

# Pre- and post-introduction patterns in neutral genetic diversity in the leafy spurge gall midge, *Spurgia capitigena* (Bremi) (Diptera: Cecidomyiidae)

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## Abstract

Many researchers have hypothesized that reduced genetic diversity in introduced populations of biological control agents can compromise the agent's ability to establish and then adapt to novel environments. Strategies such as collecting from a wide geographic area or maintaining large colony sizes through the quarantine process have been used to minimize the loss of genetic diversity. Few studies have examined how the process of collection, quarantine, and establishment could affect patterns of genetic diversity in biological control agents. In this paper, we examine patterns in neutral genetic diversity in *Spurgia capitigena*, a gall midge introduced as a biological control agent of leafy spurge (*Euphorbia esula*) from its original collection site in Italy, a newly discovered population of the midge from southern France, and in two established populations in North America. By comparing the existing populations with historical samples collected shortly after the original introductions in 1991, we found that there is evidence of a mild bottleneck in both of the introduced populations. Further, we also examined the population structure in European populations of the fly collected from leafy spurge and a closely related species, cypress spurge (*Euphorbia cyparissias*) and found evidence for local restrictions in gene flow between populations on the two plants, but no evidence to support the current taxonomy of the genus with two distinct fly species.

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## 1. Introduction

Researchers have recognized that the genetic variability within populations of biological control agents may play a central role in the success or failure of classical

biological control introductions (Hoy, 1985; Messenger and van den Bosch, 1971; Roush, 1990; Roderick and Navajas, 2003). Although genetic variation is essential for evolution to operate, and evolution may be important in success, only a few studies have examined how the process of a biological control introduction alters patterns in genetic variation (e.g., Baker et al., 2003; Hufbauer et al., 2004). In this paper, we examine the effects of collecting, quarantining, and establishing a biological control agent of leafy spurge (*Euphorbia esula* L.) on the patterns of neutral genetic variation in its populations.

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Due largely to logistical constraints, most biological control agents are collected as small sub-populations that may not represent the full genetic diversity of the source population. This reduced genetic diversity among the biological control agents could modify their effectiveness (Hopper et al., 1993). Most organisms intended for release as biological control agents also must go through one or more generations in quarantine, during which time genetic variability may be further reduced. If numbers are not sufficient for release, population sizes must be increased in insectaries. Genetic diversity may also decrease in this situation and selection pressures will favor those individuals best fit for insectary conditions (Hokkanen, 1985; Roush, 1990). If the host is sparse and the introduced insect is incapable of adequately dispersing over large distances, then gene flow between populations will likely be rare (Danforth et al., 2003) and genetic drift may further reduce the number of alleles within populations (Nei et al., 1975). Founder events can also increase the frequency of rare alleles by more rapid selection and genetic drift (Carson, 1990) or increase quantitative genetic variation through the conversion of epistatic genetic variation to additive genetic variation (Goodnight, 1988). The process of introducing biological control agents into new ranges could theoretically reduce neutral genetic variation and increase quantitative variation in important traits. Whether such changes occur is unknown and evaluations of their ecological significance have not been conducted.

To minimize potential bottlenecks in population size and founder events, early approaches to introducing new biological control agents focused on collecting at many different sites to ensure maximum genetic diversity of the species in the new range. A more sophisticated understanding of the ecology and genetics of coevolutionary interactions between plants and insects and the specificity of phytophagous insects for their hosts (reviewed in Mopper and Strauss, 1998) has refined these strategies. Several studies have illustrated that insect species have significant genetic variation that is associated with their ability to exploit different host species (e.g., Landry et al., 1993; Martel et al., 2003; Nason et al., 2002; Sunnucks et al., 1997) and even host genotypes within a species (e.g., Underwood and Rausher, 2000). When such variation among populations exists, the practice of collecting from several locations for a classical biological control introduction may unite different strains of the same species which upon mating produce less fit offspring, negatively impacting the introduced population (case studies reviewed in Hokkanen, 1985; Hoffmann et al., 2002). Thus, there can be a conflict between sampling widely to maximize genetic variation and sampling narrowly to maintain locally adapted populations.

The ability of genetic variation to predict variation in ecologically important traits is controversial (Bekessy et al., 2003; Van Tienderen et al., 2002), and meta-

analyses have been performed that support both sides of this argument. Reed and Frankham (2001) found that little correlation exists between molecular genetic variance and quantitative genetic variance; however, Merilä and Crnokrak (2001) examined data from 18 studies comparing genetic differentiation for neutral marker genes ( $F_{ST}$ ) to quantitative traits ( $Q_{ST}$ ) and found a positive relationship between  $F_{ST}$  and  $Q_{ST}$ . Another meta-analysis by Reed and Frankham (2003) found a highly significant correlation between heterozygosity and fitness. It is still unclear what role genetic variation plays in the success of introduced biological control agents. In one system, Hufbauer (2002) observed a decrease in fitness among *Aphidius ervi* Haliday (Hymenoptera: Aphidiidae) parasitoids that were introduced in New York for the control of pea aphids (*Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae)) on alfalfa. The introduced parasitoids were less successful than native French parasitoids in overcoming the aphid's physiological resistance. Further investigation into the population genetics of *A. ervi* using microsatellite loci found a mild population bottleneck and significant genetic differentiation between native and introduced parasitoids which may be correlated to the differences seen in host parasitism (Hufbauer et al., 2004).

Our objective was to determine how the process of a biological control introduction has altered patterns in neutral genetic markers within the introduced populations of the leafy spurge gall midge, *Spurgia capitigena* (Bremi) (Diptera: Cecidomyiidae). We examined mitochondrial sequence variation and variation in microsatellite loci in *S. capitigena* from native European and introduced North American populations and explored how the process of introducing the agent has altered genetic variation.

Leafy spurge (*Euphorbia esula* L.) is a noxious rangeland weed that is a major pest in the northern Great Plains of the United States and Canada. This exotic weed infests approximately one million hectare of land in North America (Whitson et al., 1999) and economic losses for North Dakota, South Dakota, Wyoming, and Montana are estimated to be almost \$130 million annually (Leitch et al., 1994).

*Spurgia esulae* was first introduced into the United States in 1987. The flies for these releases were collected from San Rosore, Italy, and had passed through one generation in quarantine at the USDA-APHIS Mission, Texas facility. These flies were released at a site near Bozeman, Montana, and at the Katie Olson Wildlife refuge in North Dakota. Subsequent releases of the flies to populations mentioned in Hansen et al. (1997) were all collected from the Bozeman, MT population (R. Hansen, USDA-APHIS-CPHST, Fort Collins, CO, personal communication).

At the time that the leafy spurge gall midge was first introduced it was named *Bayeria capitigena* Bremi (Solinas and Pecora, 1984); however, in 1990 Gagné revised this group and defined a new genus, *Spurgia*, which

includes two species, *S. capitigena* (Bremer) and *S. esulae* Gagné (= *B. capitigena* Bremer of Solinas and Pecora, 1984). In this revision, *S. esulae* is described as occurring exclusively on leafy spurge while *S. capitigena* occurs exclusively on cypress spurge, *Euphorbia cyparissias* L., a closely related species. Morphologically, the two insect species are differentiated only by several continuous characteristics of the male hypoproct (Gagné, 1990).

It has been assumed that all flies in the United States are *S. esulae* because flies that were introduced were collected only from leafy spurge in Italy (Pecora et al., 1991); however, Nelson and Carlson (1999) found that *S. capitigena* was established on leafy spurge in North Dakota. Sobhian et al. (2000) reported that *S. capitigena* was present on leafy spurge in France and had host specificity similar to *S. esulae*, inducing galls on leafy spurge as well as other plants in the subgenus *Esula* in the laboratory, but only on leafy spurge under field conditions. It is possible that if two species do exist, both fly species were unintentionally released and established in North Dakota and Canada (Sobhian et al., 2000). New data on host specificity of *S. capitigena* indicated that this species does attack leafy spurge (Sobhian et al., 2000), allowing for two possibilities: (1) either both species of flies will use leafy spurge and occur in mixed populations in Italy or (2) there is only one morphologically variable fly species. Many studies have used mitochondrial DNA sequence data to evaluate whether insects found on different hosts are differentiated enough to represent host races or cryptic species (e.g., Baer et al., 2004; Brown et al., 1996, 1997; Diegisser et al., 2004; Dobler and Farrell, 1999; Downie et al., 2001; Johnson et al., 2002; Smith et al., 2002; Wink and Legal, 2001). In addition to exploring the effects of the introductions for biological control on genetic variation in the *Spurgia* flies, we use our data to assess the degree of differentiation of flies from the native range from both leafy and cypress spurge.

## 2. Materials and methods

### 2.1. Fly collections

*Spurgia* flies were collected as larvae and pupae within host plant galls from leafy spurge and cypress

spurge in San Rossore, Italy in 1991, 2001, and 2003 and near Avignon, France in 2001. In both of these areas, leafy spurge and cypress spurge grow near each other. The French collections were all from within a ca. 6 km<sup>2</sup> area, and the Italian populations were from a ca. 3 km<sup>2</sup> area. Introduced populations were sampled from leafy spurge in the Katie Olson Wildlife Management Area, Barnes, North Dakota in 1991 and 2001 and from Bozeman, Montana in 1990. The 1990 and 1991 collections were stored at the USDA-APHIS quarantine facility in Mission, Texas. In subsequent analyses, the 2001 and 2003 Italian cypress spurge collections were treated as one population to increase sample size. This was done for the 2000 and 2001 North Dakota samples as well. Population location, sample sizes, and collection dates are presented in Table 1. Although a single gall may contain up to several dozen larvae, in the 2001 and 2003 collections we used one fly per gall in most cases. This was done to minimize the chances of sampling siblings, as larvae in a single gall are more likely to be siblings. However, due to small populations and problems keeping flies alive through quarantine, we used multiple flies from several of these galls (see Table 1); analyses using only a single individual per gall produced similar results. It is not known how many galls were sampled from the USDA-APHIS collections in 1990 and 1991. A total of 167 individual flies were used in this study.

### 2.2. DNA extraction

Adult flies or fly larvae were killed by immersion in 100% ethanol or by freezing at  $-80^{\circ}\text{C}$  and were stored at  $-80^{\circ}\text{C}$  until DNA extraction. DNA was extracted with DNeasy tissue kits (Qiagen, Valencia, CA). The DNeasy protocol for animal tissue was followed with two exceptions: 50  $\mu\text{l}$  of buffer AE was used instead of 200  $\mu\text{l}$  to increase the final DNA concentration, and after the addition of buffer AE, the mixture was incubated at room temperature for 5 min instead of 1 min.

### 2.3. Mitochondrial DNA sequencing and analysis

We sequenced a 491 base-pair section of the 16s ribosomal subunit for 94 flies, using 5'-AGTCTAACCTGCCACTG-3' and 5'-CACCGGTTTGAACCTCACATC-3'

Table 1  
*Spurgia capitigena* collection sites and dates

Population	Number of galls	Number of individuals	Collection date
Italy leafy spurge 2001	24	24	May 2001
Italy leafy spurge 1991	Unknown	9	May 1991
Italy cypress spurge 2001	20	20	May 2001 and May 2003
France leafy spurge 2001	24	24	May 2001
France cypress spurge 2001	33	36	May 2001
North Dakota leafy spurge 2001	24	24	July 2000 and July 2001
North Dakota leafy spurge 1991	Unknown	15	June 1991
Montana leafy spurge 1990	Unknown	15	April 1990

as primers. PCR products were used as templates in sequencing reactions using Applied Biosystems BigDye Terminator v1.1/3.1 chemistry and then run on an ABI 3100 capillary sequencer. These sequence data were processed in Pregap4 v. 2002.0 using the PHRED base-calling program (Ewing et al., 1998; Ewing and Green, 1998) and all trace files were examined for accuracy in the editor module of gap4 v. 2002.0 (Staden et al., 2003). We only included polymorphisms that had base-calls where PHRED estimated that there was less than a 1% chance that the call was in error. These sequence data were aligned in ClustalX v. 1.83 (Higgins and Sharp, 1988) and we then used TCS v. 1.13 to generate a gene genealogy (Clement et al., 2000).

#### 2.4. Microsatellite analyses

We used 15 polymorphic microsatellite loci to genotype the flies. A description of the microsatellites and their primers is presented in Lloyd et al. (2004). Microsatellite genotyping was performed on an ABI 3100 capillary Genetic Analyzer at Iowa State University's DNA Sequencing and Synthesis Facility, Ames, Iowa. A reference DNA sample was sent out with each order to ensure consistency in scoring. Only alleles with strong bands were scored. Extremely faint bands or nonamplifying individuals were scored as missing data. Homozygous alleles were sequenced at Davis Sequencing, LLC, Davis, California, using an ABI 3700 DNA sequencer to verify genotyping results. This was done for only 25 of the alleles because rare alleles were most often not homozygous. One locus with a GTT repeat had alleles with one or two base pair differences instead of the expected three base pair insertion/deletion within the microsatellite region. Sequencing of this allele revealed no changes to the microsatellite, indicating that mutation occurred in the flanking regions of the microsatellite and the locus was included in our analyses. Individuals with only one or two base pair differences at this locus were not coded as having variation within the microsatellite.

#### 2.5. Data analysis

Observed and expected heterozygosities, allele frequencies, and Hardy–Weinberg exact probability tests were calculated using GenePop on the Web (Raymond and Rousset, 1995) and we determined the level of neutral molecular genetic variation within and between populations of the flies and the level of inbreeding within populations by computing analyses of molecular variance (AMOVA) (Excoffier et al., 1992) using Arlequin v. 2.000 (Schneider et al., 2000). For these analyses, data were analyzed according to a stepwise mutation model (SMM) using the squared difference in repeat number between the alleles as a weight (Slatkin, 1995). Probabil-

ity values for these  $F$  statistics were estimated using permutation tests in Arlequin, and we report the probability that the null value generated from the permutations is greater than the reported value. The  $F$  statistics produced by Arlequin estimate the proportion of total genetic variance found in each level of a hierarchical structure. We examined the patterns of genetic variance in the European samples with two structures: First, with host plant (= subgroup) nested within country of origin (= group), and second with country of origin (= subgroup) nested within host plant (= group). We also examined how the process of introduction has altered patterns in variation by examining just the Italian leafy spurge and North American samples. In this analysis, collection site and date (= subgroup) was nested within country of origin (= group).

These analyses each produce four statistics:  $F_{CT}$  (variance due to group within the total variance),  $F_{SC}$  (variance due to subgroup within variance due to group),  $F_{IT}$  (variance due to individual within variance due to total variance), and  $F_{IS}$  (variance due to individual within variance due to subgroup). Finally, we used Arlequin to generate pair-wise  $F_{ST}$  values for all population locations and sample dates.

To detect signs of a recent bottleneck, we compared average heterozygosities and the average number of alleles per locus between native and introduced populations as well as between the two North Dakota populations (1991 and 2001) using two-way analyses of variance in JMP 5 (SAS Institute, Inc., 2002). We examined the hypotheses that introduced populations (North Dakota 1991, North Dakota 2001, and Montana 1991) differed in heterozygosity or the number of alleles per locus from native flies in the original collection location and collection host (IT leafy spurge 1991 and 2001). A separate comparison was made for the same response variables for ND 1991 vs. ND 2001 and IT leafy spurge 1991 vs. IT leafy spurge 2001. These analyses were run over all loci combined with locus as a random blocking variable and the numbers of alleles were log-transformed to fit the assumptions of normality. Significance levels were adjusted for multiple comparisons using the Bonferroni method when appropriate (Sokal and Rohlf, 1995).

We used the program Bottleneck and the SMM (Cornuet and Luikart, 1996) to directly test for evidence of population bottlenecks, assuming mutation-drift equilibrium and no population sub-structuring. Populations that have recently undergone a severe bottleneck in population size initially experience a greater reduction in allelic diversity than in heterozygosity. Bottleneck tests loci for higher than expected heterozygosities given the observed level of allelic diversity, assuming populations are at mutation-drift equilibrium (Cornuet and Luikart, 1996). This test examines deviations from expected heterozygosities over all loci within a population and not

against other extant populations or samples. Population bottlenecks can also be viewed graphically by sorting allele frequencies into 10 allele frequency classes following Luikart et al. (1998b). Non-bottlenecked populations have a characteristic L-shaped distribution whereas bottlenecked populations will show a mode shift with one or more intermediate frequency classes having a greater number of alleles than the low frequency class ( $\leq 0.1$ ) containing rare alleles (Luikart et al., 1998b).

To approach these data from a different perspective we used an assignment test, another way of evaluating population differentiation that is especially useful for highly variable markers such as microsatellites. Assignment tests calculate whether a given individual can be assigned to or excluded from each of a set of collection populations given the observed allele frequencies for the populations, the genotype of each individual, and a chosen probability threshold. Two individual assignment tests were implemented for all eight populations using GeneClass 1.0.02 (Cornuet et al., 1999), a program that selects or excludes populations of origin for individuals using multilocus genotypes without assuming genetic equilibrium. We used the direct self-assignment approach because the reference populations were known, with the Bayesian method and the “leave one out” procedure. The Bayesian method was chosen over the frequency method for its inclusion of rare and null alleles and the “leave one out” procedure excludes each individual to be assigned from its population sample to prevent a bias when estimating allele frequencies. Exclusion tests used the simulation option with 10,000 simulated individuals per population and populations were rejected as the source of origin for probabilities  $< 0.125$  which is the inverse of the eight populations examined.

### 3. Results

#### 3.1. MtDNA analysis

Three patterns were apparent from the haplotype network produced from the mtDNA sequences (Fig. 1). First, there was little evidence that there are two fly species, one that attacks leafy spurge and another that attacks cypress spurge. In both Italy and France, there are haplotypes that are found on both leafy and on cypress spurge. There was also little evidence that there are two, genetically distinct fly species, both of which may be found on both spurge species. For this region of the mitochondrial genome, there exists a continuous series of small changes between all haplotypes, and there are no distinct jumps between haplotypes. Second, haplotypes were grouped according to collection location: French haplotypes are more similar to other French haplotypes and Italian haplotypes are more similar to other Italian haplotypes. As expected, North American

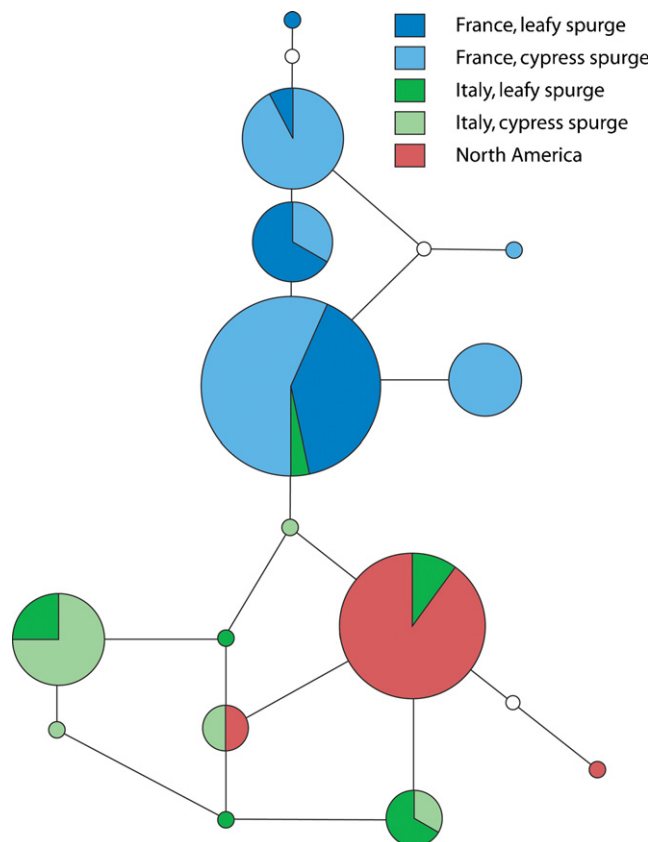


Fig. 1. mtDNA haplotype network for all sequenced flies. Each circle represents a single haplotype and is connected to other haplotypes that are one mutational step away. The size of the circle is proportional to the number of individuals with that haplotype. Inferred haplotypes are represented by open circles.

haplotypes group with those from the original collection location in Italy, but they group with cypress spurge as well as leafy spurge. Finally, there is a similar degree of sequence variation in each population of flies. There were 0.180 haplotypes per fly in the Italian samples compared to 0.109 haplotypes per fly in the French samples, and 0.115 haplotypes per fly in North America.

#### 3.2. Microsatellite analyses

Of the 112 possible locus per population combinations, 32 were monomorphic and consequently not tested for deviations from HWE. Twelve statistically significant deviations from HWE were observed, and all but one departure from HWE were caused by heterozygote deficiencies. All were found in the Italian and French populations except for one deviation in the North Dakota 2001 population.

##### 3.2.1. Population structure in European populations

The AMOVA comparing French and Italian flies collected from leafy spurge and cypress spurge support the results from the mtDNA gene genealogy. When host

plant was nested within country of origin, we found that there was large and significant structure between flies collected from France and from Italy ( $F_{CT}=0.2623$ ,  $P<0.0001$ ), and small but significant structuring due to host plant within country ( $F_{SC}=0.0271$ ,  $P<0.0001$ , Table 2). We found the same pattern when we nested country of origin within host plant: there was no evidence for structure by host plant ( $F_{CT}=-0.1486$ ,  $P=0.9902$ ), but there was structure by country within host plant ( $F_{SC}=0.2819$ ,  $P<0.0001$ , Table 3). In both of these analyses most of the genetic variance occurred within individuals within the populations ( $F_{IS}=0.2725$ ,  $P<0.0001$  for both analyses).

### 3.2.2. Population structure following biological control introductions

When AMOVA was used to compare the source populations (Italy leafy spurge samples from 1991 to 2001) and the three introduced populations (Montana 1990, North Dakota 1991, and North Dakota 2001), there was no evidence for structure between introduced and native populations ( $F_{CT}=-0.0292$ ,  $P=0.7801$ ), but there was structure between these populations within country ( $F_{SC}=0.1873$ ,  $P<0.0001$ , Table 4).

Pairwise  $F_{ST}$  values generated from the AMOVA with all populations included again demonstrate that there was significant differentiation of French populations

Table 2

AMOVA results for host plant nested within country of origin, European samples only

Source of variation	Sum of squares	Variance components	Percent of total variation
Among countries	120.213	1.20585	26.23
Among host plant within country	16.775	0.09179	2.00
Among individuals within host plant	376.928	0.89906	1.96
Within individuals	226.000	2.40038	52.22
Total	739.916	4.59708	

Average  $F$ -statistics over all loci.

$F_{IS}$  (individual within host plant): 0.27249  $P<0.00001$ .

$F_{SC}$  (host plant within country): 0.02707  $P<0.00001$ .

$F_{CT}$  (country within total): 0.26231  $P<0.00001$ .

$F_{IT}$  (individual within total): 0.47785  $P<0.00001$ .

Table 3

AMOVA results for country nested within host plant, European samples only

Source of variation	Sum of squares	Variance components	Percent of total variation
Among host plants	13.508	-0.59431	14.86
Among countries within host plant	123.480	1.29501	32.37
Among individuals within country	376.928	0.89906	22.49
Within individuals	226.000	2.40038	60.01
Total	739.916	4.00013	

Average  $F$ -statistics over all loci.

$F_{IS}$  (individual within country): 0.27249  $P<0.00001$ .

$F_{SC}$  (country within host plant): 0.28186  $P<0.00001$ .

$F_{CT}$  (host plant within total): -0.14857  $P=0.99022$ .

$F_{IT}$  (individual within total): 0.39993  $P<0.00001$ .

Table 4

AMOVA results for introduced vs. Italian samples

Source of variation	Sum of squares	Variance components	Percent of total variation
Among country	13.259	-0.06626	-2.918
Among sub-populations within country	46.985	0.43759	19.271
Among individuals within sub-populations	168.293	0.24766	10.907
Within individuals	137.500	1.65173	72.7404
Total	366.037	2.27072	

Only flies collected from leafy spurge are included in the analysis.

Average  $F$ -statistics over all loci.

$F_{IS}$  (individual within sub-population): 0.13039  $P<0.00001$ .

$F_{SC}$  (sub-population within country): 0.18725  $P<0.00001$ .

$F_{CT}$  (country within total): -0.02918  $P=0.78006$ .

$F_{IT}$  (individual within total): 0.27259  $P<0.00001$ .

from other populations sampled ( $F_{ST}$  values for these comparisons range from 0.1525 to 0.3403, all were significantly greater than zero except for France cypress 2001 and Italy leafy 1991, Table 5), but there is little evidence for population divergence by host plant within country. There was also no significant structure between the ancestral source population (Italy leafy 1991) and introduced populations, but there is evidence for divergence between the Montana population and the two North Dakota samples ( $F_{ST}=0.1706$  and 0.1408,  $P<0.0001$  for North Dakota 1991 and North Dakota 2001, respectively). There was also evidence that allele frequencies have diverged between the 2001 sample of the Italy leafy spurge population and the two North Dakota populations.  $F_{ST}$  values were 0.1217, 0.1268 for North Dakota 2001 and North Dakota 1991 respectively, and both of these values are significantly greater than zero. However, there is little evidence for divergence between the source population and the Montana collection:  $F_{ST}$  was 0.0417, not significantly different from zero after adjusting for multiple comparisons. Although there were differences between most of the populations sampled, there were no significant differences between the repeated samples from the same location. Pairwise  $F_{ST}$  values between 1991 Italy leafy spurge and 2001 Italy leafy spurge ( $F_{ST}=0.0319$ ) or North Dakota 1991 and North Dakota 2001 ( $F_{ST}=-0.0067$ ) were not significantly different from zero, indicating that effective population sizes were large enough for these two sites to resist drift effects. In summary, AMOVA analyses show a pattern of large amounts of within-population variation, evidence for structure by distance, but not host plant in Europe, and some evidence for population divergence in the introduced populations from the original collection location.

Differences in the amount of within population structure in each population was also detected with AMOVA.  $F_{IS}$  values were all significantly greater than zero for all European populations and for the North Dakota 2001 population (Fig. 2). The two historical samples from North America (sampled shortly after the original introduction of the flies) have lower  $F_{IS}$  values than those seen in the Italian populations (ANOVA with linear contrast  $F_{1,55}=6.46$ ,  $P=0.014$ ).

Introduced populations had fewer alleles per locus than any of the four contemporary samples from Europe (Fig. 3); however, only the Montana population had significantly fewer alleles than the 1991 Italian sample. As this older sample from Italy was small (only nine flies were available for analysis), this sample could be

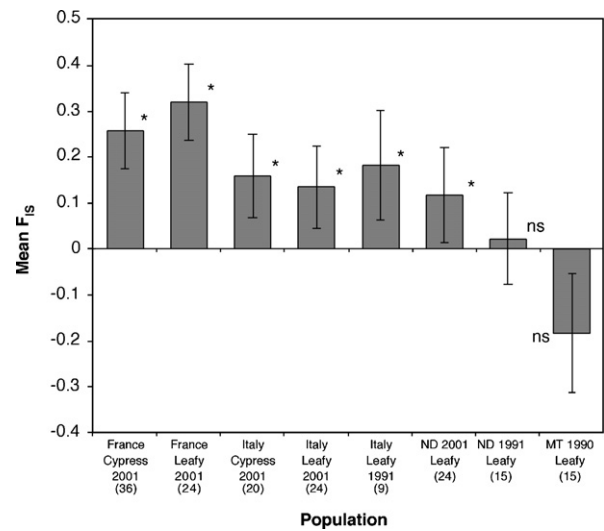


Fig. 2. Average inbreeding coefficients ( $F_{IS}$ ) for each population. Asterisks denote values that are significantly different from zero ( $P < 0.05$ ).

Table 5  
Pairwise differences in allele frequency between the populations sampled<sup>a</sup>

Source of population	Italy cypress 2001	Italy leafy 2001	Italy leafy 1991	North Dakota 2001	North Dakota 1991	Montana 1990	France leafy 2001	France cypress 2001
Italy cypress 2001	0							
Italy leafy 2001	0.0110 (0.2252)	0						
Italy leafy 1991	0.0411 (0.0901)	0.0319 (0.0720)	0					
North Dakota 2001	<b>0.1590</b> ( <b>0.0000</b> )	<b>0.1217</b> ( <b>0.0000</b> )	0.0066 (0.3063)	0				
North Dakota 1991	<b>0.1598</b> ( <b>0.0000</b> )	<b>0.1268</b> ( <b>0.0000</b> )	0.0333 (0.1171)	-0.0067 (0.4685)	0			
Montana 1990	0.0738 (0.0000)	0.0417 (0.0091)	0.0179 (0.2523)	<b>0.1408</b> ( <b>0.0000</b> )	<b>0.1706</b> ( <b>0.0000</b> )	0		
France leafy 2001	<b>0.3403</b> ( <b>0.0000</b> )	<b>0.3072</b> ( <b>0.0000</b> )	<b>0.2349</b> ( <b>0.0000</b> )	<b>0.2389</b> ( <b>0.0000</b> )	<b>0.2080</b> ( <b>0.0000</b> )	<b>0.3002</b> ( <b>0.0000</b> )	0	
France cypress 2001	<b>0.2439</b> ( <b>0.0000</b> )	<b>0.2317</b> ( <b>0.0000</b> )	0.1525 (0.0090)	<b>0.2076</b> ( <b>0.0000</b> )	<b>0.2121</b> ( <b>0.0000</b> )	<b>0.2163</b> ( <b>0.0000</b> )	0.0241 (0.0631)	0

<sup>a</sup> (Upper) Slatkin's  $R_{ST}$ , based on the squared difference in repeat numbers between allele sizes. (Lower)  $P$  values based on permutation tests. Entries in bold are significantly different from 0, based on a Bonferroni adjusted  $\alpha = 0.0021$ .

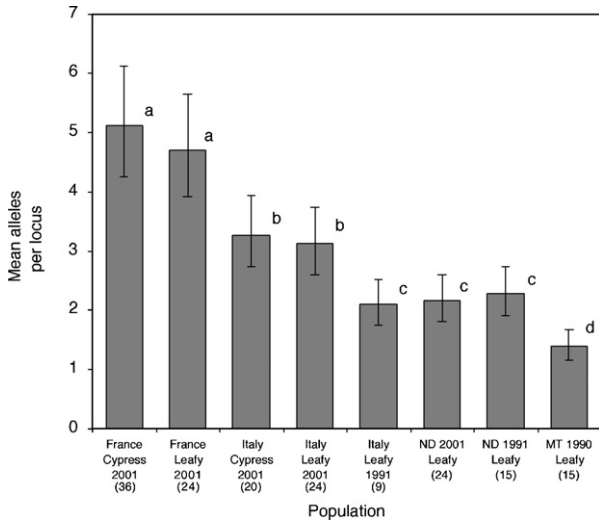


Fig. 3. Average number of alleles per locus for each of the populations sampled. Bars sharing a letter are not significantly different from each other ( $P > 0.05$ ).

underestimating the population’s allelic diversity. This seems likely as the 2001 Italian leafy spurge sample has significantly more alleles per locus than the older sample or the North American samples. A linear contrast of both Italian leafy spurge samples vs. the three introduced populations indicates that there was a significant reduction in allelic diversity in the introduced populations ( $F_{1,91} = 9.664$ ,  $P = 0.0025$ ). This was not true for heterozygosity: heterozygosity was on average slightly higher in the introduced populations when compared with the source population (Fig. 4), but this difference was not significant ( $F_{1,60} = 1.650$ ,  $P = 0.204$ ).

Correct assignment of individuals was 75% over all eight populations using the direct method, whereas the

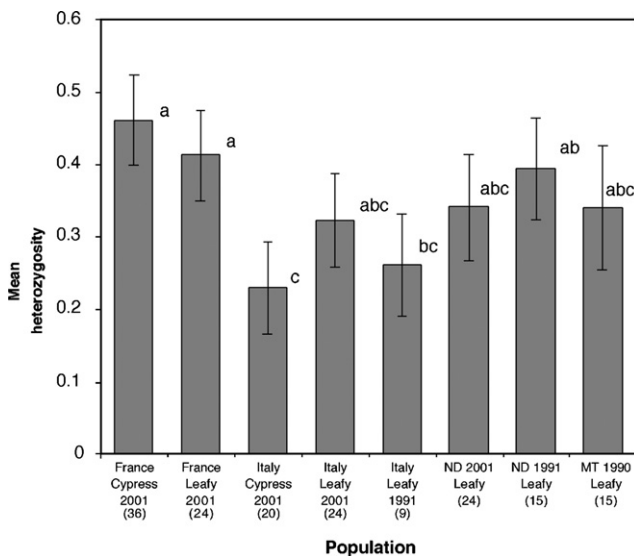


Fig. 4. Average heterozygosity for each of the populations sampled. Bars sharing a letter are not significantly different from each other ( $P > 0.05$ ).

assignment test would yield an equal probability of each population being assigned to any other population (null percentage of ~13% for all populations) if there was no genetic structure between populations (Table 6). Using the simulation method, exclusion tests (Table 7) supported the results of the AMOVA by confirming that we are unable to exclude many of the North Dakota flies as having been collected from the Italian leafy spurge and cypress spurge populations. This similarity to Italian flies was also true for several of the flies collected in France and in Montana. The test was unable to exclude most Italian and North American populations from each other, which makes sense since the introduced populations all came from Italy. On the other hand, both French populations repeatedly excluded most other populations. For both the North Dakota and Montana populations, we can confidently reject France as their origin, but not Italy. Montana could be ruled out as the likely source of origin of most individuals outside of the Montana collection.

The only population to show several indications of a population bottleneck was the Montana population (Fig. 5). The allele frequency distribution graphs show a definite mode shift in the Montana population with very common alleles (frequency >0.9) occurring over three times more than the rare alleles (frequency ≤0.1). The Bottleneck analysis indicates that there was a marginally significant ( $P = 0.055$ ) excess of heterozygotes in this population as well. Several other populations (Italy leafy 1991, North Dakota 2001, and North Dakota 1991) showed a shift to more common alleles, though rare alleles were still the most abundant. None of these populations exhibited an excess of heterozygotes ( $P$  values are all >0.85); however, this graphical method can only detect allele frequency distortions for a few dozen generations and a sample size of at least 30 is recommended (Luikart et al., 1998b).

#### 4. Discussion

Gagné’s (1990) assertion that flies attacking leafy spurge and flies attacking cypress spurge are separate species is not supported by our data. There is no evidence that mtDNA haplotypes cluster by host plant species within either the French or Italian fly populations. Further, there is little evidence that there are two groups of haplotypes representing two fly species. In European flies, haplotypes are more structured by collection location than by host plant association. Morphological differences in male genitalia (Gagné, 1990) most likely represent ecotypic variation in fly phenotypes that is not associated with genetic differentiation of the flies. Host specificity tests of the two species also indicate a degree of ecological similarity between the fly species in addition to the genetic data presented here (Pecora et al., 1991; Sobhian et al., 2000).

Table 6  
Results of the assignment tests for all eight populations using the direct, Bayesian method

Source population	n	Assigned population							
		France cypress 2001	France leafy 1991	Italy cypress 2001	Italy leafy 2001	Italy leafy 1991	ND leafy 2001	ND leafy 1991	MT leafy 1990
France cypress 2001	36	33	3	0	0	0	0	0	0
France leafy 2001	24	5	19	0	0	0	0	0	0
Italy cypress 2001	20	0	0	10	2	4	0	2	1
Italy leafy 2001	24	0	0	3	18	2	0	0	1
Italy leafy 1991	9	0	0	2	1	3	3	1	0
ND leafy 2001	24	0	0	0	0	0	17	4	0
ND leafy 1991	15	0	0	0	0	0	4	10	0
MT leafy 1990	15	0	0	0	0	0	0	0	15

Table 7  
Results of the exclusion tests for all eight populations using a simulation, Bayesian method<sup>a</sup>

Source population	n	Populations not excluded							
		France cypress 2001	France leafy 1991	Italy cypress 2001	Italy leafy 2001	Italy leafy 1991	ND leafy 2001	ND leafy 1991	MT leafy 1990
France cypress 2001	36	22	12	0	0	0	0	0	0
France leafy 2001	24	7	14	1	1	1	0	0	0
Italy cypress 2001	20	4	4	17	15	14	12	14	3
Italy leafy 2001	24	0	0	11	19	11	4	7	0
Italy leafy 1991	9	1	1	0	6	17	6	7	0
ND leafy 2001	24	1	1	11	12	9	22	22	1
ND leafy 1991	15	0	0	7	6	3	9	12	0
MT leafy 1990	15	0	0	4	11	6	3	2	15

<sup>a</sup> The exclusion threshold was 0.125.

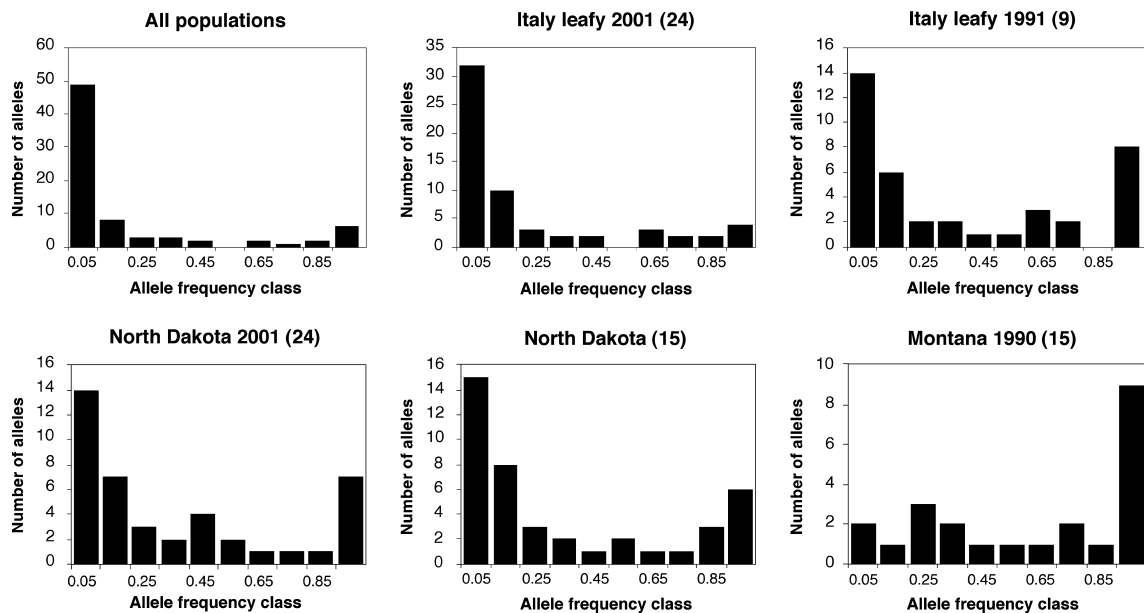


Fig. 5. Distribution of allele frequencies for native (Italy leafy spurge 2001 and 1991) and introduced (North Dakota leafy spurge 2001 and 1991, Montana leafy spurge 1990) populations into each of 10 frequency classes. Sample size in parentheses.

Although there was no significant variation between flies collected on leafy spurge or cypress spurge on average, there was genetic differentiation between the 2001 French leafy spurge and cypress spurge and some evidence from the assignment tests for a similar pattern in the 2001 Italian leafy spurge and Italian cypress spurge populations. These data suggest that although there are

not two reproductively isolated fly species specialized on leafy and cypress spurge, there is the potential that local adaptation to host plant is structuring the fly populations.

Most of the genetic variation was found among individuals within each population and between populations. The large  $F_{15}$  values found for most of the

populations are consistent with an aggregated population structure arising from multiple (presumably) sibling individuals within a single gall and a short-lived and weak-flying adult stage. As these flies have a limited life span and multiple (presumably sibling) individuals develop within an individual gall, high  $F_{IS}$  values in the original populations are not surprising, and the process of quarantining large numbers of flies from multiple galls in the same small cage might explain the lower values seen in recently established populations. Due to this fine-scale variability, assignment and exclusion tests were able to assign or exclude the likely source for any given individual based on the microsatellite data. All of the flies collected in North America are descendants of the San Rossore, Italy population, so this is not a surprising result. These results confirm that these data provide sufficient variation to distinguish populations and that the assignment tests are a valid method for determining the area of origin for introduced populations. While the data presented here are adequate for examining changes in genetic structure resulting from the introduction process, it is not clear that the limited number of European populations sampled (2) can fully describe the patterns that would be found in all populations.

We found evidence of a mild bottleneck in population size among the introduced *S. capitigena* populations. There was a statistically significant reduction in the number of alleles in the introduced *S. capitigena* populations relative to the 2001 Italy leafy spurge samples, but no significant difference in mean heterozygosity compared to the native populations. Additionally, there was no difference in the number of alleles between the 1991 Italy and the 1991 North Dakota samples. This most likely indicates that there was a mild bottleneck in population size that occurred during the original fly collections prior to processing through quarantine, as it is unlikely that the number of alleles in the source population has increased in the 10 years between samples. There is further evidence of another mild bottleneck that occurred during the release and establishment of the Montana population, as there are fewer alleles in this sample than either the North Dakota populations or in the source collection from Italy. Unfortunately, the small sample size of the Italy 1991 collection (only nine individuals were available for analysis) makes it impossible to determine if the reduction in allele frequency is the result of the small sample size or the process of collecting and quarantining the flies, and we cannot remedy this in retrospect.

The preservation of genetic diversity but loss of rare alleles that are observed here is typical of a mild bottleneck in population size (Nei et al., 1975). Recent studies have found that variance in allele frequencies and the loss of alleles are more sensitive to population bottlenecks than heterozygosity (e.g., Leberg, 1992; Luikart et al., 1998a; Spencer et al., 2000). Although there is a

reduction in the number of alleles in introduced populations and no change in heterozygosity, the Bottleneck analysis did not find evidence for a recent reduction in population size. A likely explanation for this is that the assumption that these populations are at mutation-drift equilibrium is probably not valid for these data. The fragmented nature of these populations and the likelihood of sib-mating by the flies may result in decreased heterozygosity, even in the absence of a population bottleneck. Therefore, the Bottleneck analysis (which measures departures from expected heterozygosity given the allelic diversity of a population) would be less likely to detect greater than expected heterozygosity in the introduced populations.

Other introduced biological control agents have shown similar trends. For example, Hufbauer et al. (2004) found similar results using microsatellites from the wasp *A. ervi* that was introduced in 1959 for the control of the pea aphid. They found significant genetic differentiation, fewer rare alleles, and no difference in heterozygosity between introduced and native wasps. In both Hufbauer et al. (2004) and the work presented here, there is evidence for a mild population bottleneck. Baker et al. (2003), in a study of *Diaeretiella rapae* (M'intosh) (Hymenoptera: Aphidiidae) introduced to Australia, found a narrower bottleneck in population size. As more studies focus on neutral genetic variation, such mild bottlenecks may prove to be a general pattern in biological control systems. While severe population bottlenecks can reduce genetic variation, whether this reduction negatively impacts fitness is still unclear (Mitton, 1993).

Previous research has shown leafy spurge gall midges to be highly variable in their survival on different North American leafy spurge genotypes (Lym et al., 1996). Further investigation into Italian leafy spurge genotypes compared to the naturalized North American genotypes could also help to clarify the lack of gall midge establishment and success. Several studies have proposed that molecular techniques may soon be capable of identifying local adaptation among populations (reviewed in Schlötterer, 2002), but reciprocal transplant experiments are needed to reliably determine if the flies can become locally adapted to North American leafy spurge. Recently, there has been an introduction of *S. capitigena* from southern France into western North Dakota (Kimberly Mann, USDA-ARS Northern Plains Agricultural Research Laboratory, Sidney, Montana, personal communication). This may increase the overall genetic diversity of the introduced flies, should the (currently geographically separate) populations come into contact. The consequences of this have yet to be fully evaluated. Increasing the genetic variation of established fly populations may increase the fly's ability to adapt to host genotypes; however, if there are significant fitness trade-offs in the ability to utilize separate host genotypes, it is also possible that the introduction of additional fly

genotypes will disrupt an already locally adapted system. Thus, researchers should be cautious in planning additional introductions of biological control agents solely for the purpose of increasing genetic variation.

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