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Isozyme analysis of *Aphthona* species (Coleoptera: Chrysomeidae) associated with different *Euphorbia* species (Euphorbiaceae) and environmental types in Europe[1]

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Abstract:

Isozyme analysis using starch gel electrophoresis was used to examine possible genetic differences among populations of 4 *Aphthona* species which were associated with 4 different *Euphorbia* species and 4 different environmental types in Europe. *Aphthona* species evaluated in the study included *A. cyparissiae* (Kock), *A. flava* Guillebeau, *A. lacertosa* Rosenhauer, and *A. nigriscutis* Foudras. *Euphorbia* host plant species considered in this study included *E. cyparissias* L., *E. esula* L., *E. lucida* Waldstein-Wartemberg and Kitaibel, and *E. virgata* Waldstein-Wartemberg and Kitaibel. Cluster analysis of genetic distances obtained from isozyme analysis easily distinguished populations of the 4 flea beetle species as 4 distinct groups. Based on the dendrogram generated from cluster analysis, populations of *A. lacertosa* and *A. nigriscutis* were found to be more similar genetically than either species to populations of *A. flava* or *A. cyparissiae.* The greatest genetic distance was found between populations of *A. cyparissiae* and *A. lacertosa,* which occur in different habitats and on different spurge species. A relatively high genetic distance also was found between populations of *A. cyparissiae* and *A. nigriscutis,* which are species that occur in similar habitats and on similar spurge species. Measurable degrees of genetic variability were found between populations (within a species) for 2 of the 4 *Aphthona* species sampled.

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Aphthona spp., *Euphorbia* spp., leafy spurges, isozyme-allozyme analysis, environmental type.

Leafy spurge, *Euphorbia esula* L., is a noxious, deep-rooted perennial weed of Eurasian origin that can form dense stands of up to 100% cover on pastures, prairies, rangeland, and other noncrop areas in North America. The weed reduces forage production, wildlife habitat, and causes extensive monetary losses to the livestock industry (Messersmith and Lym 1983, Lacey *et al*. 1985, Watson 1985, Nowierski and Harvey 1988). *E. esula* has infested >1 million hectares in North America since its introduction ≈200 yr ago (Alley and Messersmith 1985), and threatens many more (Lacey *et al*. 1985). Economic losses from leafy spurge for the Dakotas, Montana, and Wyoming in 1990 were estimated at >100 million dollars (Anonymous 1992) and were projected to possibly reach 144 million dollars annually by 1995 (Bangsund and Leistritz 1991).

Leafy spurge represents a genetic, chemical, and morphological mosaic, and as a consequence considerable taxonomic confusion exists (Schulz-Schaeffer and Gerhardt 1987, Harvey *et al*. 1988, Torell *et al*. 1989, Nissen *et al*. 1992). This, combined with sexual and asexual reproduction, a deep underground root system, the weed's ability to infest xeric, mesic, and even hygric sites (Nowierski and Zeng 1994), and the numerous native North America plant species in the spurge family Euphorbiaceae (Pemberton 1985) provide a great challenge for classical biological control of this weed.

The flea beetle genus *Aphthona* contains ≈40 species that are known to feed on leafy spurges, *Euphorbia* spp., in Europe and Asia. A number of species in this genus have been introduced into North America for control of leafy spurge in the United States and Canada, including *A. abdominalis* (Duftschmidt), *A. cyparissiae* (Koch), *A. czwalinae* Weise, *A. flava* Guillebeau, *A. lacertosa* Rosenhauer, and *A. nigriscutis* Foudras (Rees *et al*. 1996). Five of these *Aphthona* species – *A. cyparissiae, A. czwalinae, A. flava, A. lacertosa*, and *A. nigriscutis* – have become established successfully on leafy spurge in the United States and Canada, and in a number of cases have significantly reduced spurge density at the release sites (Pemberton and Rees 1990, Rees *et al*. 1996).

All of the established flea beetle species released against leafy spurge in the United States to date are univoltine, although some of the species show phenological differences during the course of the growing season (Hansen 1994). Early larval instars feed in or on the root hairs of the host plant, whereas later instars feed in or on the yearling roots. Adult flea beetles feed on the leaves and flower bracts of leafy spurge. *Aphthona species* overwinter as larvae and generally pupate within the spurge roots in late spring to early summer (Rees *et al*. 1996).

Genetic studies of biological control agents are becoming increasingly important because of such concerns as the release and redistribution of approved species only (Hoy *et al*. 1991), the potential existence of host races (Unruh and Goeden 1987), maximizing the rate of natural enemy adaptation and establishment in a new environment, and identifying genetic bottlenecks and poor trait selection problems associated with mass rearing efforts

(Meyers and Sabath 1981). Unruh and Goeden (1987), from studies of *Rhinocyllus conicus* Froelich, emphasized the importance of correctly matching insect biotypes, or host races, with target host plants. The failure to recognize potential hosts races in *R. conicus* in the 1960s has resulted in the undesirable attack of a number of nontarget *Cirsium* species (Turner *et al*. 1987).

Traditional taxonomic approaches used to identify *Aphthona* species in the past have included the use of such characters as size, color, markings, and the male aedeagus (Freude *et al*. 1966, Lopatin 1984). Flea beetle species such as *A. flava* can be distinguished easily from the other *Aphthona spe*cies encountered in this study because of their relatively large size and copper color. However, identification of other flea beetle species using morphological characters is much more difficult. Two of the brown species, *A. cyparissiae* and *A. nigriscutis*, are almost identical in color and size, and they commonly are found in similar habitats (often xeric) and on the same host plant species (commonly *E. cyparissias*). So close in appearance are *A. cyparissiae* and *A. nigriscutis* that when both species have been released at the same site, it is impossible to tell them apart without sacrificing individuals to examine the male aedeagus. Similarly, 2 of the black species, *A. czwalinae* and *A. lacertosa*, also are nearly identical in size and color and because they are not readily distinguishable, they are commonly considered as a mixed species population when they are redistributed in the United States (R. Hansen, APHIS-PPQ, personal communication).

Clinal variation in color, within- and between- site variability in size, and a typical slight to extremely female-biased sex ratio in the *Aphthona* species examined (e.g., 1:50 [male/female] in the *A. nigriscutis* population obtained from Batmonaster, Hungary [see Table 1] and 1:99 [male/female] in individuals *of A. nigriscutis* collected from an established population in Saskatchewan, Canada; N. Spencer, USDA-ARS, personal communication, 1996), make correct identification of the flea beetles at the species level difficult if not impossible. Hence, alternative taxonomic methods (i.e., using isozyme-allozyme analysis) were used to provide a more reliable method for identifying the *Aphthona species* considered in this research project.

In this study, isozyme analysis using starch gel electrophoresis was used to assess genetic differences among 4 flea beetle species collected from different *Euphorbia* species and different environmental types in Europe.

Materials and methods

Insect Collections. Insect and plant collection sites were located in Austria, Germany, Hungary, Italy, and Switzerland. *Aphthona* species used in this study were part of a larger study to characterize the habitat preferences of flea beetles and their associations with various *Euphorbia* species, levels of plant productivity, physical and chemical properties of the soil, micro- and macronutrients in the soil, micro- and macronutrients in the spurge foliage and roots, and 4 environmental types (ET) in Europe (R.M.N., Z.Z., D. Schroeder, and A. Gassmann, unpublished data). Environmental types were determined from the levels of plant productivity (i.e., the combined levels of cover of grasses, forbs,

Table 1. *Aphthona* species, population codes, sample size, site locations, host plants from which beetles were obtained, environmental types of the respective *Aphthona* collection sites, and sex ratio for the respective *Aphthona* species.

Species	Code	x[a]	Location	Host plant	Environmental type	Sex ratio (&&/%)[b]
Aphthona cyparissiae						
	AC1	61	Diesendorf, Austria	*Euphorbia cyparissias*	ET1	2.2
	AC2	32	Wurlma, Austria	*E. cyparissias*	ET2	–
	AC3	24	Naters, Switzerland	*E. cyparissias*	ET1	2.5
	AC4	24	Neuenberg, Germany	*E. cyparissias*	ET2	–
	AC5	33	Lobau, Austria	*E. cyparissias*	ET1	2.0
A. lacertosa						
	AL1	56	Okany, Hungary	*E. lucida*	ET4	1.3
	AL2	40	Korosszegapati, Hungary	*E. virgata*	ET3	2.6
	AL3	15	Marsolele, Hungary	*E. virgata*	ET3	1.5
	AL4	16	Komadi, Hungary	*E. virgata*	ET3	5.3
A. nigriscutis						
	AN1	25	Batmonaster, Hungary	*E. cyparissias*	ET1	50.0
	AN2	22	Lobau, Austria	*E. cyparissias*	ET1	–
A. flava						
	AF1	65	San Rossore (Pisa), Italy	*E. cyparissias*	ET2	–
	AF2	53	Bozeman, MT, USA	*E. esula*	ET2	–

AC, *Aphthona cyparissiae*; AL, *A. lacertosa*; AN, *A. nigriscutis*; and AF, *A. flava*. ET1, xeric site with 0-60% plant cover (grasses + forbs + spurge); ET2, mesic site with 61-80% plant cover; ET3, mesic site with 81-100% plant cover (no standing water or hygric indicator reeds present); ET4, hygric site with 81-100% plant cover (standing water always present and hygric indicator reeds present).

[a] Number of insects from each population used in the isozyme analysis for each of the 4 *Aphthona* species.

[b] Sex ratio for each *Aphthona* population over all sampling dates.

and spurge) found at each site, and whether or not standing water and hygric indicator reeds were present at the site. These were designated ET1 through ET4, with environmental types ET1, ET2, ET3, and ET4 containing 0-60, 61-80, 81-100% cover (no standing water or hygric indicator reeds present), and 81-100% cover (with standing water and hygric indicator reeds present), respectively. Although 7 flea beetle species in the genus *Aphthona* were obtained from sampling 6 *Euphorbia* species found in 1 or more of 4 environmental types from 17 different research sites, only 4 *Aphthona* species were collected in sufficient numbers, or were of sufficient size, to enable isozyme analysis. These included *A. cyparissiae, A. flava, A. lacertosa,* and *A. nigriscutis,* which also happen to be species that have become the most well established on leafy spurge in North America following classical biological control efforts. The *Aphthona* species were obtained from the following *Euphorbia* species: *E. cyparissias* L., *E. esula* L., *E. lucida* Waldstein-Wartemberg and Kitaibel, and *E. virgata* Waldstein-Wartemberg and Kitaibel. Information concerning the *Aphthona* species, population codes, geographic origin, host plant, environmental types, and sex ratios for the respective *Aphthona* species is presented in Table 1. Note that all of the collection sites, except for the Bozeman site, were in Europe. *A. flava* was obtained from *E. esula* near Bozeman, MT. This flea beetle population had

become established from releases made in 1987 from material collected from *E. cyparissias* near Pisa, Italy.

Flea beetle adults were aspirated from the spurge plants at the European field sites in May, June, and July 1992. Beetles were maintained on the particular spurge species from which they were collected in small insect cages until they could be processed at the International Institute of Biological Control Laboratory (IIBC), Delémont, Switzerland, for shipment to Montana State University. Adult beetles were packaged with spurge cuttings which were kept fresh by placement of the stems in hydrated flower cutting foam encased in clear pliable plastic and secured within the shipping container with wrapping tape to prevent movement and possible injury to the flea beetles. Shipping containers were sent to the Insect Quarantine Laboratory in Bozeman, MT, and upon receipt, flea beetles were frozen immediately at –80ºC for later isozyme analysis.

Electrophoretic Techniques. Electrophoresis was performed on horizontal starch gels using 12% hydrolyzed potato starch (#S-4501; Sigma, St. Louis, MO), and 500 ml gel buffer. Each individual beetle was ground in 4 drops of extraction buffer, with the supernatant being wicked into 3 filter paper wicks (5 by 10 mm), allowing each individual to be run on 3 different gels simultaneously. The power used for each gel was 10 V/cm with tris-citrate buffer pH 8.0 (TC 8.0), from Pasteur *et al*. (1988), 17.0 V/cm with a modified lithium buffer (LiOH) system from Vawter and Brussard (1975), and 13.5 V/cm for a tris-malate buffer pH 6.9 (TME 6.9) from Pasteur *et al*. (1988). Each gel was run for ≈5 hours, then sliced horizontally into 6 slices, each 1.6 mm in thickness. Each slice was subsequently stained for 1 of 13 enzyme systems. After screening the 13 enzyme systems on each of the 3 buffers, AAT, GP, ME, MPI, PEP, and SOD were run on LiOH gels; EST, GPI, HK, IDH, MDH, and XDH were run on TC 8.0 gels; and PGM was run on TME 6.9 gels. Enzyme systems, enzyme commission numbers, abbreviations, number of scorable loci for the 13 enzymes, and buffer systems are listed in Table 2. Specific buffer systems and staining recipes are available upon request from R.M.N.

Statistical Analysis. Allelic frequency data from the 4 *Aphthona* species (see *Appendix A*), was used in calculating Nei's genetic identity and Nei's genetic distance matrices (Table 3; Nei 1972). Nei's genetic distance (D_{ij}) is expressed as follows (Nei 1972):

$$D_{ij} = -\ln I_{ij} \tag{1}$$

$$\text{where, } I_{ij} = \left\{ \frac{\sum_k * x_{ki} x_{kj} *}{(\sum_k x^2_{ki} \sum_k x^2_{kj})^{1/2}} \right\} \tag{2}$$

and I_{ij} is called the genetic identity between populations i and j, which is a ratio of the proportions of loci that are alike within and between populations (Weir 1990). These calculations were performed for all pairwise combinations using the statistical software package NTSYS-PC (Rohlf 1993). Nei's genetic distance was clustered by the unweighted pair-group method using an arithmetic average to produce a dendrogram. Because the outcome of cluster analysis may be influenced by the particular algorithm chosen (Manly 1994), correspondence analysis also was conducted to provide a more objective approach for determining the genetic relationships of the flea beetle populations.

This was accomplished using a correspondence analysis ordination procedure of the allelic frequencies using the statistical software package CANOCO (Ter Braak 1988).

Table 2. Enzyme stains and buffer systems used in isozyme analysis of the 4 *Aphthona* species.

Enzyme system	Enzyme Commission no.	Abbreviation	No. loci[a]	Buffer[b]
Aspartate amino-transferase	2.6.1.1	AAT	1	LiOH
∀- and ∃-Esterase	3.1.1.1	EST	1	TC 8.0
General protein	——	GP	1	LiOH
Glucose phosphate isomerase	5.3.1.9	GPI	1	TC 8.0
Hexokinase	2.7.1.1	HK	1	TC 8.0
Isocitrate dehydrogenase (NADP)	1.1.1.42	IDH	2	TC 8.0
Malate dehydrogenase	1.1.1.37	MDH	2	TC 8.0
Malic enzyme	1.1.1.40	ME	1	LiOH
Mannose-6-phosphate isomerase	5.3.1.8	MPI	1	LiOH
Phosphoenolpyruvate carboxylase	4.1.1.31	PEP	1	LiOH
Phosphoglucomutase	2.7.5.1	PGM	1	TME 6.9
Superoxide dismutase	1.15.1.1	SOD	1	LiOH
Xanthine dehydrogenase	1.1.1.204	XDH	2	TC 8.0

[a] Number of bands per phenotype.
[b] Electrophoresis buffer.

Results

Cluster analysis of genetic distances resulted in a dendrogram, of the flea beetle populations which is shown in Fig. 1. A high degree of genetic similarity was found among 5 populations of *A. cyparissiae* (AC1, AC2, AC3, AC4, and AC5; see Table 1 for further explanation of the species population codes), 2 pairs of populations of *A. lacertosa* (AL1 and AL2, AL3 and AL4), and between the 2 populations of *A. flava* (AF1 and AF2). The consistent patterns in allelic frequencies obtained among individuals within populations for the respective flea beetle species suggested little sexual dimorphism in isozyme patterns. The lowest levels of genetic variability were found among populations of *A. cyparissiae* and between populations of *A. flava,* where populations were distinguished (within species) at a maximum Nei's genetic distance of 0.05 and 0.02, respectively (Fig. 1; Table 3). The highest degree of genetic variation between populations was found for *A. nigriscutis,* whose 2 populations were distinguished from each other at a Nei's genetic distance of 0.25. Populations of *A. lacertosa* were separated into 2 genetic groups, which were distinguished from each other at a Nei's genetic distance of 0.10.

Geographic distance did not appear to have any bearing on the levels of genetic variability found among populations within a species based on the 16 loci. For example, nearly identical Nei's genetic identities were found among the 5 populations of *A. cyparissiae,* although populations of this species were obtained from eastern Austria, Switzerland, and western Germany, and all populations were obtained from the same *Euphorbia* species (Tables 1 and 3).

Measurable degrees of genetic variability were found between populations for 2 of the *Aphthona* species sampled: *A. flava* populations AL1 and AL2, from populations AL3 and AL4, and *A. nigriscutis* population AN1 from AN2 (Fig. 1; Tables 1 and 3). Again, the degree of genetic difference did not seem to be related to geographic distance (e.g., populations AL1 and AL4 which showed some genetic divergence from each other were collected from sites only 40 km apart), or the *Euphorbia* species from which the beetles were collected (e.g., populations AL2 and AL4, which were both collected from *E. virgata,* showed almost as much genetic divergence from each other as populations AL1 and AL4, which were collected from *E. lucida* and *E. virgata,* respectively).

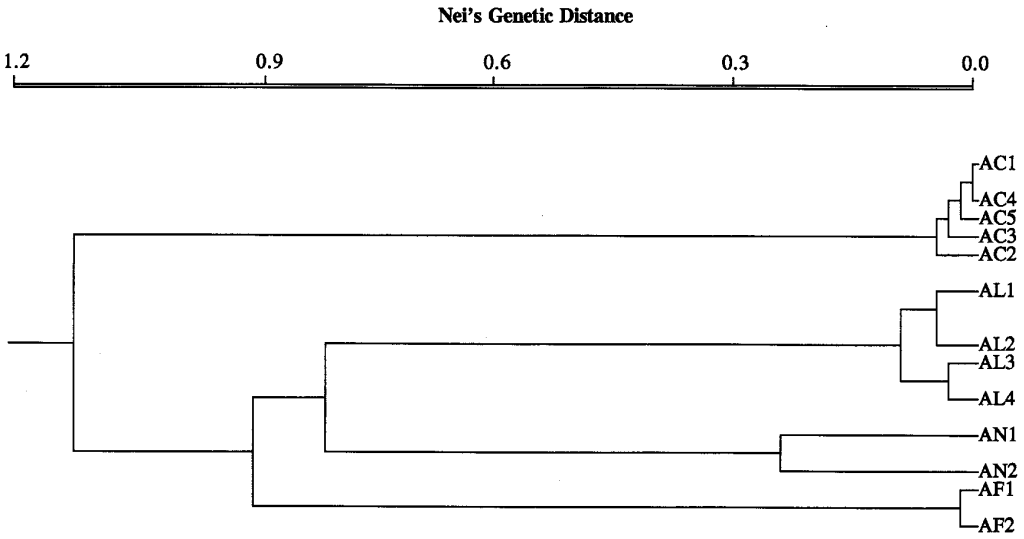

Fig. 1. Unweighted pair-group dendrogram of the *Aphthona* populations generated from Nei's (1972) genetic distance matrix. See Table 1 for explanation of *Aphthona* population codes. Software package NTSYS-PC (Rohlf 1993) was used to conduct the data analyses.

It is interesting to note that 2 of the *A. lacertosa* populations which showed a relatively high degree of genetic similarity (AL1 and AL2; Fig. 1) were collected from different *Euphorbia* species (*E. lucida* and *E. virgata,* respectively; Table 1), and from different environmental types (ET4 and ET3, respectively). The other populations showing a high degree of genetic similarity (AC1, AC2, AC3, AC4, and AC5; AF1 and AF2) were collected from either the same (*E. cyparissias* for populations ACI-AC5) or different *Euphorbia* species (*E. cyparissias* and *E. esula* for populations AF1 and AF2, respectively), and from either different or similar environmental types (ET1, ET2, ET1, ET2, and ET1, for populations AC1-AC5, respectively; ET2 for both populations AF1 and AF2; Table 1; Fig. 1).

The results of cluster analysis showed that the allelic frequencies obtained from the isozyme analysis were sufficient to distinguish among the 4 flea beetle species (Fig. 1). Based on the relative positions of the flea beetle populations within the genetic distance dendrogram, populations of *A. nigriscutis* and *A. lacertosa* were found to be more similar genetically (i.e., they shared more common alleles) than with populations of *A. cyparissiae* and *A. flava.*

At the species level, populations of *A. lacertosa* and *A. nigriscutis* were distinguished from each other at a Nei's genetic distance of 0.83, and populations of *A. lacertosa, A. nigriscutis,* and *A. flava* at a Nei's genetic distance of 0.92 (Fig. 1). *A. cyparissiae* was distinguished from the other 3 flea beetle species at a Nei's genetic distance of 1.15.

Based on the results of cluster analysis, the 2 most genetically divergent species were *A. cyparissiae* and *A. lacertosa.* This result was not unexpected, because these 2 species occur in different habitats and on different *Euphorbia* species. Two other species that showed a relatively high genetic distance were *A. cyparissiae* and *A. nigriscutis.* This result also might be expected because these 2 species often are found in the same environmental types (ET1 and ET2) and often on the same *Euphorbia* species (typically *E. cyparissias*). Hence, interspecific competition between these 2 species may select for such genetic divergence.

A correspondence analysis ordination of the allelic frequencies among the 4 *Aphthona* species is shown in Fig. 2. Populations from each of the 4 flea beetle species are represented by 4 distinct clusters, with a total of 91.1% of the variation explained by the 1st 3 coordinate axes. This result was consistent with that obtained for the 4 *Aphthona* species using cluster analysis.

Discussion

In general, the *Euphorbia* species and environmental types showed little relationship with the levels of genetic variability estimated among populations of the respective *Aphthona* species. Flea beetle species that were associated with similar environmental types (ET1 and ET2) and the same *Euphorbia* species, *E. cyparissias* (i.e., flea beetle species *A. cyparissiae* and *A. nigriscutis*), showed almost as great a genetic distance from each other

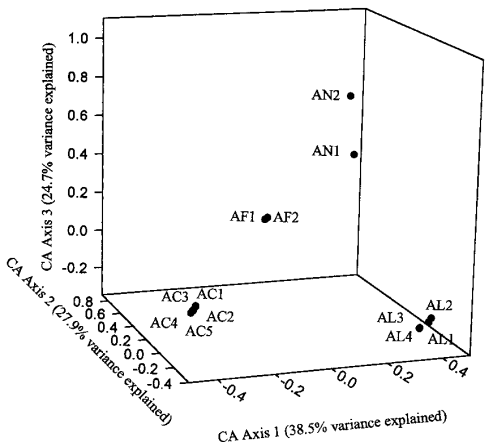

Fig. 2. Three-dimensional plot of correspondence analysis ordination of the *Aphthona* populations based on the frequency of allelic bands. Coordinate axis 1, axis 2, and axis 3 (eigenvalues) accounted for 38.5, 27.9, and 24.7%, respectively, of the variation in the data. See Table 1 for an explanation of the *Aphthona* population codes. Statistical package used: CANOCO (Ter Braak 1988).

as from another species (*A. lacertosa*), which was obtained from entirely different habitat types (i.e., ET3 and ET4) and from different spurge species (*E. lucida* and *E. virgata*), irrespective of the geographic distance of the collection sites. From an evolutionary standpoint, intraspecific competition, and interspecific competition for similar resources, may select for such genetic divergence (Bush and Hoy 1984, Feder *et al*. 1988, Smith 1988) and for such traits as temporal or seasonal asynchrony (Smith 1988, Hansen 1994), resource partitioning, oviposition behavior, and host selection (Zwölfer and Preiss 1983, Zwölfer 1988). This, in part, may explain our results.

Isozyme analysis using starch gel electrophoresis, and the analysis of genetic distance and allelic frequencies by cluster analysis and correspondence analysis, respectively, were found to be effective techniques for distinguishing between the 4 *Aphthona* species examined. The consistent patterns found between cluster analysis and correspondence analysis suggested that the unweighted pair-group method, based on genetic distance, was an appropriate approach to use for generating the dendrogram in this study.

Although measurable degrees of genetic variability were found between populations (within a species) for 2 of the 4 flea beetle species sampled, the resolving power of the isozyme technique for discerning individual populations or races within a species appeared to be somewhat limited. Some improvement in resolution power between populations might be obtained through the use of other genetic analysis techniques such as polyacrylamide gel electrophoresis (PAGE; J.E.B., unpublished data), or randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) approaches (McDonald and McDermott 1993, Haymer 1995).

The baseline isozyme data generated by the current study will be useful in the identification of these 4 *Aphthona* species in the future. Where multiple species have been released against leafy spurge in the United States and Canada, isozyme analysis will be helpful in estimating the relative abundances of the respective flea beetle species, when a release site may contain mixed brown species (e.g., *A. cyparissiae* and *A. nigriscutis*) or mixed black species (e.g., *A. lacertosa* and *A. czwalinae*). A check of the isozyme patterns also could help ensure that the correct flea beetle species is being released.

Isozyme analysis also could be helpful in identifying natural enemy populations for a given species that show a high degree of genetic similarity. Should natural enemy numbers be too low at one particular site to enable collection, alternative sites could be identified which could provide additional natural enemy material to use in classical biological control with hopefully a minimal risk of them showing different levels of host specificity.

The current study also presents evidence of at least 1 population within 2 of the 4 *Aphthona* species examined that stands out diagnostically, and may suggest the possible existence of biotype or host race differences between populations. The importance of such information in the context of biological control strategies and efficacy has been addressed in the literature (e.g., Berlocher 1979, Gonzalez *et al*. 1979, Bush and Hoy 1984, Goeden *et al*. 1985). These findings are of relevance to evolutionary biology as well as to biological control and systematics, because patterns over time in host race formation (or reduction) may be of primary significance to speciation processes (Bush 1969, Bush and Hoy 1984).

Table 3. Matrix of Nei's genetic identity (above diagonal lines) and Nei's genetic distance (below diagonal lines; Nei 1972) for populations of the 4 *Aphthona* species.

Population and location	Host plant	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *A. cyparissiae* Diesindorf, Austria	*E. cyparissias*	–	0.996	0.996	0.999	0.998	0.265	0.244	0.309	0.316	0.377	0.371	0.387	0.389
2. *A. cyparissiae* Wurlma, Austria	*E. cyparissias*	0.004	–	0.991	0.995	0.990	0.274	0.253	0.319	0.327	0.388	0.391	0.398	0.401
3. *A. cyparissiae* Naters, Switzerland	*E. cyparissias*	0.004	0.009	–	0.997	0.996	0.260	0.241	0.300	0.308	0.387	0.376	0.385	0.385
4. *A. cyparissiae* Neuenberg, Germany	*E. cyparissias*	0.001	0.005	0.003	–	0.998	0.263	0.244	0.303	0.311	0.384	0.374	0.389	0.390
5. *A. cyparissiae* Lobau, Austria	*E. cyparissias*	0.002	0.010	0.004	0.002	–	0.260	0.239	0.304	0.311	0.368	0.362	0.378	0.378
6. *A. lacertosa* Okany, Hungary	*E. lucida*	1.329	1.295	1.348	1.334	1.349	–	0.994	0.983	0.981	0.940	0.912	0.431	0.429
7. *A. lacertosa* Korosszegapati, Hungary	*E. virgata*	1.411	1.375	1.424	1.410	1.432	0.006	–	0.964	0.960	0.547	0.407	0.450	0.446
8. *A. lacertosa* Marsolele, Hungary	*E. virgata*	1.175	1.142	1.203	1.193	1.191	0.017	0.037	–	0.999	0.506	0.377	0.413	0.412
9. *A. 1acertoda* Komadi, Hungary	*E. virgata*	1.151	1.119	1.179	1.168	1.167	0.019	0.041	0.002	–	0.508	0.385	0.409	0.409
10. *A. nigriscutis* Batmonaster, Hungary	*E. cyparissias*	0.976	0.947	0.949	0.956	1.000	0.062	0.064	0.681	0.677	–	0.815	0.459	0.469
11. *A. nigriscutis* Lobau, Austria	*E. cyparissias*	0.992	0.938	0.979	0.984	1.016	0.092	0.898	0.976	0.955	0.205	–	0.319	0.332
12. *A. flava* San Rossore, Italy	*E. cyparissias*	0.950	0.921	0.954	0.944	0.974	0.841	0.799	0.885	0.894	0.778	1.142	–	0.992
13. *A. flava* Bozeman, MT	*E. esula*	0.9455	0.9127	0.9539	0.9406	0.9718	0.8461	0.8086	0.8865	0.8939	0.7562	1.102	0.0084	–

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Appendix A. Allelic frequencies for 16 loci from the 4 *Aphthona* species.

Locus	Allele	ACl[a]	AC2	AC3	AC4	AC5	AL1	AL2	AL3	AL4	AN1	AN2	AF1	AF2
AAT	1	1.00	0.84	1.00	1.00	0.97	–	–	–	–	0.92	–	0.03	–
	2	–	–	–	–	–	1.00	1.00	1.00	1.00	–	–	–	–
	3	–	–	–	–	–	–	–	–	–	–	–	0.58	0.75
	4	–	0.06	–	–	–	–	–	–	–	0.08	1.00	–	–
	5	–	0.10	–	–	0.03	–	–	–	–	–	–	–	–
	6	–	–	–	–	–	–	–	–	–	–	–	0.39	0.25
	n	61	32	24	24	33	56	40	15	16	25	22	65	53
EST	1	–	–	0.06	–	–	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	1.00	1.00	1.00	1.00	–	–	1.00	1.00
	3	1.00	1.00	0.88	1.00	1.00	–	–	–	–	–	–	–	–
	4	–	–	0.06	–	–	–	–	–	–	1.00	1.00	–	–
	n	25	16	17	16	22	8	8	8	8	4	9	24	24
GP	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	n	29	24	8	8	21	8	16	8	8	19	9	40	36
GPI	1	–	–	–	–	–	1.00	1.00	1.00	0.88	–	–	–	–
	2	–	–	–	–	–	–	–	–	–	–	–	0.05	–
	3	1.00	0.94	0.96	1.00	1.00	–	–	–	–	–	–	0.79	0.80
	4	–	–	–	–	–	–	–	–	–	1.00	1.00	–	0.07
	5	–	–	–	–	–	–	–	–	0.12	–	–	–	–
	6	–	–	0.04	–	–	–	–	–	–	–	–	0.16	–
	7	–	0.06	–	–	–	–	–	–	–	–	–	–	0.13
	n	60	32	24	24	33	50	40	15	16	23	22	44	30
HK	1	0.81	0.59	0.88	0.81	1.00	–	–	–	–	–	–	0.09	–
	2	0.05	0.13	–	0.06	–	–	–	–	–	1.00	1.00	0.82	1.00
	3	–	–	–	–	–	1.00	0.88	1.00	1.00	–	–	–	–
	4	0.14	0.28	0.12	0.13	–	–	–	–	–	–	–	0.09	–
	5	–	–	–	–	–	–	0.12	–	–	–	–	–	–
	n	37	32	16	16	22	8	8	8	8	4	9	22	20
IDH–1	1	–	0.03	–	–	–	1.00	1.00	0.92	0.87	1.00	–	1.00	1.00
	2	1.00	0.94	0.88	0.96	1.00	–	–	–	0.065	–	1.00	–	–
	3	–	0.03	0.12	–	–	–	–	0.08	0.065	–	–	–	–
	4	–	–	–	0.04	–	–	–	–	–	–	–	–	–
	n	60	32	24	24	33	56	40	12	15	25	22	65	53
IDH–2	1	0.98	0.94	0.96	1.00	1.00	–	–	–	–	–	–	0.73	0.83
	2	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	0.95	0.03	–
	3	0.02	0.06	0.04	–	–	–	–	–	–	–	0.05	0.24	0.17
	n	61	32	24	24	33	56	40	15	16	25	22	64	53
MDH–1	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	n	61	32	24	24	33	56	40	15	16	25	22	65	53

Locus	Allele	ACl[a]	AC2	AC3	AC4	AC5	AL1	AL2	AL3	AL4	AN1	AN2	AF1	AF2
MDH–2	1	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	–	–	–	1.00	1.00
	3	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	–	–
	n	61	32	24	24	33	54	40	15	16	25	22	65	53
ME	1	–	–	–	–	–	–	–	–	–	–	–	0.92	0.74
	2	1.00	1.00	0.92	0.92	1.00	0.50	0.31	1.00	1.00	–	–	0.04	0.09
	3	–	–	–	0.04	–	0.19	0.25	–	–	1.00	1.00	–	–
	4	–	–	0.04	–	–	–	–	–	–	–	–	0.04	0.17
	5	–	–	0.04	0.04	–	0.31	0.44	–	–	–	–	–	–
	n	52	32	24	24	33	16	16	7	8	25	22	52	43
MPI	1	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	–	1.00	1.00
	2	0.98	0.94	0.96	1.00	1.00	–	–	–	–	–	–	–	–
	3	0.02	0.06	0.04	–	–	–	–	–	–	–	1.00	–	–
	n	55	32	24	24	33	18	24	15	16	10	22	59	50
PEP	1	–	–	–	–	–	–	–	–	–	–	–	1.00	1.00
	2	0.95	1.00	1.00	1.00	1.00	–	–	–	–	0.96	0.82	–	–
	3	–	–	–	–	–	1.00	0.98	1.00	1.00	–	–	–	–
	4	0.03	–	–	–	–	–	–	–	–	0.04	0.18	–	–
	5	0.02	–	–	–	–	–	–	–	–	–	–	–	–
	6	–	–	–	–	–	–	0.02	–	–	–	–	–	–
	n	61	32	24	24	33	56	40	15	16	25	22	65	53
PGM	1	–	–	–	–	–	0.18	0.40	0.13	0.065	–	–	0.96	0.87
	2	0.98	0.97	0.88	0.96	1.00	0.50	0.33	0.80	0.80	–	–	0.02	0.02
	3	–	–	0.04	–	–	–	–	–	–	1.00	1.00	–	–
	4	0.02	0.03	0.04	–	–	0.23	0.27	0.07	0.065	–	–	0.02	0.11
	5	–	–	0.04	0.04	–	0.09	–	–	0.06	–	–	–	–
	n	43	32	24	24	.33	22	15	15	15	22	22	59	45
SOD	1	1.00	1.00	0.88	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.86	1.00	1.00
	2	–	–	0.12	–	0.03	–	–	–	–	–	0.14	–	–
	n	29	8	8	16	33	16	16	15	16	25	22	8	8
XDH–1	1	–	–	–	–	–	1.00	1.00	1.00	1.00	–	–	–	–
	2	1.00	1.00	1.00	1.00	1.00	–	–	–	–	1.00	1.00	1.00	1.00
	n	22	16	24	24	33	30	32	15	16	25	22	65	53
XDH–2	1	1.00	1.00	1.00	1.00	1.00	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	–	–	–	1.00	1.00
	3	–	–	–	–	–	1.00	1.00	1.00	1.00	1.00	1.00	–	–
	n	22	16	24	24	33	30	32	15	16	25	22	65	53

n, number of specimens analyzed for a given *Aphthona* population.

[a] Refer to Table 1 to determine the *Aphthona* species, population codes, sample size, collection site location, host plants from which beetles were obtained, environmental types of the respective *Aphthona* collection sites, and sex ratios for the respective *Aphthona* species.