

Evidence for multiple introductions of *Centaurea stoebe micranthos* (spotted knapweed, Asteraceae) to North America

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Abstract

Invasive species' success may depend strongly on the genetic resources they maintain through the invasion process. We ask how many introductions have occurred in the North American weed *Centaurea stoebe micranthos* (Asteraceae), and explore whether genetic diversity and population structure have changed as a result of introduction. We surveyed individuals from 15 European native range sites and 11 North American introduced range sites at six polymorphic microsatellite loci. No significant difference existed in the total number of alleles or in the number of private alleles found in each range. Shannon–Weaver diversity of phenotype frequencies was also not significantly different between the ranges, while expected heterozygosity was significantly higher in the invasive range. Population structure was similar between the native range and the invasive range, and isolation by distance was not significant in either range. Traditional assignment methods did not allocate any North American individuals to the sampled European populations, while Bayesian assignment methods grouped individuals into nine genetic clusters, with three of them shared between North America and Europe. Invasive individuals tended to have genetically admixed profiles, while natives tended to assign more strongly to a single cluster. Many North American individuals share assignment with Romania and Bulgaria, suggesting two separate invasions that have undergone gene flow in North America. Samples from three other invasive range sites were genetically distinct, possibly representing three other unique introductions. Multiple introductions and the maintenance of high genetic diversity through the introduction process may be partially responsible for the invasive success of *C. stoebe micranthos*.

Keywords: *Centaurea maculosa*, *Centaurea stoebe*, invasive species, microsatellites, multiple introductions, spotted knapweed

Received 17 April 2008; revision received 16 July 2008; accepted 17 July 2008

Introduction

Biological invasions are initiated by one or more introductions of a species into an area where it previously was not present. Introduction events can consist of many or only a few individuals (Gaskin *et al.* 2005), and both the number of introductions and the number of propagules introduced during each event can have a large effect on the genetic

outcome of an invasion. Because genetic diversity provides the raw materials necessary for adaptive evolution, it has been hypothesized that multiple introductions may lead to especially problematic invaders capable of swift evolutionary response to selection pressure (Kolar & Lodge 2001; Dlugosch & Parker 2008).

When a small number of individuals are introduced, genetic diversity can become markedly decreased in the invasive range of a species relative to its native range. Bottlenecks in population size, founder effects, and evolution via genetic drift in small populations all can contribute to reductions in variation (Nei *et al.* 1975; Husband & Barrett 1991). For

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example, genetically depauperate invasive populations have been observed in the Colorado potato beetle (*Leptinotarsa decemlineata*, Grapputo *et al.* 2005) and the soap bush (*Clidemia hirta*, DeWalt & Hamrick 2004). The lack of genetic variation is thought to be caused by few introductions and small founding population sizes, although an apomictic reproductive strategy may also contribute to low diversity (Poulin *et al.* 2005). Additionally, successive, nested founder events can result in cumulative losses of genetic diversity, as in the giant bramble (*Rubus alceifolius*, Amsellem *et al.* 2000).

Alternatively, invasive populations can be founded multiple times, and founding population sizes can be relatively large (Brown & Marshall 1981). In such cases, genetic diversity tends to be slightly reduced in the invasive range, but not significantly so. This pattern has been observed in the mustards *Capsella bursa-pastoris* (Neuffer & Hurka 1999) and *Alliaria petiolata* (Durka *et al.* 2005), in the Brazilian peppertree (*Schinus terebinthifolius*, Williams *et al.* 2005), and in the freshwater mussel (*Dreissena rostriformis bugensis*, Theriault *et al.* 2005). Even if initial founding population sizes are small, genetic diversity can be maintained if population size rebounds quickly after introduction, as observed in the European rabbit (*Oryctolagus cuniculus*, Zenger *et al.* 2003). If multiple introductions occur from genetically differentiated lineages in the native range, intraspecific crossing can lead to increased heterozygosity or novel combinations of alleles in the new range (Ellstrand & Schierenbeck 2000). This can lead to diversity being higher within populations in the invasive range than within populations in the native range, a pattern seen in the brown anole (*Anolis sagrei*, Kolbe *et al.* 2004), thiarid snails (*Melanoides tuberculata*, Facon *et al.* 2008) and in the common ragweed (*Ambrosia artemisiifolia*, Genton *et al.* 2005). Genetic isolation by distance may not be observed in species' invasive ranges because relatively little time has passed to allow genetic drift to overcome the effects of multiple introductions, as seen in *Centaurea diffusa* (Marrs *et al.* 2008).

Spotted knapweed, *Centaurea stoebe* subspecies *micranthos* L. (Asteraceae, also referred to as *Centaurea maculosa* Lam. and *Centaurea biebersteinii* DC), is an especially problematic invader in North America for which little information on genetic diversity and population structure exists. It was first recorded in North America in Victoria, British Columbia in 1893 and was probably imported as a contaminant of alfalfa seed or soil carried as ships' ballast (Groh 1944; Watson & Renney 1974). After over 110 years of invasion, *C. stoebe micranthos* has spread over much of North America, and is considered noxious in 15 US states (USDA Natural Resources Conservation Service Plants Database). Spotted knapweed is a species of great concern in the USA and Canada because of its ability to displace native grass species (Kedzie-Webb *et al.* 2001) and invade undisturbed natural systems (Tyser & Key 1988), reducing the forage quality of rangelands and changing the ecosystems of wildlands. The ecological and economic impact of spotted knapweed has encouraged inten-

sive study into possible management options, including biological control. Studying the genetic diversity and population structure of *C. stoebe micranthos* will help us understand the introduction history of the species. Additionally, information about probable native range origins of *C. stoebe micranthos* may help focus future explorations for potential biological control agents.

Polymorphic molecular markers from presumably neutrally evolving areas of the genome, such as microsatellites, can be used to elucidate genetic diversity and population structure in native and introduced populations. Two recent meta-analyses examined whether a relationship exists between neutral molecular marker diversity and quantitative trait variation; Merilä & Crnokrak (2001) found a strong correlation between F_{ST} and Q_{ST} at the level of population differentiation, while Reed & Frankham (2001) found a weak correlation of genetic variation within populations. Although the correlation is imperfect and care should be exercised in interpretation (Hufbauer 2004), neutral marker variation is a good, relatively easy-to-measure proxy for overall more ecologically relevant variation (Reed & Frankham 2003), and is often used to draw conclusions about how genetic diversity has changed following an introduction (see Durka *et al.* 2005; Genton *et al.* 2005; Grapputo *et al.* 2005; for current examples). Here we use multilocus microsatellite genotypes of individuals from the native and introduced ranges of spotted knapweed to investigate three sets of questions regarding the invasion of this species: (i) Is genetic diversity higher in the native range than in its introduced range? (ii) How is genetic variation structured among sampling sites in the European and North American ranges of *C. stoebe micranthos*? Is isolation by distance significant in either range? (iii) Are multiple introductions likely to have occurred, and can we infer native range origins of the invasive populations we sampled?

Materials and methods

Study species

The genus *Centaurea* L. (Asteraceae) contains about 300 species (Garcia-Jacas *et al.* 2006), many of which are indistinguishable morphologically (Ochsmann 2000). In North America, 34 *Centaurea* species are reported to be introduced (USDA Natural Resources Conservation Service Plants Database), 14 of which are defined as noxious weeds in one or more states. Relationships of taxa within the genus are not well resolved (Garcia-Jacas *et al.* 2006). Ochsmann (2000) considers *Centaurea stoebe* s.l. to be a group of about 33 taxa (both species and subspecies that are differentiated by morphological characteristics), including spotted knapweed. The nomenclature for spotted knapweed is complex. There are two cytotypes, a diploid ($2n = 2x = 18$) and a tetraploid ($2n = 4x = 36$). The cytotypes are morphologically indistinguishable

Location	Site code	N	GPS	H_E	A_A	A_P
Europe					23.7	51
<i>Centaurea stoebe stoebe</i> (2×)						
Basel, Switzerland	CH1	21	47.550N 7.583E	0.559	3.0	0
Kembs, France	F3	20	48.139N 7.083E	0.373	3.2	1
<i>Centaurea stoebe micranthos</i> (4×)						
Jundola, Bulgaria	BG4	20	42.056N 23.834E	0.856	12.5	7
Monastery Route, Bulgaria	BG8	10	42.123N 23.268E	0.740	5.7	3
Rüse, Bulgaria	BG27	28	43.716N 25.918E	0.705	7.8	2
Bohonye, Hungary	HU12	29	46.403N 17.468E	0.668	6.8	4
*Batmonostor, Hungary	HU17	27	46.114N 18.931E	0.642	4.7	4
Baia Mare, Romania	RO20	25	47.407N 23.500E	0.650	5.8	0
Valea Argovei, Romania	RO25	3	44.368N 26.810E	0.756	4.3	1
Maribor, Slovenia	SLO6	21	46.447N 15.794E	0.686	4.7	5
Kholodne Jar, Ukraine	UA7	9	49.016N 32.210E	0.700	4.5	1
Bila Tserkva, Ukraine	UA15	16	49.794N 30.050E	0.703	5.5	0
Kamjanac Podilsky, Ukraine	UA19	4	48.658N 26.577E	0.763	3.0	1
Ostrag, Ukraine	UA31	8	50.201N 26.319E	0.521	5.2	0
Berdiciv, Ukraine	UA35	6	49.826N 28.630E	0.563	2.7	1
North America					20.8	28
<i>Centaurea stoebe micranthos</i> (4×)						
Big Bend, California	SCA1	9	41.007N 121.958W	0.762	4.7	0
Vail, Colorado	USCO3	30	39.650N 106.450W	0.669	6.2	2
Grimes Creek, Idaho	USID1	28	43.850N 115.750W	0.732	6.7	0
Couer D'Alene, Idaho	USID2	18	47.700N 116.800W	0.805	8.8	3
Florence, Montana	USMT1	13	46.633N 114.200W	0.782	8.3	3
Hamilton, Montana	USMT2	21	46.231N 114.150W	0.798	8.3	0
Seeley, Montana	USMT3	10	47.217N 113.500W	0.809	6.2	1
Bend, Oregon	USOR1	30	44.050N 121.300W	0.791	7.0	3
Keene Valley, New York	USNY1	21	44.206N 73.767W	0.616	5.7	2
Middletown, Virginia	USVA1	29	39.000N 78.250W	0.761	10.0	5
Bayfield, Wisconsin	USWI1	10	46.800N 90.817W	0.795	6.5	2

Table 1 *Centaurea stoebe* sampling locations, site codes used in Fig. 3, number of individuals sampled per site (N), approximate GPS coordinates of sampling sites, average gene diversity over all loci (H_E , expected heterozygosity), average number of alleles per locus (A_A), and number of private alleles (A_P , the numbers of alleles private to each sampling site do not total to the number of alleles private to the range, because some alleles private to Europe or North America are found in more than one site within the range). The sampling location indicated with an asterisk (*) may have contained both diploid and tetraploid individuals

and together come under *C. stoebe* L., a name that takes precedence over *Centaurea maculosa* Lam., which has been used commonly to refer to both cytotypes (Ochsmann 2000). The monocarpic diploid is designated *Centaurea stoebe* ssp. *stoebe* L., and the polycarpic tetraploid is designated *Centaurea stoebe* ssp. *micranthos* (S.G. Gmelin ex Gugler) Hayek (for which *Centaurea biebersteinii* DC is a synonym). Diploid *C. stoebe stoebe* are native to Western Europe, and tetraploid *C. stoebe micranthos* are native to Eastern Europe and Asia. The spotted knapweed plants in North America that have been surveyed are tetraploids (Watson & Renney 1974; Muller 1989; Ochsmann 2000; H. Müller-Schärer, University of Fribourg, personal communication), and have up to four alleles at microsatellite loci (Marrs *et al.* 2006). Thus, we call our North American samples *C. stoebe micranthos*. Hereafter, when we use the name *C. stoebe*, we are referring to both *C. stoebe micranthos* and *C. stoebe stoebe*.

Sample collection and preparation

We sampled from 15 European and 11 North American locations (Table 1, Fig. 1). European sampling sites were

focused in the Eastern European area of the species' range, where the tetraploid subspecies *C. stoebe micranthos* is indigenous, since North American plants are thought to be entirely tetraploid (Ochsmann 2000). Several Western European (likely diploid *C. stoebe stoebe*) locations were sampled as well. North American sites were spread across the invasive range of *C. stoebe micranthos* within the US, including Western, Midwestern, and East Coast locations.

Sites with fewer than 30 individuals were sampled exhaustively, while 30 or more plants were sampled at locations containing many individuals. At these larger sites, plants were sampled at least 1 m apart to reduce the chance of sampling siblings, and to sample the range of genetic variation present at each site. Naturally, the likelihood of sampling siblings is greater in the smaller sampling sites. We aimed to genotype 30 individuals per sampling site, and in the end we obtained data on 3–30 individuals per site (Table 1), with sites for which we had data on few individuals being excluded when appropriate from the below analyses as indicated. Leaf tissue or mature seedheads were collected from individual plants, depending on the season of collection. Leaf tissue was dried and stored on desiccant for transport

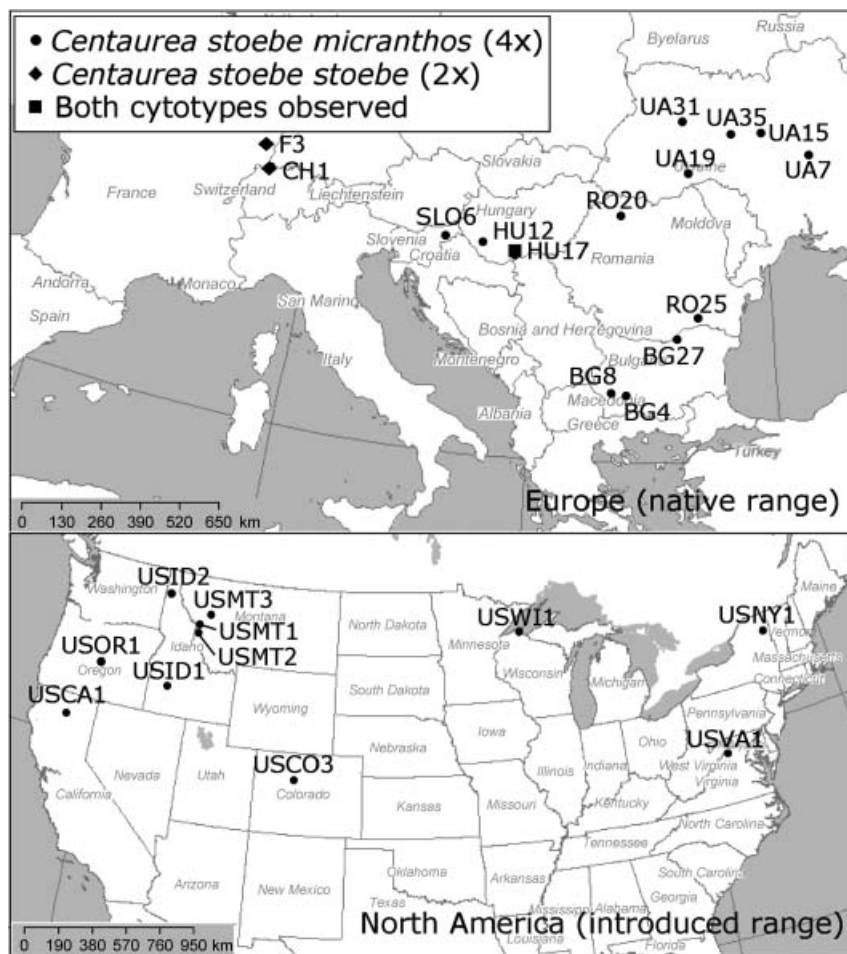


Fig. 1 Map of sampling locations. *Centaurea stoebe micranthos* sample sites are indicated with circles, *Centaurea stoebe stoebe* sites are indicated with diamonds, and the potentially mixed ploidy site is shown as a square. Site codes are given in Table 1. Note the difference in scale between the European and North American maps.

to the laboratory, while seeds were kept separate by maternal plant, then germinated to provide fresh leaf tissue for DNA extraction. Genomic DNA was extracted from desiccated and fresh leaves using QIAGEN Plant Mini kits and stored at -20°C until genotyping.

Microsatellite analysis

We genotyped the samples using six microsatellite loci (21 CM36, 42 CM27, 38 CM22, CM15, CM26, and CM17) from Marrs *et al.* (2006). Polymerase chain reaction conditions, allele visualization techniques, and band scoring followed Marrs *et al.* (2006). Once all individuals from a sampling site were genotyped, we examined the genotypes to determine whether each site was likely to contain diploid or tetraploid individuals. If a site had no individuals with more than two alleles at any of the six loci, we inferred that the site contained only the diploid subspecies, *C. stoebe stoebe*. If three or more alleles were observed at any locus for any individual, the site was assumed to contain the tetraploid subspecies, *C. stoebe micranthos*. The analysis methods are described in detail below.

When microsatellite loci are surveyed in polyploid species, they are not completely codominant as they are in diploid

species. It is not possible to know the copy number of each allele when an individual is not homozygous or fully heterozygous (for example, when one observes the allelic phenotype A, B in a tetraploid individual, the genotype could be ABBB, AABB, or AAAB). While dosage effects (revealed, for example, by differences in peak height) can in some cases be used to determine the genotype of polyploid species (Landergrott *et al.* 2006), we were not able to do so with our raw data. Thus, the full genotypes for individuals that were not homozygous (e.g. showed only allele A) or fully heterozygous (e.g. showed four different alleles, A, B, C, and D) could not be determined. In such cases, each allele observed in an individual was recorded once, and the remainder of the genotype was coded as missing data. For example, when alleles A and B were observed, the genotype recorded was AB??, where '?' represents missing data. Although some bias may be introduced by this method of coding genotypes, it is the only way to treat raw polyploid data when dosage effects are not present, and any method of making assumptions as to the copy number of each allele in a genotype would bias results much more. Coding incomplete genotypes in this manner may bias estimates of population genetic statistics such as expected heterozygosity and allelic richness, and may

result in fewer well-supported relationships in assignment test results. Incomplete genotype coding will not bias measures of genetic diversity like total number of alleles or number of private alleles, however. All individuals were coded and analysed as tetraploids, even those from the two likely diploid Western European sites, to be able to compare across cytotypes directly. The incomplete genotype codes were used in all statistical analyses described below, except in the traditional assignment tests which required the data to be formatted as a binary matrix.

The way that microsatellite data from tetraploids must be coded, as outlined above, prevents straightforward calculation of several common statistics such as allelic richness. Additionally, most software packages for analysis of population genetic data do not allow input of polyploid data. Thus, the analyses described below are limited accordingly to approaches suitable for polyploid data.

Comparing genetic diversity in native and introduced ranges

To examine genetic diversity in the European and North American ranges of *C. stoebe*, we computed expected heterozygosity, the average number of alleles, and the number of private alleles (Neel 1973; Slatkin 1985) in each sampling location using SPAGeDi version 1.2 (Hardy & Vekemans 2002), a software program that computes statistics and permutation tests of relatedness and differentiation between populations for organisms of any ploidy level (Hardy & Vekemans 2002). SPAGeDi version 1.2 assumes polysomic inheritance, as seen in autopolyploids. As it is unknown whether *C. stoebe micranthos* is auto- or allopolyploid, results should be interpreted with some caution. We used analysis of covariance to compare expected heterozygosity, the average number of alleles per locus, and the number of private alleles per site between European tetraploid *C. stoebe micranthos* and North American invasive *C. stoebe micranthos* with continent as the predictor variable and sample size of each population as the covariate (JMP version 5.0, SAS Institute). The data on both expected heterozygosity and the average number of alleles per locus were normally distributed, while the number of private alleles was square-root transformed to meet the assumptions of ANCOVA. To determine if allelic diversity varied significantly between the two ranges, we computed Shannon–Weaver phenotype diversity indices for each range and compared them using permutation tests (10 000 permutations) with the program F-DASH (Obbard *et al.* 2006). A genetic phenotype is simply the observed alleles seen in an individual without any assumptions made about the copy number of each allele, and is used here because we do not have full genotype information for the majority of individuals in the study. The analyses with F-DASH were performed two different ways. First, we compared all European *C. stoebe* to the North American samples. Then, we

compared only the European samples observed to be tetraploid (*C. stoebe micranthos*) to the North American samples. The program F-DASH was also used to compare the average number of alleles carried by each individual in each range, both including and excluding the likely diploid European samples.

Comparing population structure in the native and introduced ranges

We compared F_{ST} -statistics (F_{ST}), which estimate the amount of among-population variation in a sample, within European *C. stoebe micranthos*, within North American *C. stoebe micranthos*, and between European and North American *C. stoebe micranthos* using SPAGeDi (Hardy & Vekemans 2002). As little gene flow is expected to occur between diploids and tetraploids, two apparently diploid *C. stoebe stoebe* sites and one apparently mixed ploidy site (see Results, below) were excluded from this analysis to prevent overestimation of population structure in Europe. Permutation tests (20 000 permutations) were implemented to provide 95% confidence intervals around estimates of F_{ST} for each of these comparisons. Confidence intervals allowed us to determine whether population structure was greater in either the native or introduced range, and to determine whether continent-level structuring was significantly different from zero. We calculated pairwise F_{ST} values (Weir & Cockerham 1984) to determine genetic distance between sample locations using SPAGeDi 1.2 (Hardy & Vekemans 2002). Pairwise F_{ST} was plotted against the ln of spatial distance between sampling locations to visualize the correlation between genetic and geographical distances. We calculated Slatkin's similarity measure {or linearized F_{ST} , $M = [(1/F_{ST}) - 1]/4$, Slatkin 1993} for each population comparison and used Mantel tests and reduced major axis (RMA) regression implemented in the program IBD (Bohonak 2002) to determine if isolation by distance trends seen in the pairwise F_{ST} plots were significant.

We implemented two types of assignment tests. First, we coded the data as one would for a dominant marker data set (e.g. for each of the six loci, all alleles were coded as a 1 if present and a 0 if absent), and ran assignment tests using AFLPOP version 1.1 (Duchesne & Bernatchez 2002). This test enables us to evaluate whether North American individuals could be directly assigned to any of the sampled European populations. This method is an adaptation of Paetkau *et al.* (1995), and was used because the data from tetraploid *C. stoebe micranthos* are not completely codominant as described above. The second assignment test was performed on the full data set using Structure version 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003; Falush *et al.* 2007). The power of this approach is that one can acknowledge that a sample site does not necessarily represent a true genetic population. Structure (version 2.2) can be used to infer the number of

genetic clusters present in the data set (K), and to assign individuals to these clusters. The model uses a Bayesian approach, beginning with prior distributions of model parameters. It updates these with observed data by the Markov-chain Monte Carlo method, then finds population groupings that are in equilibrium and assigns individuals to these populations. Bayesian methods assume that observations are randomly drawn from each cluster and that all potential source populations are predefined (Manel *et al.* 2005). Structure also assumes that Hardy–Weinberg equilibrium is present within populations and that there is complete linkage equilibrium between loci within populations (Pritchard *et al.* 2000). Individuals are therefore grouped with other individuals to form clusters that are in Hardy–Weinberg and linkage equilibrium, and K is simply the number of those clusters. In addition to determining the number of genetic clusters, Structure also determines percentage assignment of each individual to each genetic cluster. Although the program has been recompiled for polyploids (Falush *et al.* 2003; Falush *et al.* 2007), it is important to realize that Hardy–Weinberg equilibrium applies to diploids and the Structure results should be interpreted conservatively. Nonetheless, Falush *et al.* (2007) tested version 2.2 and found it performed well, even with fully dominant amplified fragment length polymorphism (AFLP) data. We used Pritchard *et al.*'s (2000) ad hoc method for determining the approximate number of genetic clusters present in the complete data set by giving the program a range of values for K as priors and determining which one gave the highest estimated log probability of the data. Structure analyses may also be useful in distinguishing diploid *C. stoebe stoebe* from tetraploid *C. stoebe micranthos*. We computed three independent runs for each possible K from 2 to 30 using a burn-in of 500 000 followed by 750 000 data collection repetitions, sufficient to reach a stable α and estimated log probability of the data. Results between runs were consistent. All iterations were run with the admixture model, which assumes that individuals may have mixed ancestry, because of the likelihood of interpopulation and interspecific crossing in the *C. stoebe* system. We also selected to model allele frequencies as independent between populations, a prior that expects allele frequencies in different populations to be somewhat different from one another (Falush *et al.* 2003).

Alleles found in a single European sampling site may also contribute information about possible European origins of North American *C. stoebe*. We examined the data set for European private alleles (e.g. found at only a single location within Europe) that were shared with North American individuals. To evaluate average genetic similarity between sampling sites, we constructed a phenetic tree using Nei's standard genetic distance (1972) with the unweighted pair group method with arithmetic mean (UPGMA) using the program Populations (Langella 1999) with 10 000 bootstrap replications on the full data set.

Results

Ploidy

All North American sites contained individuals whose genotypes were consistent with tetraploidy (e.g. three or more alleles in at least one locus). Most European sites also appeared to be tetraploid, except the two Western European sites, Kembs, France (F3) and Basel, Switzerland (CH1). Most individuals in the Batmonostor, Hungary (HU17) site had genotypes with two or fewer alleles at each locus (consistent with diploidy), but a few individuals had more than two alleles at a locus, indicating they were likely to be tetraploid, and suggesting the site either contained tetraploids of low diversity or individuals of both cytotypes. For the analyses, we classed this site as tetraploid to be conservative, but that determination remains tentative without detailed cytological analyses.

Genetic diversity

The microsatellite loci examined in this study were highly polymorphic. All six loci were polymorphic in every sampling location except one; Kembs, France (F3) was monomorphic at locus CM17. We recorded a total of 176 alleles over all six loci and 26 sampling locations, of which 148 were found in the native European range of the species (EU), and 125 were found in the invasive North American range (NA). Overall, European *Centaurea stoebe* sites averaged 23.7 alleles per locus, while North American sites averaged 20.8 alleles per locus. For *Centaurea stoebe micranthos*, average numbers of alleles per locus within individual sampling sites ranged from 2.7 to 12.5 in Europe, and from 4.7 to 10.0 in North America (Table 1), and did not differ significantly between the ranges (EU mean = 5.82, NA mean = 6.90, $F_{1,21} = 1.7$, $P = 0.198$), although sample size had a significant effect on the average number of alleles ($F_{1,21} = 5.05$, $P = 0.035$). The diploid *Centaurea stoebe stoebe* had significantly fewer alleles per locus than the tetraploid *C. stoebe micranthos* (diploid mean = 3.01, tetraploid mean = 6.47, $F_{1,23} = 8.97$, $P = 0.007$). These results did not change qualitatively if we excluded Batmonostor, Hungary (the site of possibly mixed ploidy or low diversity tetraploids) from analyses entirely.

Private alleles (found only within a single sample location) were found in both ranges. At the continental scale, European *C. stoebe* had more alleles unique to the continent (51) than North America (28). Of the 51 alleles found only in European *C. stoebe*, 30 (58.8%) were private to individual sites (Table 1), while the remainder were shared among two or more European sites. Twenty-one of the 28 (75%) total private alleles in North America were unique to individual sites, and seven alleles were shared between two or more North American sites. At the level of sampling site, there was not a significant difference in the number of private alleles

per site between the ranges (EU raw mean = 2.00, NA raw mean = 1.91, $F_{1,21} = 0.50$, $P = 0.487$). There was a marginally significant effect of sample size on the number of private alleles per site ($F_{1,21} = 3.6$, $P = 0.072$).

Expected heterozygosity varied from a low of 0.373 at the likely diploid Kembs, France (F3) site to a high of 0.856 at the Jundola, Bulgaria (BG4) site. Mean expected heterozygosity within sample locations was significantly higher in the invasive range than in native range *C. stoebe micranthos* (NA mean = 0.760, EU mean = 0.656, $F_{1,21} = 6.76$, $P = 0.016$), while sample size had no effect on expected heterozygosity ($F_{1,21} = 0.70$, $P = 0.412$). Shannon-Weaver diversity of phenotype frequencies was higher in North American samples (3.40) than in European *C. stoebe* samples (3.08), but the difference was not significant ($P = 0.123$). When diploid Western European samples (from Kembs, France and Basel, Switzerland) were excluded from the data set, North America still had a slightly, but not significantly, higher phenotypic diversity (NA = 3.40, EU = 3.13, $P = 0.134$).

The average number of different alleles carried by each individual was significantly higher in North America than in Europe (NA = 1.92, EU = 1.72, $P = 0.023$) when the likely diploid European samples were included. When the diploids were removed, the invasive range still had more different alleles per individual, although the difference was no longer significant (NA = 1.92, EU = 1.79, $P = 0.089$).

Population structure

We compared F_{ST} over all *C. stoebe micranthos* sites, among European *C. stoebe micranthos* sites, among North American sites, and between European and North American *C. stoebe micranthos* sites to determine the scale of population structure in our data set (Fig. 2). All four F_{ST} values were significantly greater than zero, demonstrating population structure at each hierarchical level. The global average F_{ST} (among-site variation over all sites) was 0.098 (95% confidence interval 0.069–0.128), indicating a highly significant amount of population structure overall. Among-site variation was lower in North America ($F_{ST} = 0.081$, 95% confidence interval 0.058–0.103) than in Europe ($F_{ST} = 0.121$, 95% confidence interval 0.072–0.171), but this difference was not significant. Differences between regions (EU vs. NA) explained a small but significant amount of the variation ($F_{ST} = 0.017$, 95% confidence interval 0.010–0.025). When population pairwise F_{ST} values were plotted against the geographical distance separating the *C. stoebe micranthos* sampling sites, no relationship was apparent between genetic and geographical distance in Europe or North America. The program *IBD* (Bohonak 2002) confirmed these patterns. Slatkin's similarity measure (Slatkin 1993) was not significantly negatively correlated to geographical distance in Europe ($Z = 5360.1160$, $r = 0.2042$, $P = 0.0996$). In North America, there was also no significant

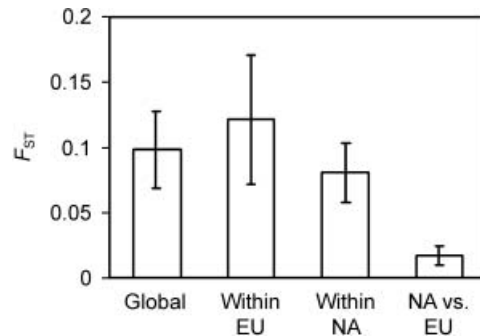


Fig. 2 Estimates of F_{ST} over all *Centaurea stoebe micranthos* sites (global avg.), among European *C. stoebe micranthos* sites (within EU), among North American sites (within NA), and between *C. stoebe micranthos* on the two continents (NA vs. EU). Error bars are 95% confidence intervals.

relationship between Slatkin's similarity measure and geographical distance ($Z = 357828.70$, $r = -0.0942$, $P = 0.277$).

Multiple introductions and origins

Traditional assignment tests implemented as for fully dominant data using AFLPOP (Paetkau *et al.* 1995; Duchesne & Bernatchez 2002) did not allocate any North American individuals to sampled European sites, even at the lowest stringency. Using Pritchard *et al.*'s (2000) method for estimating the number of genetic clusters in a data set, we found the data set was consistent with $K = 9$ with over 99% probability. We then plotted each individual's percentage assignment to each of these nine genetic clusters (Fig. 3). While individuals from many sampling sites assigned to multiple clusters, at some sites most individuals assigned strongly to the same genetic cluster, indicating a higher degree of within-population similarity. Most European individuals assigned strongly to the same genetic cluster as the other individuals from their sampling site, a visual indication of population structure, while the North American individuals and sites tended to be more genetically mixed. Within Europe, we found that most individuals from Basel, Switzerland (CH1) and Kembs, France (F3) assigned strongly to a cluster that was not well represented in any other individuals in our study (represented by light blue in Fig. 3). The microsatellite genotypes of these individuals suggest they are the diploid subspecies *C. stoebe stoebe* (\leq two alleles per locus per individual). Individuals from the site that contained just a few individuals with more than two alleles per locus per individual, Batmonostor, Hungary (HU17), did not share assignment to this cluster, but assigned strongly to a cluster, shown in royal blue, that was also predominant in many Ukrainian individuals. Most Jundola, Bulgaria (BG4) and Monastery Ridge, Bulgaria (BG8) individuals were strongly assigned to the genetic cluster shown in red

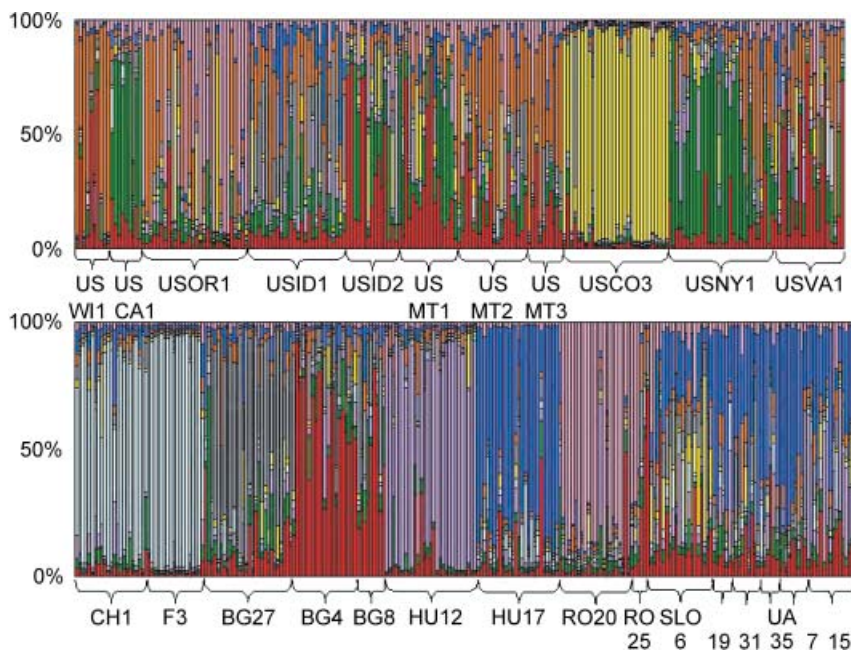


Fig. 3 Assignment test results: percentage assignment of each North American (top graph) and European (bottom graph) individual (represented by vertical bars) to each of the nine genetic clusters (represented by different colours) inferred by the program Structure (Pritchard *et al.* 2000). Site codes (Table 1) indicate the geographical location of individuals along the x-axis.

in Fig. 3. Several North American individuals, particularly from Idaho and Montana, also assigned to this cluster. Most samples from Bohonye, Hungary (HU12) assigned very strongly to the genetic cluster shown in lavender, which was not well represented in North America. Rüse, Bulgaria (BG27) individuals assigned fairly strongly to the cluster shown in grey, a cluster that many Grimes Creek, Idaho (USID1) individuals assigned strongly to as well. The samples from Baia Mare, Romania (RO20) mostly assigned to the cluster shown in pink, a cluster that is strongly represented in many Bend, Oregon (USOR1) individuals as well as other North American individuals. The European sites from Slovenia and the Ukraine were much more genetically admixed than the other native range sites. We also observed genetic clusters that were virtually unique to North America. The Keene Valley, New York (USNY1) site showed a large proportion assignment to the green cluster, which we also found in Trinity, California (USCA1) but did not see in the native range. There was also a cluster (shown in orange) that was predominant in individuals from Bayfield, Wisconsin (USWI1) and in some individuals from Oregon and Montana. Vail, Colorado (USCO3) showed the strongest assignments in the invasive range of the species, to the cluster shown in yellow; this cluster was also not well represented in the native range of *C. stoebe*.

Examination of the data set for alleles private to a single European sampling site, but shared by North American individuals showed several connections. Overall, five European sampling sites contained private alleles also found throughout North America. Romanian, Bulgarian (BG4 and BG8), and Slovenian private alleles were widespread in the invasive range. The putative mixed ploidy site, Batmonostor, Hungary (HU17), shared one private allele at low frequency

with several North American regions, as well as with 17 individuals from the Vail, Colorado (USCO3) site. The Vail site also contained rare European alleles at high frequency from Slovenia (SLO6) and Valea Argovei, Romania (RO25).

Population similarity analysis by UPGMA tree produced a tree with very weak bootstrap support at all nodes (details not presented).

Discussion

Ploidy

The microsatellite loci used in this study differentiated between diploid and tetraploid *Centaurea stoebe* for most sample sites. Individuals from the range of the diploid subspecies *Centaurea stoebe stoebe* assigned strongly to a distinct genetic cluster (Fig. 3), and also possessed microsatellite allelic phenotypes consistent with diploidy (two or fewer alleles at each locus). One site located in the area of overlap of the subspecies' ranges included individuals with diploid allelic phenotypes, but several individuals had more than two alleles at a locus, suggesting that the site might contain both cytotypes, or is a tetraploid site with relatively low diversity. That a single location can have both diploids and tetraploids has been confirmed with flow cytometry (H. Müller-Schärer, University of Fribourg, personal communication).

Genetic diversity is comparable between ranges

Invasive North American sampling locations had fewer total and private alleles than native European sampling locations, but those reductions in numbers of alleles were not significant, suggesting that invasive *Centaurea stoebe micranthos*

was not subjected to a severe demographic bottleneck during its introduction. Indeed, several other lines of evidence also suggest there was no founder effect or bottleneck during the invasion process. Expected heterozygosities of sample locations were significantly higher in the invasive populations than in the native populations. Shannon–Weaver phenotype diversity, which explicitly corrects for sample size, also did not differ significantly between ranges. A similar pattern was found in a study using cpDNA sequence data (Hufbauer & Sforza 2008): haplotype diversity did not differ significantly between North America and Europe, supporting the notion that *C. stoebe micranthos* was not subjected to a severe bottleneck in population size upon introduction to North America.

While the native range had more total and private alleles, the invasive range had more combinations of alleles. This corresponded with individuals on average carrying more different alleles in North America than in Europe. This outcome may be related to the allele frequencies. European sample locations had some alleles that were dominant, and other rare alleles that occurred only at low frequency, while North American sample locations had a more even distribution of allele frequencies, and fewer rare alleles. The high degree of invasive range diversity seen in this study is similar to results seen by others and is consistent with an invasion pattern of multiple introductions (Durka *et al.* 2005; Gaskin *et al.* 2005; Genton *et al.* 2005; Facon *et al.* 2008), or of a quick return to large population sizes, allowing retention of genetic diversity after a demographic bottleneck (Zenger *et al.* 2003).

Population structure is similar between ranges

Population genetic structure (measured as F_{ST}) was similar between Europe and North America, with a trend towards higher structuring in the native range (Fig. 2). Our sampling within the native range covered a smaller geographical area than within the introduced range, thus with more comparable sampling, this trend might become significant. An explanation for this general pattern is simply that there has not been enough time since introduction into North America for genetic drift to lead to differentiation of isolated populations. Although most variation occurred within populations in both ranges, there was a significant among-population component to genetic variation in each range. Thus, in both *C. stoebe*'s native European and its introduced North American range, population structure is apparent. This contrasts strongly with patterns observed for the congener *Centaurea diffusa*, where there was no significant population structure in the native European range, but strong population structure within the introduced North American range (Marrs *et al.* 2008). In addition to structure among populations in *C. stoebe*, we saw a small but significant amount of population differentiation at the continent level. This continent level effect could

be simply because we have not captured all of the source populations in our sampling, but it could also be the result of genetic drift that occurred during the introduction process, population differentiation due to drift since introduction, or the result of selection on different traits in the introduced range (Blossey & Notzold 1995; Mooney & Cleland 2001; Maron *et al.* 2004).

We did not see significant correlations between genetic and geographical distance in either range of the species. This pattern is different from the one of native range isolation by distance that has been found in other systems and attributed to the long history of undisturbed European populations (Genton *et al.* 2005). Our result suggests that native range *C. stoebe micranthos* populations may have undergone recent disturbance by human transport of propagules, which matches the patterns seen for *C. diffusa* across some of the same portions of the native range (Marrs *et al.* 2008).

Although population genetic structure is significant in *C. stoebe*, it seems to be operating on a larger scale than the one at which we sampled. If all of the collection locations truly corresponded to genetically distinct clusters, we would expect to find a K (the number of clusters inferred using Structure, Pritchard *et al.* 2000) close to the number of sites in the study (26). Instead, we estimated the number of genetic clusters in the data set at just nine.

Multiple introductions to North America

Traditional assignment tests did not allocate any North American individuals to European populations. Even at the lowest stringency, high interpopulation genetic variation in Europe prevented positive assignment of invasive individuals to sampled native populations. Additionally, coding the data as fully dominant for use in AFLPOP version 1.1 (Duchesne & Bernatchez 2002) loses information, making these tests inherently less powerful than the Bayesian approach to assignment as implemented in Structure version 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007). The Bayesian assignment test (Fig. 3) inferred nine genetic clusters. Overall, most individuals within a sampling site in Europe assigned fairly strongly to a single genetic cluster, resulting in blocks of colour that corresponded to sample site in the bar plot (Fig. 3). In the invasive range, however, most individuals were not strongly assigned to a single cluster; genetically admixed *C. stoebe micranthos* is spread across the invasive range. Alleles private to a single European location were also often found in several widespread North American sites, indicating a high degree of gene flow and admixture in the invasive range. Many North American individuals of mixed assignments have affinity with the clusters represented by orange, green, grey, pink and red in Fig. 3. Orange and green are not represented within the native range. Gray links Grimes Creek, Idaho (USID1) with

Rüse, Bulgaria (BG27). The cluster represented by pink is predominant in the Baia Mare, Romania (RO20) site, which would indicate a Romanian origin for an introduction event. Meanwhile the red cluster is found in several Bulgarian sites (BG4 and BG8), suggesting that another introduction occurred from this region. Hypotheses of Romanian and Bulgarian origins are further supported by the relatively high frequency of private alleles from these sites that were observed in North American individuals.

Our data suggest several other introduction events as well, although they cannot be linked to any of our sampled European sites. The Vail, Colorado (USCO3) site is genetically distinct from all other North American sites in the Structure plots, and also contained high frequencies of alleles that were rare even in their native range populations of origin. The cluster that dominates the genetic assignment of the Vail site is not well represented in the Structure plot of the European samples, except in several Slovenian individuals. The lack of broad assignment of European individuals to this yellow cluster suggests that at least some genetic sources of invasive populations are not included in our samples, or that this site is the product of a highly unique mixture of rare native range genotypes. The fact that we found 28 private alleles in North America also supports the conclusion that our sampling regime missed some native sources of our invasive populations. The North American site from Keene Valley, New York assigns strongly to the green Structure cluster, which is also common in Trinity, California (USCA1) and not well represented in Europe (Fig. 3). Finally, there is a cluster, represented with orange in Fig. 3, that dominates the Bayfield, Wisconsin (USWI1) site and is present in some of the Montana sites which may represent yet another introduction that we cannot directly connect to our sampled European sites. Given the complete lack of positive assignment of North American individuals to European populations in traditional assignment tests, similarities between North American and European sites revealed by the Bayesian assignment method should be viewed as tentative. Nonetheless, the Bayesian method results do strongly suggest a history of multiple introductions of *C. stoebe* to North America, a result corroborated by chloroplast sequence data (Hufbauer & Sforza 2008).

Conclusions

Diversity reducing processes such as selection, genetic drift by bottleneck, or founder effect do not seem to have played an important role in the invasion of this weed. Indeed, invasive *C. stoebe* are more heterozygous than, or at least as heterozygous as their native counterparts. Population genetic structuring was greatest among European sites, although it was also significant among North American sites and at the continent level, between Europe and North America. Isolation by distance was not significant in either range of

the species. We found evidence for multiple separate introductions of this species to North America. It seems likely that we have had at least one introduction from Romania, which contributed to many of the genetically admixed North American individuals. The other area that may have contributed to the mixed invasive populations is Bulgaria. Three other introductions may have founded the Vail, Colorado; Keene Valley, New York; and Bayfield, Wisconsin sites. These genetic clusters are not well represented in Europe and are likely to correspond to introductions from areas of the native range that we did not sample. Alternately, these clusters may represent genetic groups that originated in the invasive range, either by genetic drift or as a result of adaptive evolution.

Centaurea stoebe micranthos has been able to maintain its genetic diversity through the invasion process as a result of multiple introductions. Most North American sites appear to contain individuals with highly genetically admixed assignment profiles, likely the result of gene flow between separately introduced genotypes of *C. stoebe micranthos*. A minority of sites contain individuals that share a strong co-assignment and are genetically unique from other sites. From a management perspective, these results suggest that *C. stoebe micranthos* will continue to be difficult to control. North American *C. stoebe micranthos* has several native range origins and some of these genotypes appear to have hybridized in the new range. If biological control agents are specialized at the level of host genotypes, then samples from any single one of the areas of origin within the native range are unlikely to be effective on all invasive genotypes of *C. stoebe micranthos*.

Acknowledgements

This work was funded by USDA NRI grant 2002-35320-12137, USDA ARS EBCL Cooperative Agreement 5840121141, the Colorado State University Agricultural Experiment Station, and NSF DEB grant 0515743. The authors thank Dr William C. Black IV, Steven Rauth, Dr Amy Blair, Dr Rebecca Kao, and four anonymous reviewers for suggestions that greatly improved the manuscript. Dr Andrew Norton and Dale Woods collected samples for us, and Dr Susan Knudson helped with operating the ABI instrument.

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This study is part of R. Marrs' PhD dissertation which examined the population genetics of *Centaurea stoebe* and *Centaurea diffusa* in their introduced and native ranges. Her primary research interest is biological invasions. R. Sforza studies plant-insect interactions and biological control of weeds, and spends much of his time evaluating the herbivores of invasive weeds in their native ranges in search of potential biological control agents. R. A. Hufbauer is an evolutionary ecologist whose current research is focused mainly on biological invasions, plant-insect interactions, and biological control.
