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New DNA markers reveal presence of *Aphthona* species (Coleoptera: Chrysomelidae) believed to have failed to establish after release into leafy spurge

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ABSTRACT

Six species of *Aphthona* flea beetles from Europe have been introduced in North America for the purpose of controlling a noxious weed, leafy spurge (*Euphorbia esula*). In the years following the releases, five of the species have been recorded as being established at various locations. There is no evidence that the sixth species ever became established. A molecular marker key that can identify the DNA of the five established species is described. The key relies on restriction site differences found in PCR amplicons of a portion of the mitochondrial cytochrome oxidase I gene. Three restriction enzymes are required to separate the immature specimens which are not visually separable. Adults which can be quickly separated into the two black species and three brown species require only two restriction enzymes to resolve the species. Many of the original releases and relocations of the flea beetles used populations containing mixed species that were often not thoroughly characterized as to species. The markers showed the presence of two *Aphthona* species in North Dakota that were believed to have been absent from the state for the past decade. Without the marker assay these populations would probably have been overlooked.
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1. Introduction

Chrysomelid flea beetles from the genus *Aphthona* have been introduced into North America since the late 1980s in an effort to control the invasive weed, leafy spurge (*Euphorbia esula* L) (Rees and Spencer, 1991; Spencer, 1994; Hansen et al., 1997; Kirby et al., 2000; Bourchier et al., 2002; Nowierski and Pemberton, 2003). The weed and *Aphthona* are both native to Eurasia (Wendel et al., 1992). Six *Aphthona* species, *Aphthona flava* Guillebeau, *Aphthona cyparissiae* Koch, *Aphthona abdominalis* Duftschmid, *Aphthona nigriscutis* Foudras, *Aphthona czwalinae* Weise, and *Aphthona lacertosa* Rosenheim were released in the Northern Great Plains states during the course of this biological control program (Carlson and Mundal, 1990). The first four species are various shades of brown to gold in color, while the latter two are predominantly black (Olson and Hansen, 2006). Where flea beetles have become successfully established in North America, they have regularly reduced leafy spurge stands by up to 90% (Anderson et al., 1999; Mundal et al., 1999; Kalischuk et al., 2004). In the years since the initial releases, *Aphthona* flea beetles have been collected from sites of establishment and relocated to new sites. During the course of these redistribution efforts, the relative abundance of the species at collection sites has changed (Mundal et al., 1999).

Aphthona lacertosa and *A. nigriscutis* have come to dominate the extant populations (Lym and Olson, 1999). They comprise most of the *Aphthona* beetles currently found in North Dakota and Minnesota and are common in Alberta as well. *A. flava* and *A. cyparissiae* have become so scarce in North Dakota and Minnesota that collections of brown beetles are often assumed to be all *A. nigriscutis*. This is partly a matter of convenience because these three species are not easily distinguishable by their external morphology and color (Fauske, 2003). *A. flava* and *A. cyparissiae* can still be found as the dominant species at some of original release sites in Alberta, but their relative abundance at a large number of sites has not been assessed. *A. czwalinae* was thought to have been a major component in the early years of the biocontrol program until it was discovered that most of what was being called *A. czwalinae* was in fact *A. lacertosa.* With that revelation, *A. czwalinae* ceased to be a factor in leafy spurge biocontrol. There have not been verifiable sightings of *A. czwalinae* in either North Dakota or Minnesota for several years (Mundal et al., 1999). Despite being released in several states, there is no record that *A. abdominalis* ever became

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established in leafy spurge stands (Anon, 2006). *A. abdominalis* has never been released in Canada (Bourchier et al., 2002).

A reliable means of identifying the individual species in this group of biological control agents is needed for monitoring their dispersal in different habitats and to understand sympatric interactions among the species. We have developed a relatively simple DNA-based assay that can identify five *Aphthona* species (not *A. abdominalis*) that have been part of the leafy spurge biocontrol effort. A phylogenetic analysis of these species is forthcoming. In the process of testing beetles from 10 populations, we discovered a location in eastern North Dakota that supports substantial numbers of three species, *A. czwalinae*, *A. flava* and *A. cyparissiae*, which have not been observed in North Dakota in recent years.

2. Materials and methods

Adult flea beetles were obtained from leafy spurge infested sites in North Dakota (collected 2001–02, 2006), Minnesota (collected 2003–04, 2006–07), Montana (collected 2002) in the USA, and Alberta (collected 2003–04), Canada. Beetles from the United States were brought to Fargo live and frozen at −80 °C for future use. Beetles from Canada were frozen in Canada and shipped to Fargo packed on dry ice.

The four North Dakota (ND) collection areas were near Medora, near Lisbon, near Minot in Ward County, and in Fargo at the USDA Lab research plot and along railroad tracks about 0.7 km to the west. There were two widely separated collection areas in Minnesota (MN). One cluster of sites was along the Minnesota River just southwest of Minneapolis. A second cluster of sites was in Clay County, MN about 20 mi east of Fargo, ND. Montana (MT) samples were from several locations in the southern part of the state. Canadian collection sites were in the Cardston/Lethbrige region of southern Alberta (AB). A listing of the specific sites and their abbreviations is in Table 2. Site coordinates can be found in Roehrdanz et al. (2006). Nine specimens of *A. flava* were obtained from unknown locations in MT. Six DNA samples from 2000 and identified as *A. czwalinae* from MT were obtained from R. Nowierski.

Morphological species assignment was based on external and internal morphology. Adult beetles can be quickly sorted into black beetles, *A. lacertosa* plus *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). The brown beetles required detailed dissection and examination of genitalia to confirm species status (Lesage and Paquin, 1996).

2.1. DNA preparation and PCR

Total DNA was extracted from either whole insects using the high salt procedure of Cheung et al. (1993) or from one of the hind legs using the Dneasy Tissue kit (Qiagen Corp.). 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. A total of 589 specimens, representing all five species, were subjected to long PCR-RFLP using a ~9000 bp segment of the mt genome (Primers: C2R; C2-J-3684 5′-GGTCAAT GTTCAGAAATTTGTGG-3′ and 16S2; LR-N-12945 5′-GCGACCTCG ATGTTGGATTAA-3′). The amplicons were digested with seven restriction enzymes and the RFLPs were analyzed as described in Roehrdanz et al. (2006).

Portions of the mitochondrial *cox*1-*tRNA*Leu-*cox*2 region were amplified from specimens with differing PCR-RFLP haplotypes using the primer pairs C1-RLR (5′-TTGATTTTTTGGTCATCCAGA AGT-3′) and C2 (5′-CCACAAATTTCTGAACATTGACC-3′) or C1-2797 (5′-CCTCGACGTTATTCAGATTACC-3′) and C2-3380 (5′-TCAATATCA TTGATGACCAAT-3′). PCR products of about 1503 and 617 bp, respectively, were sequenced. GenBank accession numbers of the sequences are: *A. lacertosa* (DQ381553–DQ381562), *A. cyparissiae* (DQ386423–DQ386434, EU449966–EU449978), *A. flava* (EU440 532–EU440540), *A. nigriscutis* (EU448964–EU448988), *A. czwalinae* (EF090277–EF090281).

Computer evaluation of the sequences was used to identify restriction sites that were not polymorphic within species. The minimum subset of these sites that would permit identification of the five species was chosen. The mitochondrial primers FB-C1 (5′-TACTCAGATTACCCTGATGTATTT-3′) and FB-C2 (5′-TATCATTGA TGTCCAATTGTTTTAATT-3′) were designed to encompass these restriction sites and improve the amplification from all five species. The new primers amplify a portion of the mtDNA *cox*1-*tRNA*Leu-*cox*2 region that is 605 bp in length. Reaction components were from the Applied Biosciences Taq Gold kit and the PCR conditions were 35 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 3 min. Three restriction enzymes that yielded simple patterns (*Hin*f I, *Pst* I, and *Rsa* I) were used to create profiles that define the species. These can be used for identification of immature and adult individuals.

3. Results

A total of 65 PCR-RFLP haplotypes were identified from the five species of flea beetles. The haplotypes formed five clades with the sequence divergence between the clades ranging from about 4.5% to 6.0% (data not shown). The number of RFLP haplotypes per species ranged from 5 (*A. czwalinae*) to 24 (*A. nigriscutis*). The five clades and the morphological species assignments were coincident. Representatives of 60 of the PCR-RFLP haplotypes were sequenced for the *cox*1-*cox*2 region. Sequences were also obtained from an additional 13 specimens that were not scored for PCR-RFLP haplotype. The GenBank accession numbers for all of the sequences are reported in Section 2. A Neighbor-joining tree indicated that sequence clades also correspond to species (data not shown).

Table 1 shows the restriction fragment sizes obtained with the three enzymes that were used. *Rsa* I separates the black species, *A. lacertosa* and *A. czwalinae* from the three brown species, *A. nigriscutis*, *A. cyparissiae*, and *A. flava*. The black species contain a single *Rsa* I site that produces two fragments (398 and 207 bp). All but one of the brown species specimens lacked this site and the DNA remained uncut. The one exception, an *A. flava* specimen, produced two unique *Rsa* I fragments (312 and 290 bp).

In step two, *Pst* I was used to distinguish between the specimens with the *Rsa* I 398/207 pattern. *A. czwalinae* has one *Pst* I site, whereas *A. lacertosa* has none. *Pst* I by itself is not informative for the brown species. The step two digestion for beetles lacking the *Rsa* I 398/207 pattern used *Hinf* I. Neither *A. cyparissiae* nor *A. flava* contain a *Hinf* I site. *A. nigriscutis* DNA was cleaved with *Hinf* I and two different patterns were recovered, some with a single *Hinf* I site and others with two sites. In step three the samples that did not have a *Hinf* I site can be digested with *Pst* I. *A. flava* has a *Pst* I site, but *A. cyparissiae* does not. A flow chart for the digestions is presented in Fig. 1. Table 1 includes the fragment sizes for double digests and the triple digest. Fig. 2 shows some examples of both single and double digestions including two patterns for *A. nigriscutis*.

All of the collection sites had a mixture of black and brown beetles. Use of the DNA markers enabled assessment of the species composition of the brown beetles and the black beetles at each site (Fig. 1 and Table 2). *A. cyparissiae* was the most common brown *Aphthona* at Alberta sites AB1 and AB3, whereas *A. nigriscutis* was the most common brown species present at site AB4. An additional

Table 1
Aphthona species diagnostic RFLP fragments from mtDNA 605 bp *cox*1-*cox*2 amplicon.

	Brown to gold adults						Black adults	
Restriction enzyme	*A. cyparissiae*	*A. flava*		*A. nigriscutis*			*A. lacertosa*	*A. czwalinae*
Number[a]	27	8[b]	1[b]	6[b]	9[b]	9[b]	11	5
Rsa I	605[c]	605	312 290	605	605	605	398 207	398 207
Pst I	605	419 186		419 186	419 186	605	605	419 186
Hinf I	605	605		325 164 116	441 164	441 164	605	605
Pst I/*Rsa*	605	419 186	290 186 126	419 186	419 186	605	605	212 207 186
Rsa I/*Hinf*	605	605	312 290	325 164 116	441 164	441 164	398 207	398 207
Pst I/*Hinf* I	605	419 186		303 164 116 22	419 164 22	441 164	605	419 186
Pst I/*Rsa* I/ *Hinf* I	605	419 186	290 186 126	303 164 116 22	419 164 22	441 164	398 207	212 207 186

[a] Number of sequences with each pattern.
[b] Multiple RFLP patterns within *A. nigriscutis* and *A. flava*.
[c] Fragment sizes in bp.

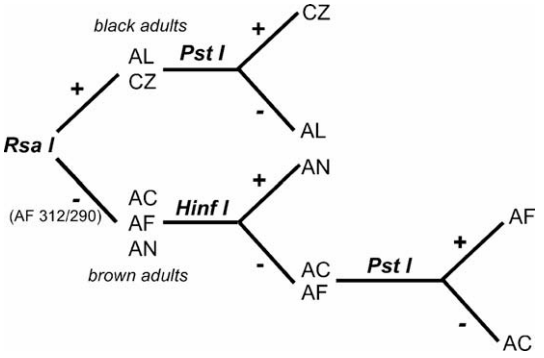

Fig. 1. Key to introduced species of *Aphthona* using PCR-RFLP. See Table 2 for fragment sizes. AC, *A. cyparissiae*; AF, *A. flava*; AN, *A. nigriscutis*; AL, *A. lacertosa*; CZ, *A. czwalinae*.

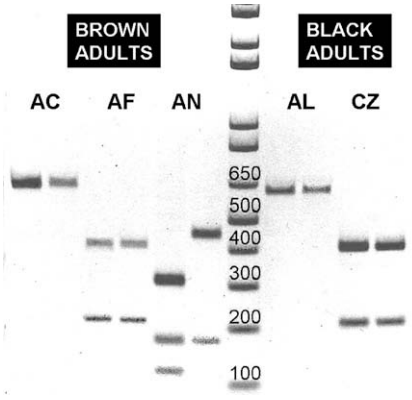

Fig. 2. Restriction digest of *Aphthona* PCR products. Two individuals of each species. Lanes 1–6, DNA from brown beetles cleaved with *Pst* 1 and *Hinf* 1. Lane 7, DNA size marker in base pairs. Lanes 8–11, DNA from black beetles cleaved with *Pst* 1. Lanes 1–2, AC, *A. cyparissiae*; Lanes 3–4, AF, *A. flava*; Lane 5, AN, *A. nigriscutis* (first pattern in Table 1); Lane 6, AN, *A. nigriscutis* (third pattern in Table 1); Lanes 8–9, AL, *A. lacertosa*; Lanes 10–11, CZ, *A. czwalinae*.

AB site had substantial numbers of *A. flava* based on morphological observation, but the DNA obtained from them was low quality and the data is not included. Three ND sites and the southern MN collections had 227 brown beetles that tested as *A. nigriscutis* and

Table 2
Comparative numbers of *Aphthona* species at different collection sites.

Beetle color	Beetle species	Alberta, Canada			North Dakota, USA				Minnesota, USA		Montana
		AB1	AB3	AB4	MED	WAR	LIS	FAR	EPS	CLA	Var[a]
Brown	AN	1	1	47	19	78	20		111	19	20
	AC	17	35	13				24 (491)[b]	1	71 (593)[b]	11
	AF		1					19			14
Black	AL	77	58	13	26	26	17 (71)	6 (104)	9 (471)	(512)	(11)
	CZ							8 (134)			

Species identification based on DNA markers except numbers in parentheses which were identified from adult morphology.
Species: AN, *A. nigriscutis*; AC, *A. cyparissiae*; AF, *A. flava*; AL, *A. lacertosa*; CZ, *A. czwalinae*.
Locations: Alberta – AB1 and AB3, Cardston, AB; AB4, Lethbridge, AB; North Dakota – MED, Medora, ND; WAR, Ward County, ND; LIS, Lisbon, ND; FAR, Fargo, ND; Minnesota – EPS, Eden Prairie/Shakopee, MN; CLA, Clay County, MN; Montana – various sites combined. See Section 2 for additional description.
[a] Only brown adults were tested.
[b] Brown adults.

there was a single *A. cyparissiae*. Species identification of the black beetles was performed by both DNA analysis and external coloration of the femur. *A. lacertosa* was the only black beetle collected from collection sites other than FAR (1280 beetles in total). The MT collections contained *A. lacertosa* and all three brown species.

The species composition at the FAR site was unique. In the summer of 2006, brown *Aphthona* beetles were first observed on leafy spurge at the USDA-ARS Biosciences Research Laboratory in Fargo. Prior to this, no *Aphthona* beetles had been observed in this plot but due to the large number of adults found in 2006, some beetles were probably present, at least in 2005 if not earlier. Leafy spurge had been at this location for five years. These individuals were expected to be *A. nigriscutis*, because nearly all of the brown beetles from ND had previously been identified through DNA and morphological analyses as *A. nigriscutis*. However, DNA examination of a sample of the beetles showed that there was a mixture of *A. cyparissiae* and *A. flava*. Since these were not the species commonly found in ND and they had recently colonized the research plot, a search was conducted to find the potential source of these beetles.

A total of 786 beetles were collected from the research plot and a second location 0.6 km away which consisted of 534 brown (68%) and 252 black (32%) specimens. Among the 45 brown beetles that were examined for their DNA profile, 24 were scored as *A. cyparissiae* (56%), 19 as *A. flava* (44%), and none as *A. nigriscutis*. Visual or DNA examination of the black beetles found 110 *A. lacertosa* (44%), and 142 *A. czwalinae* (56%).

4. Discussion

The fact that individuals carefully identified as to species are associated with distinct clades with respect to both mtDNA sequences and extended RFLP data gives us confidence in the reliability of the simple PCR-RFLP test described here. The genus *Aphthona* is native to Eurasia and the only species found in North America are those that have become established via introductions for biocontrol. Both the adults and larvae feed on leafy spurge, whereas other native flea beetles do not. Adults collected from sweeps of leafy spurge stands and larvae obtained from leafy spurge roots will be *Aphthona*.

The screening protocol in Fig. 1 provides a means to identify immature stages of the beetles. If adult beetles are being surveyed, the *Rsa* I digestion is unnecessary because it is quicker to visually separate the black beetles from the brown beetles before DNA extraction. Double digestions could be used to save time in situations where an initial survey has revealed that not all species are present, but a single restriction enzyme is not sufficient for species discrimination. In principle, the triple digest can identify all five species in a single step. When using patterns from the triple digestion, caution would be advised because some of these patterns contain fragments that are similar in size to other patterns (441 & 164, 419 & 186, 419 & 164, 398 & 207). Gel conditions should be adjusted to be certain that differences in similar sized bands can be recognized.

Finding a significant population of *A. czwalinae* was unexpected. This discovery, coupled with the history of the black *Aphthona* releases in ND, is important for determining how *A. czwalinae* arrived at its current distribution in the state. Black beetles labeled as *A. czwalinae* were obtained from USDA-APHIS and were released at a location northwest of Valley City, ND in 1989 (Lym and Nelson, 2000). Monitoring for beetles for two years after their release did not reveal any beetles and it was assumed that they had failed to establish. In the third summer after release a bare patch was detected in the stand of leafy spurge and close observation revealed a population of black beetles. Beetles from this population were collected and transferred to other leafy spurge infested sites. Descendants of the Valley City release became the source of all the subsequent releases and transfers of black beetles in ND and MN. In the mid 1990s, a batch of these beetles was sent to Canada for release and established in AB. Upon examination of these beetles, it was concluded that the vast majority of them were not *A. czwalinae*, but were instead a different species, *A. lacertosa* (P. Harris, personal communication). Ten voucher specimens remaining from the original 1989 Valley City release were examined and were determined to comprise four *A. czwalinae* and six *A. lacertosa*. For several years in the mid 1990s the early translocations of black beetles in ND and MN were referred to as *A. czwalinae/A. lacertosa* mixed populations. As it became apparent that *A. czwalinae* was not present at any of the new sites, the populations began to be referred to as *A. lacertosa* alone.

The early releases of the brown *Aphthona* beetles were often described as multi-species releases. Exact species compositions were not recorded in release reports once the program was operational. Non-identified flea beetles were collected from successful sites and used to seed new sites. It is also possible that there are some errors in published reports that cataloged the species released if only adult body color was used as the distinguishing character, instead of dissection and examination of genitalia. In much of ND and MN, *A. nigriscutis* became the dominant brown species. In retrospect it is difficult to determine if *A. nigriscutis* is really the most effective brown *Aphthona* or if it just happened to take over a location that became the main source for many subsequent releases.

In MN, *A. nigriscutis* populations released in the early 1990s remained fairly small and localized to specific areas within a release site. When *A. lacertosa* was introduced to the state in the mid 1990s, it began populating sites quickly and thoroughly. In fact, most of the early success with biological control in the state was attributed to this species alone. However, *A. nigriscutis* continues to build its populations at many sites throughout MN and is having a much larger observed impact on leafy spurge. For example, over the past five or six years, beetle harvest records from sites that were seeded with an *A. lacertosa/A. nigriscutis* mixture typically show that the dominant species collected is *A. lacertosa*, and approximately 30–45% of the collected individuals consist of *A. nigriscutis* (A. Cortilet, unpublished observation). *A. cyparissiae* populations remain strong in Clay County of west-central MN and area that was one of the first in MN to receive beetles.

The FAR collection site does not appear to be a location that had intentional releases of flea beetles. The site is adjacent to a heavily-trafficked Burlington Northern Santa Fe Railroad line. Inadvertent movement of beetles by train would be a possible mechanism for dispersing the beetles into new patches of leafy spurge. Alternatively, in the early 1990s almost every biocontrol insect being tested against leafy spurge could be found in a stand of leafy spurge that was located behind a cemetery and adjacent to an Air National Guard base and regional airport in Fargo. This location was used in attempts to establish nursery sites for all species. It is located about 3 km northeast of the 2006 FAR collection site and beetles may have moved from the cemetery site to the FAR site at some point. A survey of the cemetery in 2006 revealed the presence of leafy spurge but no beetles were apparent.

We believe the cemetery location is the most likely candidate as a source site for the FAR population. We do not know what route the insects may have taken to get to their present location. What is most compelling is that three species, *A. czwalinae*, *A. flava*, and *A. cyparissiae*, that have become rare in the active biocontrol program in ND, have survived about 15 years beyond their original release in ND without being knowingly collected and transported. Two of these species, *A. flava* and *A. cyparissiae* are also still present at early release locations in other regions, such as AB.

Using the DNA key developed in this paper for a wider scale survey would be useful to determine if *A. czwalinae*, *A. flava*, and *A. cyparissiae*, are more common than previously thought, and in

what habitats throughout the North American range of leafy spurge. The DNA assay has the added advantage that sampling could be extended to immature stages of the beetles and could be used to study competition between larvae of different species. In addition, given that *A. czwalinae* was initially selected for release because of a possible preference for shaded habitats where spurge control is currently not effective, it would be worth reexamining the biology and interspecific competition of these species at established North American sites, and their potential for possible redistribution programs. Successful biocontrol of leafy spurge has been dominated by *A. lacertosa* and *A. nigriscutis* (Larson and Grace, 2004); however, these species have not been effective biocontrol agents in all locations. Perhaps it is time to give the other three species a chance to prove themselves in situations where they are not in direct competition with the former two species. The DNA markers described here can be used to monitor the purity of such releases.

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