Phylogeny and Genetic Diversity of Flea Beetles (*Aphthona* sp.)
Introduced to North America as Biological Control Agents for Leafy Spurge

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ABSTRACT A molecular phylogeny is presented for the five main species of *Aphthona* flea beetles that were introduced to North America in conjunction with the leafy spurge (*Euphorbia esula* L.) biological control program. The mitochondrial genome was examined using polymerase chain reaction-restriction fragment length polymorphism (RFLP) of a 9,000-bp segment and nucleotide sequencing of a 575-bp piece of *cox1*-*cox2*. A neighbor-joining tree of the RFLP data, along with neighbor-joining and maximum parsimony trees of the sequence alignments, all had the same major branching pattern. Each of the recognized species was a well defined clade. Three within species subbranches had very limited mitochondrial DNA diversity. One was a *Wolbachia*-infected lineage of *A. nigriscutis* most likely generated by a *Wolbachia* sweep where the spreading *Wolbachia* infection brought along the infected mitochondrial haplotype. Two of three subclades of *A. lacertosa* also had very little genetic diversity. One of these subclades also displayed a divergence from the other that was analogous to the divergence observed between some of the other species pairs, suggesting it may be a cryptic species. Its distribution was restricted to Canada. The other genetically depauperate *A. lacertosa* line was the only lineage recovered in the United States. The geographically restricted nature of some of the genetic lines could be exploited to possibly improve biological control in some habitats through redistribution to other locations. It is not obvious that either *Wolbachia* infection or a narrow genetic base has had any detrimental effect on biological control.

KEY WORDS mitochondrial DNA, invasion genetics, Euphorbia, cryptic species, rangeland weeds
leafy spurge control and is viewed favorably by landowners (Hodur et al. 2006). On the negative side is the fact that the beetles do poorly on very sandy soils (Butler et al. 2006, Richardson et al. 2008). Detailed records of which species were introduced at any specific location were not kept, and during the course of these redistribution efforts, the relative abundance of the species at established sites changed (Mundal et al. 1999). Through either the accident of redistribution or the nature of their own adaptability, *A. lacertosa* and *A. nigriscutis* have become the dominant species at most control locations (Lym and Olson 1999). They comprise most of the *Aphthona* beetles currently found in North Dakota and Minnesota and are common in Alberta, Canada, as well. *A. czwalinae* is scarce and has never been an important contributor to leafy spurge control. Some pockets of *A. flava* and *A. cyparissiae*, and a small population of *A. czwalinae* that had been overlooked or forgotten since the early years of introduction, were recently discovered in eastern North Dakota and west central Minnesota (Roehrdanz et al. 2009). The most numerous of these seem to be the populations of *A. cyparissiae* in eastern Clay Co., MN. Recognized populations of *A. flava* and *A. cyparissiae* also have survived since their release at a few sites in Alberta. Despite being released in several states, there is no record that *A. abdominalis* ever became established in leafy spurge stands (Gassmann and Schroeder 1995, USDA 2006). Another species, *A. venustula*, was collected in Europe and underwent some preliminary tests in the United States, but it was never approved for release (Gassmann 1996).

At the time that *Aphthona* species were being collected in Europe, tested for host plant specificity, passed through quarantines, released in North America, and becoming established at some of the release sites, there was no concerted effort to assess the breadth of genetic diversity within and among populations. Any of the steps, from unintentional collection of a highly inbred population to selection at the time of establishment, could have introduced significant genetic bottlenecks into the North American populations. The reduced genetic diversity that often accompanies bottlenecks is usually considered to be detrimental to the survival of a transplanted species regardless of whether the transplant is orchestrated or invasive. The major effects are inbreeding and the reduction of ability to evolve in the new environment (Keller and Waller 2002, Lee 2002, Allendorf and Lundquist 2003, Crawford and Whitney 2010). The adverse consequences of bottlenecks can be overcome when the founding population contains an admixture of genotypes from different sources (Colautti et al. 2005, Lachmuth et al. 2010). Although such an event may or may not have an impact on the ultimate success of a release, the problem cannot be considered without an assessment of which species may have been genetically restricted in the process.

We seek to determine the phylogenetic relationship of the species currently used in biological control and to assess the genetic variability of these species 15+ years after introduction. We have collected *Aphthona* beetles from various North American locations to measure the genetic diversity both within and between the released species. The extent of intraspecific diversity, including a geographic dispersal component, was measured primarily by using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) of a large section of the mitochondrial DNA (mtDNA). Phylogenetic relationships were confirmed by DNA sequencing of a smaller mtDNA segment from the PCR-RFLP haplotypes. Although PCR-RFLP has been mostly replaced by low-cost DNA sequencing, the data obtained can be quite informative. We have reported previously on a molecular identification key for these species by using PCR-RFLPs (Roehrdanz et al. 2009).

**Materials and Methods**

Adult flea beetles *A. flava* (AF), *A. cyparissiae* (AC), *A. nigriscutis* (AN), *A. czwalinae* (AZ), and *A. lacertosa* (AL) were obtained from leafy spurge infested sites in North Dakota (collected 2001–2002, 2006), Minnesota (collected 2003–2004, 2006–2007), and Montana (collected 2002) in the United States, and Alberta.
Canada (collected 2003–2004) (Fig. 1). Beetles from the United States were brought alive to Fargo and frozen at −80°C for future use. Beetles from Canada were frozen in Canada and shipped to Fargo packed on dry ice. North American collection sites are described in Roehrdanz et al. (2006, 2009). In Roehrdanz et al. (2006), several sites along the Minnesota River southwest of Minneapolis, MN, received separate listing. The data from these sites were pooled for Roehrdanz et al. (2009) and are also pooled here. It is shown as EFS MN (Eden Prairie/Shakopee). Five specimens, two A. venustula (AV) collected near Dueseldorf, Austria, and A. czwalinae (AZ 4), A. flava (AF 3984), and A. nigriscutis (AN 3907), all from Montana, were provided as frozen samples by R. Nowierski as part of his research collection from Montana State University, Bozeman, MT. Flea beetles Phyllotreta cruciferae Goeze from western North Dakota were provided by J. Knodel (North Dakota State University, Fargo, ND) and used as an outgroup.

Morphological species assignment was based on external and internal morphology. Adult beetles can be quickly sorted into black beetles (A. lacertosa and A. czwalinae) and brownish to gold-brown beetles (A. nigriscutis, A. cyparissiae, and A. flava). The two black species are easily distinguished under a dissecting microscope based on hind femur color and by dissection of the reproductive organs (LeSage and Paquin 1996, Fauske 2003). The brown beetles required detailed dissection and examination of genitalia to confirm species status because colors are unreliable (LeSage and Paquin 1996).

Total DNA was extracted from either whole insects by using the high salt procedure of Cheung et al. (1993) or from one of the hind legs by using the DNeasy tissue kit (QIAGEN, Valencia, CA). When only a partial beetle was used, the remainder of the insect was returned to the freezer at the USDA–ARS Biosciences Research Laboratory in Fargo as a voucher specimen. In total, 595 specimens, representing five species (not A. venustula), were subjected to long PCR-RFLP by using an ~9,000-bp segment of the mt genome (primers were C2R [C2-J-3684, 5′-GGTCAAATGTTCAGAAATTTGTGG-3′] and HsS2 [LR-N-12945, 5′-GCGACCTTCAGATGTGCAATTAA-3′]). The fragment contains coding regions, tRNAs, and a small piece of the large rRNA, but it does not include any part of the control region. Reaction components were from the XL kit (Applied Biosciences, Foster City, CA) and the long PCR conditions were as described previously (Roehrdanz 1995, Roehrdanz and Degruillier 1998). The amplicons were digested with seven restriction enzymes (XbaI, Asel, AluI, SpfI, DraI, DpnII, or HinflI), and the RFLPs were analyzed as described in Roehrdanz et al. (2006). RFLP patterns were determined using agarose gel electrophoresis and ethidium bromide staining in the company of molecular size standards. Individual RFLP patterns were given an alphabetical designation, whereas the composite haplotypes were numbered in the order they were discovered. Some initial haplotype numbers in sequence were discarded when those individuals were determined to belong to one of the other existing haplotypes.

Once we had established a level of polymorphism, we proceeded to sequence the diversity. Specimens were chosen for their diversity and a small piece of the mitochondrial cox1–rRNA–cox2 region was amplified from specimens with differing PCR–RFLP haplotypes along with a few individuals from more recent collections that had not been tested for RFLPs. The primer pairs were as follows: C1–RLR (C1-J–2572, 5′–TTGATTTTTTGTGATGCCAGAAGT–3′) and C2 (C2-N–3662, 5′–CCCAAAATTTGCTACACCATGAC–3′) or C1–2776 (C1-J–2776, 5′–CCTCGACGTATTCAGATTACC–3′) and C2–3380 (C2-N–3380, 5′–TCAATCTCAGTAGGCAAT–3′).

PCR products of ~1,503 bp and 617 bp, respectively, were sequenced. The mitochondrial primers FB–C1 (C1-J–2785, 5′–TACTCGAGTACCTTAGATT–TT–3′) and FB–C2 (C2-N–3370, 5′–TATCGATTGCTCAGATT–TTTT–3′) were designed to improve the amplification from all species (Roehrdanz et al. 2009). The new primers amplify a portion of the mtDNA cox1–rRNA–cox2 region that is 605 bp. PCR reaction conditions were 35 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 3 min by using the Taq Gold kit (Applied Biosciences). Direct sequencing of the PCR products was performed by the DNA Facility, Iowa State University, Ames, IA. The sequences were all trimmed to 575 bp and aligned for analysis using the AlignX component of Vector NTI Advance 11 software (Invitrogen, Carlsbad, CA).

Data Analysis. The genetic relationships of the Aphthona RFLP haplotypes were determined using the RestSite software (Miller 1991) that relies on Nei’s measure of genetic distance. The software has the advantage of being able to analyze restriction fragment data generated by enzymes with different recognition site lengths in a single operation. The absolute size of the fragments is unimportant. What matters is whether a fragment is the same size or different from fragments in other patterns. The fragments were assigned a position number beginning with the largest (as 1) and continuing to the smallest that was scored, which was usually not <100 bp. The position numbers were formatted for the software. Bootstrapping is used within the program, but specific bootstrap numbers are not part of the output. The genetic distance matrix from RestSite was used for tree construction both within RestSite and also with the neighbor program of PHYLIP (Felsenstein 1993). The Newick tree was transferred to MEGA version 4 (Tamura et al. 2007) to draw and edit the tree. Phylogenetic analyses of nucleotide sequences including neighbor-joining, minimum evolution, maximum likelihood, and maximum parsimony were performed in MEGA 4. Neighbor-joining is based on the method of Saitou and Nei (1987), with a bootstrap test using 500 replicates. Branch lengths on the tree are proportional to evolutionary distances and gaps in the data were eliminated. The maximum parsimony method also used 500 replicates for the bootstrap test, and the gaps were eliminated.
Table 1. Summary of the numbers of restriction fragments and fragment patterns for each restriction enzyme

| Restriction enzyme | Restriction fragments (N) | Nucleotides screened (N)* | Fragment patterns (N) | Fragments/pattern (avg) |
|--------------------|--------------------------|--------------------------|----------------------|-------------------------|
| XbaI               | 19                       | 114                      | 16                   | 4.7                     |
| AseI               | 52                       | 312                      | 16                   | 16.8                    |
| Aul                | 45                       | 192                      | 18                   | 16.2                    |
| SpeI               | 45                       | 270                      | 15                   | 15.2                    |
| DraI               | 30                       | 234                      | 13                   | 15.2                    |
| DpnI               | 49                       | 196                      | 19                   | 11.2                    |
| HanII              | 50                       | 200                      | 22                   | 9.3                     |
| Total              | 302                      | 1518                     | 119                  | 88.6                    |

* Nucleotides screened is the number of restriction fragments multiplied by the nucleotide length of the recognition sequence of the corresponding enzyme.

GenBank accessions of the coxl-cox2 sequences are as follows: A. lacertosa (DQ381553-DQ381562), A. cyparissiae (DQ386423-DQ386434, EU449966–EU449978), A. flava (EU440532-EU440540), A. nigriscutis (EU448964–EU448988), A. czaivalinae (EF909277–EF909281), A. venustula (EU440542–EU440543), and P. cruciferae (EU498311, EU440541).

Results

The long PCR-RFLP approach was applied to 594 specimens representing the five species (A. flava, A. cyparissiae, A. nigriscutis, A. czaivalinae, and A. lacertosa). The PCR fragment was ≈9 kb, slightly more than one-half of the typical insect mitochondrial genome. The fragments were scored and their approximate sizes are indicated for each fragment pattern from each of the seven restriction enzymes in Supp Table 1 ([online only]). A summary of the restriction fragments and patterns is shown in Table 1. In total, 302 restriction fragments were identified. The number of fragments for each enzyme ranged from 10 for XbaI up to 52 for AseI. When the length of the recognition sites is factored in this represents 1,518 bp sampled. In total, 119 fragment patterns were observed with the number of patterns per enzyme varying from 13 to 22. The average number of fragments per fragment pattern was calculated for each restriction enzyme, and the sum of these averages indicates that 88 to 89 fragments were scored per specimen (Table 1).

Restriction fragment patterns were combined into haplotypes for each of the five species (Supp Table 2 [online only]). The numbers of individuals of each RFLP haplotype are presented in Supp Table 3 ([online only]). Sixty-six haplotypes were identified, of which 31 (47%) were singletons. By far the greatest frequency of singletons occurred in A. nigriscutis, where 69% of the haplotypes were in that category. Often one or a few haplotypes comprised the majority of specimens for each species. A. nigriscutis haplotype N1 was found in 75% of the individual beetles. A. lacertosa haplotype L1 accounted for 49% of the samples, and haplotypes L1, L2, and L3 combined comprised 86%. In A. flava, two haplotypes, F1 and F4, made up 70% of the individuals. The situation was somewhat different in A. cyparissiae where six haplotypes made up 76% of the total, but none was found in >20% of individuals. There were insufficient sample numbers of A. czaivalinae to make any generalization about haplotype frequencies.

Supp Table 3 ([online only]) also presents the collection sites associated with each of the haplotypes. Some patterns of distribution were noted. The most widely shared haplotype is N1, which was obtained from seven collection sites in Minnesota (MN), North Dakota (ND), and Alberta (AB). Haplotypes C5 and C9 also were found across a wide geographic range that encompassed Fargo, ND, Montana (MT) and AB. Among the rare haplotypes (singletons) of A. nigriscutis 61% were from the EPS MN collection, although that collection supplied only 31% of the beetles analyzed. Private or near private haplotypes also were observed. Four haplotypes of A. cyparissiae (C16, C18, C19, and C20) comprising 18 insects were found only in Fargo, ND. Sixteen of 17 A. nigriscutis haplotype N3 came from EPS MN. The geographic distribution of haplotypes is perhaps the most irregular for A. lacertosa. Haplotype L1 was the only A. lacertosa haplotype found in ND and MN. L1 also was found at AB site 3. All of the other eight haplotypes were recovered only from the AB samples. Within AB the 14 L8 haplotypes all originated from AB site 3. In contrast 46/47 L2 haplotypes came from AB site 1. There were no black beetles at AB site 4.

The RFLP data were compiled and formatted for pairwise distance by analysis using the RestSite program of Miller (1991). The distance matrix was used in the neighbor program of PHYLIP (Felsenstein 1993) to produce a neighbor-joining tree. The tree is presented in Fig. 2. All five Aphthona species form their own clade. Based on the data, A. nigriscutis and A. czaivalinae are the two most closely related taxa. A. cyparissiae and A. flava might also be considered to be sister species, although they seem to be somewhat more deeply diverged than the former pair (3.3 versus 2.7%). There are some interesting demarcations within the species as well. A. nigriscutis is split into two clades, one of which has very few haplotypes and the other has many. The haplotype poor branch is primarily haplotype AN1 (187/190). The other A. nigriscutis clade splits into two groups in this analysis, but the divergence between them is small and there is no geographic sorting of these lineages. The branching pattern of A. lacertosa is quite striking. Haplotype AL1, which is exclusive to AB, is diverged from the other AL haplotypes by ≈2.5%. This is very similar to the 2.7% divergence between A. nigriscutis and A. czaivalinae. The remaining A. lacertosa further split into two groups with a divergence >1.2%. A summary of the geographic distribution of the RFLP clades is presented in Table 2.

Samples were chosen for sequencing based on rRFLP haplotype differences and a few examples from locations that were not tested with RFLPs. The sequence collection sites, GenBank numbers and the
Sequence haplotypes are located in Supp Table 4 (online only). Sequences were obtained from 86 specimens, 85 *Aphthona* plus the crucifer flea beetle. From these, 59 haplotypes were detected. Sequence haplotypes for the 575-bp sequence are differentiated from the RFLP haplotypes by the addition of the letter S in the identifier. Sequences were given the lowest RFLP haplotype number they were associated with. The same sequence could be linked to more than one RFLP haplotype. For example in *A. cyparissiae* RFLP haplotypes C3, C4, C10, C13, and C19 all produced the same sequence which was labeled CS3. Because the highest number RFLP haplotype for *A. cyparissiae* was C20, new sequences that were not associated with an RFLP haplotype began with CS21. There were also examples where a single RFLP haplotype yielded more than one sequence. Thus a second sequence for *A. lacertosa* L2 was given the number LS10.

Phylogenetic analysis of the sequence alignments was done using the neighbor-joining, minimum evolution, maximum likelihood, and maximum parsimony protocols. The topology of all of the resulting trees was so similar that only the neighbor-joining tree is presented here (Fig. 3). Trees were constructed with and without the inclusion of the crucifer flea beetle sequence. The presence of the outgroup served only to visually crowd the *Aphthona* branches. Therefore, the trees are shown without the crucifer flea beetle.

The neighbor-joining sequence-based tree used the 85 *Aphthona* sequences. Bootstrap numbers are based on 500 replicates and are indicated for the more significant nodes. The individual sequences are labeled with a species indicator followed by the GenBank accession number followed by the sequence haplotype number. The species are monophyletic with respect to the other recognized species. Bootstrap support for all of the species clades except *A. flava* is >95%. Bootstrap support for the *A. flava* branch is 75%. The topology of the tree is not significantly different than the one derived from the RFLP data. The branch containing *A. nigriscutis* haplotype NS1 corresponding to the high frequency N1 RFLP haplotype has a divergence value of 0.035 (3.5%) from the remaining *A. nigriscutis* and has 100% bootstrap support. The other *A. nigriscutis* show the same two subbranches as the previous data. The cluster containing NS3 as the most frequent haplotype has minimal divergence from the cluster containing NS5, but it is supported by a 90% bootstrap number. The *A. lacertosa* branch also has the same three elements as the RFLP tree. All three subbranches have high bootstrap numbers. The branch containing the AB restricted LS2 (RFLP-L2) haplotype is 0.061 diverged from the cluster with *A. lacertosa* LS1. This compares with a 0.045 divergence between *A. czwalinae* and NS1 of *A. nigriscutis*. It is also greater than the divergence of the *A. flava* and *A. cyparissiae* branches (0.044). The other two *A. lacertosa* subclades have a diversity of 0.019. The divergence between the *A. lacertosa* and the *A. venustula* lineages is 0.168.

The maximum parsimony tree topology matches the previous ones. All of the same clades are indicated. The number of mutational steps from the most recent common ancestor to *A. lacertosa* LS2 (15) is slightly less than the number of steps to *A. nigriscutis* LS1. This compares with a 0.045 divergence between *A. czwalinae* and NS1 of *A. nigriscutis*. It is also greater than the divergence of the *A. flava* and *A. cyparissiae* branches (0.044). The other two *A. lacertosa* subclades have a diversity of 0.019. The divergence between the *A. lacertosa* and the *A. venustula* lineages is 0.168.

The maximum parsimony tree topology matches the previous ones. All of the same clades are indicated. The number of mutational steps from the most recent common ancestor to *A. lacertosa* LS2 (15) is slightly less than the number of steps to *A. nigriscutis* NS1 (16.7) from its common ancestor with *A. czwalinae*.

**Discussion**

For the RFLP component, restriction enzymes were chosen for their ability to produce 5–16 fragments on the gel, enough bands to obtain sufficient data but not so many that overlapping bands would be a serious problem. It was also important that the banding patterns display some polymorphism. The seven restriction enzymes used met those criteria (Table 1).
Table 2. Geographical distribution of *Aphthona* collections used for RFLP haplotypes

| Species a | RFLP haplotype b | AB site 1 | AB site 3 | AB site 4 | MT | MN EPS | MN CLAY | ND FAR | ND LIS | ND WARD | ND MED | Total |
|-----------|------------------|----------|----------|----------|----|--------|---------|--------|--------|---------|-------|-------|
| AC        | All              | 17       | 35       | 12       | 5  | 1      | 1       | 24     | 9      | 10      | 29    | 94    |
| AZ        | All              | 1        | 1        | 9        | 2  | 77     | 27      | 6      | 20     | 9       | 190   | 248   |
| AF        | All              |           |          |          | 2  | 51     | 26      | 5      | 15     | 7       | 58    | 55    |
| AN        | All              | 1        | 46       |          | 8  | 6      | 17      | 26     | 26     | 120     | 47    | 596   |
| AL        | All              | 77       |          |          | 8  | 6      | 17      | 26     | 26     | 120     | 47    | 596   |
| AL Clade 1| L1, L5, L8      |          |          |          | 37 | 8      | 17      | 26     | 26     | 120     | 47    | 596   |
| AL Clade 2| L2              | 46       |          |          | 1  | 8      | 17      | 26     | 26     | 120     | 47    | 596   |
| AL Clade 3| L3, L4, L6, L7, L9 | 31   | 16     |          | 17 | 8      | 17      | 26     | 26     | 120     | 47    | 596   |
| Totals    |                  | 95       | 91       | 58       | 58 | 17     | 86      | 56     | 37     | 112     | 35    | 596   |

Alberta: AB, sites 1 and 3; Cardston, site 4, Lethbridge; ND, North Dakota; MED, Medora; WARD, Ward Co.; LIS, Lisbon; FAR, Fargo; MN, Minnesota; EPS, Eden Prairie/Shakopee; CLAY, Clay Co.; MT, Montana, various sites combined (see map in Fig. 1).

a AC, *A. cyparissiae*; AZ, *A. czwalinae*; AF, *A. flavia*; AL, *A. lacertosa*; and AN, *A. nigriscutis*.
b RFLP haplotypes contain the single species abbreviation letter followed by a number.

The number of nucleotides screened in this process, ≈1,500, is equivalent to a modest sequencing project.

As Figs. 2 and 3 demonstrate, the phylogenies generated by fragment comparisons and sequence alignments have the same basic topology. The species clades encompass the same samples with both sets of data, and the species groups are in agreement with the morphological taxonomy. The major secondary branches within *A. nigriscutis* and *A. lacertosa* also are observed in both trees (and the other three analyses of the sequences). This greatly increases the confidence that these branches are not an artifact of the type of data or the method of analysis.

The brown species, *A. cyparissiae*, produced 19 RFLP haplotypes from 94 individuals, with an overall diversity of 0.012. However, the main cluster of 15 haplotypes from 88 individuals was not nearly so diverse (≈0.002). The 14 *cox1-cox2* haplotypes had an average base substitution per site of 0.006, intermediate between the two extreme values for the RFLP samples. Two of the sequence haplotypes covered eight of the RFLP haplotypes (Table 3). Existing concentrations of this species seem to be in the western part of the release range (MT and AB) and in west central MN, but there is no apparent genetic divergence between these widely separated populations. Among the fourteen *cox1-cox2* haplotypes seven were found in AB and six in Clay Co. The species was quite rare in EPS MN (Roehrdanz et al. 2009). Two of the common RFLP haplotypes (C5 and C9) were recovered from ND, MT, and AB, a spread of ≈800 km. No *A. cyparissiae* were collected from the three heavily sampled locations west of the Red River in ND.

A second brownish colored species, *A. flavia*, yielded seven RFLP haplotypes from 29 specimens. All but one of the samples examined came from Fargo, ND, or MT. The MT group had six haplotypes in nine insects. The Fargo collection had two dominant haplotypes that were also found in MT and a minor haplotype that was unique to Fargo. The maximum diversity within this group was ≈0.005, somewhat less than the overall level for *A. cyparissiae*. By comparison, the *cox1-cox2* sequences had an average of 0.49 substitutions per site, 8× greater than *A. cyparissiae*. Five of the nine sequence haplotypes also carry one of two different 3-bp deletions. All seven of the RFLP haplotypes yielded unique sequences (Table 3), which reinforces the point that both types of data can detect similar levels of diversity.

The third brown species, *A. nigriscutis*, presents an interesting pattern of haplotype distribution. RFLP haplotype N1 makes up 75% of the individuals tested. N1 was found at every site that had *A. nigriscutis*, including two AB sites that each produced a single...
specimen, both of which were N1. The clade that includes this dominant haplotype is infected with the bacterial endosymbiont Wolbachia, as reported previously (Roehrdanz et al. 2006). Wolbachia infection is typically accompanied by severely reduced mtDNA variability. Four RFLP haplotypes were observed in the infected branch, 187 N1s, and three singletons—N10, N17, and N18. The RFLP diversity and sequence diversities within this clade are low and similar, 0.005 and 0.003, respectively. In the DNA sequences this clade has 100% bootstrap support. The cox1-cox2 divergence of this branch from the rest of A. nigriscutis is 0.042, which is virtually identical to the divergence between the species A. cyparissiae and A. nigriscutis as a whole. This branch corresponds to Clade A of Roehrdanz et al. 2006.

The remaining uninfected A. nigriscutis (Clade B of Roehrdanz et al. 2006) display extensive polymorphism. The 85 individuals exhibit 22 RFLP haplotypes, but two thirds of the haplotypes are represented by single individuals. The branch has 90% bootstrap support and a within group cox1-cox2 diversity of 0.01. In both data sets and all analyses, this branch bifurcates into the same two subbranches. The bootstrap number for one of these branches is 90%. The evolutionary divergence between these two branches is 0.015, and the within branch diversities are 0.006 and 0.004. The greater divergence of the Wolbachia-infected mitochondrial lineage from these latter two groups suggests that the infected lineage originated from a different geographic region and subsequently spread through the collection region via common maternal inheritance with the Wolbachia. An allozyme study of A. nigriscutis exhibited genetic differentiation of populations from different collection sites (Nowierski et al. 1996). PCR-based Wolbachia detection was not available at that time.

Only 10 specimens of the black A. czwalinae were tested. The 10 RFLP samples yielded five haplotypes and the sequences from each haplotype were different (Table 3). Despite the polymorphism, the within species divergence numbers were fairly low, 0.002, for the RFLP set and 0.004 for the sequences. This species has never had an impact on the biological control program. The black A. czwalinae and the brown A. nigriscutis appear as sister taxa, with between-species diversities of 0.022 (RFLP) and 0.041 (cox1-cox2). The between-species diversity for the sequences is less than the within species number for A. flava.

The most unusual array of haplotypes belongs to the other black species, A. lacertosa. The RFLP, NJ, and MP trees all place this species well separated from the previous four species. The RFLP distance to A. nigriscutis is 0.379, whereas the NJ distance is 0.191 and the NJ divergence within the large group is 0.030. Only A. flava’s is greater. However, the A. lacertosa haplotype array clearly separates into three distinct branches anchored by L1 (LS1), L2 (LS2), and L3 (LS3). Within these groups the divergence is much less, 0.003 for L1 and 0.005 for both L2 and L3. In the RFLP tree, the distance between L2 and L1 or L3 is ~0.022, or approximately the same as the distance between the A. nigriscutis and A. czwalinae. In the NJ results, the separation of L2 from the others is greater than the A. nigriscutis and A. czwalinae divergence and nearly at the level separating A. flava from A. cyparissiae. Table 2 shows that L1 is the sole haplotype recovered in MN and ND, whereas nearly all of L2 was from one of the AB sites. L1 (along with L5, which is a slight variant of L1) was found in significant numbers at a different AB site from L2. Haplotype L3 and its variant L7 were absent.

The black A. czwalinae and the brown A. nigriscutis appear as sister taxa, with between-species diversities of 0.022 (RFLP) and 0.041 (cox1-cox2). The between-species diversity for the sequences is less than the within species number for A. flava.

The most unusual array of haplotypes belongs to the other black species, A. lacertosa. The RFLP, NJ, and MP trees all place this species well separated from the previous four species. The RFLP distance to A. nigriscutis is 0.379, whereas the NJ distance is 0.191 and the NJ divergence within the large group is 0.030. Only A. flava’s is greater. However, the A. lacertosa haplotype array clearly separates into three distinct branches anchored by L1 (LS1), L2 (LS2), and L3 (LS3). Within these groups the divergence is much less, 0.003 for L1 and 0.005 for both L2 and L3. In the RFLP tree, the distance between L2 and L1 or L3 is ~0.022, or approximately the same as the distance between the A. nigriscutis and A. czwalinae. In the NJ results, the separation of L2 from the others is greater than the A. nigriscutis and A. czwalinae divergence and nearly at the level separating A. flava from A. cyparissiae. Table 2 shows that L1 is the sole haplotype recovered in MN and ND, whereas nearly all of L2 was from one of the AB sites. L1 (along with L5, which is a slight variant of L1) was found in significant numbers at a different AB site from L2. Haplotype L3 and its variant L7 were absent.
between other species in the same genus. The three terminal clades all have very high bootstrap support. A strong argument can be made that the clade with L2 should probably be elevated to species status or at the minimum be described as a cryptic species within an *A. lacertosa* species complex. The level of the L3 clade is more ambiguous. The difference between L1 and L3 is less than that observed for other species in the genus but it is greater than the *Wolbachia*-enforced divergence in *A. nigricutis*. Members of all three *A. lacertosa* clades were examined for *Wolbachia* and none were found, so that possibility is eliminated. It could be considered as another cryptic species; however, it also could be part of a broadly diverse species with intermediates existing in Europe.

The narrow genetic base of L1 has a plausible explanation. In 1989 black *Aphthona* believed to be *A. czwalinae* were imported and released at a single site in ND. It was later discovered that the releases were a mixture of *A. czwalinae* and *A. lacertosa*. It took a couple years before any progeny were noticed. That single point introduction ultimately became the source of all of the black flea beetles redistributed in ND and MN (Lym and Nelson 2000). *A. czwalinae* eventually disappeared from this population. The potential for multiple genetic bottlenecks in this process is substantial. Some of these beetles made their way to Canada (Roehrdanz et al. 2009), which accounts for the presence of L1 in AB. Earlier in the 1980s, *A. lacertosa* from a different European source were brought to Canada and their descendents survived around the original release site in AB that produced L2 and L3. Nowierski et al. (1996) observed some allozyme differentiation in European populations of *A. lacertosa*. Ironically, it seems that the Canadian introduction also may have involved two species. Examination of *A. lacertosa* samples from all three clades to see whether physical differences between them can be linked to the existing *Aphthona* taxonomy would be very beneficial and enable further clarification of the situation at Canadian field sites.

The molecular phylogenetic analysis of *Aphthona* flea beetle species in North America establishes the genetic relationships in this group that has become an important component of biological control of leafy spurge. Two of the species, *A. nigricutis* and *A. czwalinae* seem to be the most recently diverged. The three brown groups that have caused numerous identification problems are clearly well defined species, just as the original taxonomic work said they were. For the most part, the genetic diversity within the species is not severely restricted. A subgroup of *A. nigricutis* has narrow mtDNA diversity as the result of a *Wolbachia* infection. The question of whether a *Wolbachia* free strain should be released to locations where the existing genotypes have not thrived remains open. *A. lacertosa* is the biggest puzzle. The three clades are divergent enough to merit consideration of new species or cryptic species. Further examination of other DNA sequences, physical traits, ecology, geographic origins, and reproductive isolation would be in order. Defining a new taxon usually requires an additional piece of corroborating evidence from at least one of these categories (Danm et al. 2010).

Two of the clades, one *A. nigricutis* and one *A. lacertosa*, also have very narrow mtDNA bases. There are no data to indicate whether this could be a drawback to biocontrol. Most studies of invasive species or colonization indicated that a lack of genetic diversity has a negative impact on the establishment and spread of species in alien environments (Lee 2002, Crawford and Whitney 2010, Lachmuth et al. 2010). However, lack of mtDNA diversity does not doom a species but rather serves as an indicator that further examination is warranted. Clade L1 has performed quite well in the United States, despite its mitochondrial uniformity. Other species have succeeded with a narrow mtDNA base. The western corn rootworm (*Diabrotica virgifera virgifera* LeConte) spread from New Mexico to the Atlantic Coast with very little discernible mtDNA diversity (Szalanski et al. 1999). The solitary bee *Lasioglossum leucozonium* Schrank has successfully invaded North America from Europe with no discernible mtDNA diversity (Zayed et al. 2007). Similarly the gall wasp *Quadristichus erythrinae* Kim has spread across an area from India to Hawaii in 2 yr, while being monomorphic for both a mitochondrial and nuclear sequence (Rubinoff et al. 2010). The distinction between the introduction of a biological control agent and an invasive species is really a matter of contrasting human desires concerning the outcome. It is possible that biological control could be enhanced with the acquisition of a *Wolbachia*-free population of *A. nigricutis* or by wider dispersal of the Canadian variant of *A. lacertosa*. Perhaps the most important point to be made is that it would be much preferable to examine the genetic background of potential biocontrol agents before they are released rather than rely on luck.

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References Cited

Allendorf, F. W., and L. L. Lundquist. 2003. Introduction: population biology, evolution and control of invasive species. Conserv. Biol. 17: 24–30.

Anderson, G. L., E. S. DelFosse, N. R. Spencer, C. W. Prosser, and R. D. Richard. 2000. Biological control of leafy spurge: an emerging success story, pp. 15–25. In Proceedings, X International Symposium on Biological Control of Weeds, 4–14 July 1999, Bozeman, MT. Montana State University, Bozeman, MT.

Bourchier, R. S., S. Erb, A. S. McClay, and A. Gassmann. 2002. *Euphorbia esula*. (L.) leafy spurge and *Euphorbia cyparissias* (L.) cypress spurge (Euphorbiaceae). pp. 346–358. In P. Mason and J. Haber (eds.), Biological control programmes against insect and weeds in Canada 1981–2000. Commonwealth Agricultural Bureaux, Slough, United Kingdom.
Butler, J. L., M. S. Parker, and J. T. Murphy. 2006. Efficacy of flea beetle control of leafy spurge in Montana and South Dakota. Rangeland Ecol. Manage. 59: 453–461.

Carlson, R. B., and D. Mundal. 1996. Introduction of insects for the biological control of leafy spurge in North Dakota. N D Farm Res. 47: 7–8.

Cheung, W. Y., N. Hubert, and B. S. Landry. 1993. A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and other PCR analysis. Genome Res. 3: 69–70.

Colautti, R. I., M. Manca, M. Viljanen, H. Ketelaars, H. Birgi, H. Maeluaae, and D. Heath. 2005. Invasion genetics of the Eurasian spiny waterflea: evidence for bottlenecks and gene flow using microsatellites. Mol. Ecol. 14: 1569–1579.

Crawford, K. M., and K. D. Whitney. 2010. Population genetic diversity influences colonization success. Mol. Ecol. 19: 1253–1263.

Damm, S., B. Schierwater, and H. Hadrums. 2010. An integrative approach to species discovery in odonates from character-based DNA barcoding to ecology. Mol. Ecol. 19: 3851–3893.

Fauske, G. M. 2003. Character-based DNA barcoding to ecology. Mol. Ecol. 19: 3881–3893.

Gassmann, A. 1996. Life history and host specificity of Aphthona cemistula Kutsch. (Coleoptera: Chrysomelidae), a candidate for the biological control of leafy spurge (Euphorbia esula L.) in North America. J. Appl. Entomol. 120: 405–411.

Gassmann, A., and D. Schroeder. 1995. The search for effective biological control agents in Europe: history and lessons from leafy spurge (Euphorbia esula L.) and cypress spurge (Euphorbia cyparissias L.). Biol. Control 5: 466–477.

Gassmann, A., D. Schroeder, E. Mav, and G. Sommer. 1996. Biology, ecology, and host specificity of European Aphthona spp. (Coleoptera: Chrysomelidae) used as biocontrol agents for leafy spurge, Euphorbia esula (Euphorbiaceae), in North America. Biol. Control 6: 105–113.

Hansen, R. W., R. D. Richard, P. E. Parker, and L. E. Wendel. 1996. Distribution of biological control agents of leafy spurge (Euphorbia esula L.) in the United States: 1985–1996. Biol. Control 10: 129–142.

Hodur, N. M., F. L. Leistritz, and D. A. Bangsund. 2006. Biological control of leafy spurge: utilization and implementation. Rangeland Ecol. Manage. 59: 445–452.

Kalischuk, R. S., B. Bourchier, and A. S. McClay. 2004. Post hoc assessment of an operational biocontrol program: efficacy of the flea beetle Aphthona lacertosa Rosenhauer (Chrysomelidae: Coleoptera), an introduced biocontrol agent for leafy spurge. Biol. Control 29: 418–426.

Keller, L. F., and D. M. Waller. 2002. Inbreeding effects in wild populations. Trends Ecol. Evol. 17: 230–241.

Kirby, D. R., R. B. Carlson, K. D. Krabbenhoft, D. Mundal, and M. M. Kirby. 2000. Biological control of leafy spurge with introduced flea beetles (Aphthona spp.). J. Range Manage. 53: 305–308.

Lachmuth, S., W. Durka, and F. M. Schurr. 2010. The making of a rapid plant invader: genetic diversity and differentiation in the native and invaded range of Senecio inaequidens. Mol. Ecol. 19: 3952–3967.

Lee, C. E. 2002. Evolutionary genetics of invasive species. Trends Ecol. Evol. 17: 386–391.

LeSage, L., and P. Paquin. 1996. Identification keys for Aphthona flea beetles (Coleoptera: Chrysomelidae) introduced in Canada for the control of spurge (Euphorbia spp., Euphorbiaceae). Can. Entomol. 128: 593–603.

Lym, R. G., and J. A. Nelson. 2000. Biological Control of Leafy Spurge (Euphorbia esula) with Aphthona spp. along railroad right-of-ways. Weed Technol. 14: 642–646.

Lym, R. G., and D. L. Olson. 1999. Leafy spurge control using flea beetles. North Dakota State University Extension Service Publication 1183 (revised 2002).

Miller, J. C. 1991. RESTSITE, version 1.2. Institute of Molecular Evolutionary Genetics, The Pennsylvania State University, University Park, PA.

Mundal, D. A., D. L. Olson, and R. B. Carlson. 1999. The effect of Aphthona spp. flea beetle (Coleoptera: Chrysomelidae) larval feeding on leafy spurge, Euphorbia esula L., root systems and stem density in North Dakota: 1986–96. Team leafy spurge publications. (http://www.ars.usda.gov/teampub/teampublications.html).

Nowierski, R. M., Z. Zheng, D. Schroeder, A. Gassmann, B. C. FitzGerald, and M. Cristofaro. 2002. Habitat associations of Euphorbia and Aphthona species from Europe: development of predictive models for natural enemy release with ordination analysis. Biol. Control 23: 1–17.

Nowierski, R. M., and R. W. Pemberton. 2003. Invasive plants of the eastern United States, leafy spurge. (http://www.invasive.org/eastern/biocontrol/14LeafySpurge.html).

Nowierski, R. M., G. J. McDermott, J. E. Bunnell, B. C. FitzGerald, and Z. Zeng. 1996. Isozyme analysis of Aphthona species (Coleoptera: Chrysomelidae) associated with different Euphorbia species (Euphorbiaceae) and environmental types in Europe. Ann. Entomol. Soc. Am. 89: 585–586.

Olson, D., and R. Hansen. 2006. Biology of leafy spurge biological control agents. In R. Bourchier, R. Hansen, R. Lym, A. Norton, D. Olson, C. Randall, M. Schwarlander, and L. Skinner (eds.), Biology and biological control of leafy spurge. Forest Health Technology Enterprise Team, USDA Forest Service.

Rees, N. E., and N. R. Spencer. 1991. Biological control of leafy spurge, pp. 181–192. In L. James, J. Evans, M. Raiphs, and R. Child (eds.), Noxious range weeds. Westview Press, Boulder, CO.

Richardson, L. A., C. J. Juricek, R. G. Lym, D. R. Kirby, and D. A. Tober. 2008. Integrated leafy spurge (Euphorbia esula) control using Imazapic, Aphthona spp. biological control agents, and seeded native grasses. Invasive Plant Sci. Manage. 1: 255–264.

Roehrdanz, R. L. 1995. Amplification of complete insect mitochondrial genome in two easy pieces. Insect Mol. Biol. 4: 169–172.

Roehrdanz, R. L., and M. E. Degrugillier. 1998. Long sections of mitochondrial DNA amplified from fourteen orders of insects using conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 91: 771–778.

Roehrdanz, R. L., D. Olson, B. Bourchier, S. Sears, A. Cortilet, and G. Fauske. 2006. Mitochondrial DNA diversity and Wolbachia infection in the flea beetle Aphthona nigriscutis (Coleoptera: Chrysomelidae) an introduced biocontrol agent for leafy spurge. Biol. Control 37: 1–8.

Roehrdanz, R. L., D. Olson, G. Fauske, B. Bourchier, A. Cortilet, and S. Sears. 2009. New DNA markers reveal presence of Aphthona species (Coleoptera: Chrysomelidae) believed to have failed to establish after release into leafy spurge. Biol. Control 49: 1–5.

Rubinoff, D., B. S. Holland, A. Shibata, R. H. Messing, and M. G. Wright. 2010. Rapid invasion despite lack of genetic variation in the Erythrina gall wasp (Quadrastichus erythrinae Kim). Pac. Sci. 64: 23–31.
Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Spencer, N. R. 1994. Exotic insects for leafy spurge control, pp. 63–65. In Proceedings, Leafy Spurge Strategic Planning Workshop, 29–30 March 1994, Dickinson, ND. North Dakota State University Agricultural Extension Service, Fargo, ND.

Szalanski, A. L., R. L. Roehrdanz, D. B. Taylor, and L. C. Chandler. 1999. Genetic variation in geographical populations of western and Mexican corn rootworm. Insect Mol. Biol. 8: 519–526.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596–1599.

[USDA] U.S. Department of Agriculture. 2006. Ecology and management of leafy spurge (Euphorbia esula L.). USDA, Natural Resources Conservation Service, Montana. Invasive Species Technical Note No. MT-2. (http://www.ars.usda.gov/SP2UserFiles/Place/53254300/Reports/USA-BCW(forpdf)v5.pdf).

Wendel, L. E., R. Hanson, P. Parker, and R. Richard. 1992. Leafy spurge overview and summary of federal regulatory effort, pp. 3–34. In Proceedings, Symposium: leafy spurge, 22–24 July Lincoln, NE.

Zayed, A., S. A. Constantin, and L. Packer. 2007. Successful biological invasion despite a severe genetic load. PLoS ONE 2:e868.

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