^{A,B}$	48.4 ± 4.2^{B}	**58.3 ± 7.6**

Different letters denote significantly different means (Tukey–Kramer $P < 0.05$). Values represent means ± 1 SE.

*Marginally significant at the $\alpha < 0.10$ level.

†Root biomass and above + below-ground biomass were not assayed for plants grown in competition with western wheatgrass.

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species (Ellstrand and Schierenbeck 2000; Gaskin and Schaal 2002, Rieseberg et al. 2007). In this study, we evaluated the hypothesis with both artificial and natural *C. diffusa* × *C. stoebe* subs. *stoebe* hybrids (*C.* x *psammogena*). Because the *Centaurea* genus contains multiple problematic invaders (http://plants.usda.gov/), and a number of species within the genus hybridize, we believe this system is particularly well suited to examine the hybridization hypothesis, as the findings may have real-world implications.

Our first question focused on whether North American DK outperforms pure European DK, DK from sites in Europe that contain plants with hybrid morphology, or both. The fact that the three plant classes did not differ for any of the measured morphological or life history traits suggests that neither preinvasion hybridization nor post-introduction evolution has played a large role in the success of DK. Blumenthal and Hufbauer (2007) included four populations of DK in a multispecies common garden and similarly found no evidence for increased growth. Blair and Hufbauer (2009a) conducted field surveys of DK in its native and introduced range and found more robust plants (i.e., taller with greater diameter) in North America. As individuals from some of those field surveys were included in this common garden, it seems likely that these patterns are driven by environmental field conditions rather than post-introduction evolutionary change. If introgression by SK conferred some advantage to the DK in North America, we did not detect it in this experiment. Of course, there are many traits that we did not measure in this study (trichome density, palatability, etc.). Additionally, the similar phenotypic variance detected among the three plant groups suggests that if hybridization initially caused increased variance, it has already been removed by natural selection.

Second, we compared artificially created BC1 hybrids and North American DK to determine whether early generation hybrids differ from DK (Question 2 above) and might influence knapweed invasion if diploid SK is introduced to North America. BC1 plants had significantly greater leaf production throughout the life cycle and also had a greater number of reproductive shoots at the end of the experiment. These patterns are not likely caused by transient heterosis, as BC1 individuals were used rather than F1 individuals. Indeed, the hybrids performed much like SK for these performance traits (Table 4), in spite of the fact that 75% of the genome was derived from DK, while only 25% was from SK. Significantly more BC1 seeds germinated, although the BC1 seeds took, on average, approximately 1.5 days longer to germinate. BC1 plants also demonstrated increased phenotypic variance for eight of 12 morphological and life history traits. Such elevated levels of phenotypic variance could help such hybrids colonize novel environments encountered in the invaded range. Overall, these findings imply that future hybridization events could lead to even more robust DK plants with a greater potential for responding to selection.

Backcross 1 plants did not differ in their response to competition compared to European DK. However, one BC1 line, which had higher leaf production, also led to greater reductions in biomass of the competitor grass, suggesting that this specific line may be a superior competitor. Unique genetic recombination and the individual plants in each cross could profoundly alter the effects of hybridization on an invasion. This was further confirmed by comparing the three BC1 lines for all measured traits (Table 5). Trait means of individual hybrid lines were above, below, or similar to that of the plants from the parental DK population (Vantage, WA). As our study contained only a subset of potential backcrosses, these findings suggest that the inclusion of additional BC1 lines generated from different crosses could have led to different results.

It was surprising that we did not detect transgression for any of the measured traits. In a review of 171 hybridization studies, Rieseberg et al. (1999) found that 155 (91%) reported at least one transgressive trait, leading them to conclude that 'transgression is the rule rather than the exception.' Rather than demonstrating transgression, the hybrids in this study tended to acquire a more SK-like phenotype. This is in line with the observation that in Europe, where hybridization occurs, backcrossing will produce more *C. stoebe* subsp. *stoebe*-like individuals in the long run (J. Ochsmann, personal communication).

Recent studies lend additional support to Ellstrand and Schierenbeck's (2000) seminal paper: A number of invasive species are indeed of hybrid origin (Gaskin et al. 2009; Gaskin and Kazmer 2009; Blair and Hufbauer 2010; but see Whitney et al. 2009), and hybridization seems to increase the success of some species (Ridley and Ellstrand 2009; Grosholz 2010). Our results suggest that the inclusion of hybrids from the native range may not have altered the course of *C. diffusa* invasion in North America (Question 1), but that further hybridization could potentially do so (Question 2) via increasing phenotypic variation and enhancing leaf production and potentially competitive ability (as observed in one of three crosses). With the ever-increasing shuffling of the world's biota, hybridization events are likely to become more common; a greater understanding of this evolutionary mechanism in relation to invasion may aid in the management and eventual control of some invasive species.

Acknowledgements

We thank Stephen Meyer, Nichole Streifel, Joseph Hansen, and Eric Hardy for help with plant care and biomass

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data collection; Silas Davidson patiently assisted with the hand pollinations; Dirk Baker helped to make the site maps; P. Häfliger, O. Kornyenko, S. Mosyakin, H. Müller-Schärer, U. Schaffner, A. Shipunov, and U. Treier helped with the European seed collection and V. Blair, E. Coombs, and R. Marrs-McGinn helped with seed collection in North America; Heinz Müller-Schärer generously shared ploidy data and welcomed us into his laboratory for use of his flow cytometer at the University of Fribourg in Switzerland; and three anonymous reviewers greatly improved this paper. Funding for this research was provided by the Colorado Agricultural Experiment Station, an EPA STAR fellowship FP-91676001-0, CIPM G257-05-W0094, NSF #0508922, NSF DDIG #0607974, and USDA #2002-35320-12137.

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Appendix 1 – diffuse knapweed ploidy assays

While research suggested only the presence of diploid diffuse knapweed (DK) in North America (Moore and Frankton 1954; Ochsmann 2001a; Marrs et al. 2008a), we wanted to further confirm this with ploidy assays using flow cytometry.

Flow cytometry

We conducted chromosome counts with a flow cytometer (CyFlow; Partek, Munster, Germany) on individuals from sites composed of DK and morphologically intermediate hybrid individuals in CO (three sites), OR (one site), WY (one site), and WA (one site). We assayed from one to 14 maternal plants at each site. Additionally, we assayed 22 individuals from a site in Hood River, OR, which contained a mix of spotted knapweed (SK) and DK and intermediate hybrids. This research was conducted in 2006 at the University of Fribourg, Switzerland, and was separated from the greenhouse common garden experiment. Thus, individuals from these sites were not included in the common garden experiment.

Plants were approximately 6 weeks old when assayed for ploidy. Briefly, the technique was as follows. Approximately 0.5 cm^2 (hole punch #4) of leaf tissue was put in a 55-mm plastic petri dish. We then added 200 μL of the extraction buffer (kit - Cystain®, Partec, Munster, Germany; PI Absolute P, Catalog Number 05-5022) and immediately chopped the tissue rapidly for 30–60 s with a sharp razor blade. We then added another 300 μL of the extraction buffer, incubated the solution for 90 s at room temperature, and filtered the liquid portion of the sample with a disposable filter (Partek- Celltrics®, 30 μm). We then added 2 mL of the freshly made staining solution,

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containing per sample, 2 mL staining buffer, 12 μL Propidium Iodide, and 6 μL of RNAse A stock solution (5 mg Rnase combined with 1.5 mL water) (all solutions included in the kit), and incubated the sample for at least 30 min, shielded from light. Samples were then ready to be analyzed with the flow cytometer. Standards were diploid and tetraploid SK plants that were confirmed with root squashes. We periodically ran a standard and a blank (approximately every 10 samples).

As we found that all of the North American hybrid/DK sites contained only diploids (see results below), we crossed diploid DK with diploid SK to create relevant hybrids. Thus, we later also assayed the ploidy of all individuals from all of the European sites of DK included in this study, as described above. The European SK sites had previously been confirmed as diploid (H. Müller-Schärer, personal communication).

Results

All of the plants that exhibited SK morphology from Hood River, OR, were tetraploid; all plants that exhibited either diffuse or hybrid morphology, across all sites assayed in North America, were diploid [fluorescence, respectively (mean ± SE): $n = 9$, 238.9 ± 3.2; $n = 34$, 126.6 ± 1.7; and $n = 20$, 124.7 ± 2.1]. Triploids might have been expected to be present in the Hood River site because both spotted and diffuse occurred together there. Although there was spread around the peak fluorescence for diploid (113.7–135.6 fluorescence) and tetraploid (227.5–252.7 fluorescence) individuals, there was no evidence for triploidy. This further supports the idea that hybrids are not created post-introduction, as currently only tetraploid SK and diploid DK are thought to occur in North America, and we do not have evidence that these cytotypes hybridize. All of the European sites were later confirmed as diploid (Table 1). Additionally, the parent plants used to generate the hybrids were confirmed as diploids.

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