Genetic and behavioral variability in the ovary-feeding Nitidulid Brachypterolus pulicarius collected from Dalmatian and yellow toadflax
by Kelly Lynn Hering

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Entomology
Montana State University

Abstract:
Braychypteroluspulicarius is an ovary-feeding beetle in the family Nitidulidae. The species is found on Dalmatian and yellow toadflax, two non-native, invasive weeds. The beetle is native to Eurasia and is considered an important natural enemy and biological control agent for toadflax. Because B. pulicarius is found, at varying densities, on both yellow toadflax and Dalmatian toadflax, questions have been raised about the potential existence of host races in the species. Amplified fragment length polymorphism (AFLP) molecular genetic techniques are commonly used in studies of population genetics. Because it is a relatively easy and reliable method that does not require previous knowledge about the beetles' genome, the AFLP technique was utilized to examine the patterns of variability of populations ofB. pulicarius. Patterns of observed variability that corresponded with commonality of host plant could serve as evidence for host races in B. pulicarius. Insects were collected from both yellow and Dalmatian toadflax at a total of 12 locations in the northwestern US, British Columbia, and Europe. Volatile collections were made from host plants to characterize their chemical emissions and to look for species-specific plant differences. Behavioral assays were attempted to determine if beetles showed a preference for the species of host plant from which they were collected. Volatile collections revealed variability in volatile production within and between host plant species. Behavioral trials were highly variable and preference results were not obtained. AFLP analyses revealed variation that did not correspond to host plant commonality. Overall, the study revealed the dynamic nature and a high level of uncertainty surrounding the fundamental knowledge of this biological system. No evidence was found for host race existence in B. pulicarius. Alternative explanations for the observed variabilities are discussed.

GENETIC AND BEHAVIORAL VARIABILITY IN THE OVARY-FEEDING NITIDULID *BRACHYPTEROLUS PULICARIUS* COLLECTED FROM DALMATIAN AND YELLOW TOADFLAX

by

Kelly Lynn Hering

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Entomology

MONTANA STATE UNIVERSITY
Bozeman, Montana

November 2002

APPROVAL

of a thesis submitted by

Kelly Lynn Hering

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Dr. David Weaver David t. Weaver Date 11/7/2002

Approved for the Department of Entomology

Dr. Greg Johnson [signature] Date 11-7-02

Approved for the College of Graduate Studies

Dr. Bruce McLeod Bruce R. McLeod Date 11-7-02

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature Kelly L Hing

Date 11/6/02

ACKNOWLEDGEMENTS

I would like to acknowledge the many people who made the completion of this thesis possible. Dr. Robert Nowierski, my original advisor, gave me the opportunity to come to Montana State University to work in biological control of weeds and on this project. Dr. David Weaver served as a dedicated committee member and 'adopted' me when Dr. Nowierski had an opportunity to move on in his career - for that I am extremely grateful. Dr. Bill Dyer sacrificed time and provided helpful insight as a committee member. Thank you to Bryan FitzGerald, who not only took me to the nicest places in Montana, but helped me through every step of my research and gave me countless, necessary 'nudges' of encouragement. Funding for this project was provided by the Blackfoot Challenge, the Bureau of Indian Affairs, and the Bureau of Land Management. The National Park service approved my requests for a collecting permit in Yellowstone National Park, giving me access to important study sites. Thanks also to all of the entomology faculty who were always eager to help when asked and to my fellow graduate students, past and present, for your truly invaluable humor, support, and shared suffering! (I wouldn't have made it without you guys.) These thanks would not be complete without mentioning my family who sent an endless supply of love and encouragement long-distance throughout this process. And finally, thank you to Chad for living with me during my thesis work and still liking me at the end.

TABLE OF CONTENTS

TABLE OF CONTENTS - CONTINUED

LIST OF TABLES

LIST OF TABLES - CONTINUED

LIST OF TABLES - CONTINUED

LIST OF FIGURES

LIST OF FIGURES - CONTINUED

LIST OF EQUATIONS

ABSTRACT

Braychypterolus pulicarius is an ovary-feeding beetle in the family Nitidulidae. The species is found on Dalmatian and yellow toadflax, two non-native, invasive weeds. The beetle is native to Eurasia and is considered an important natural enemy and biological control agent for toadflax. Because *B. pulicarius* is found, at varying densities, on both yellow toadflax and Dalmatian toadflax, questions have been raised about the potential existence of host races in the species. Amplified fragment length polymorphism (AFLP) molecular genetic techniques are commonly used in studies of population genetics. Because it is a relatively easy and reliable method that does not require previous knowledge about the beetles' genome, the AFLP technique was utilized to examine the patterns of variability of populations of *B. pulicarius*. Patterns of observed variability that corresponded with commonality of host plant could serve as evidence for host races in *B. pulicarius*. Insects were collected from both yellow and Dalmatian toadflax at a total of 12 locations in the northwestern US, British Columbia, and Europe. Volatile collections were made from host plants to characterize their chemical emissions and to look for species-specific plant differences. Behavioral assays were attempted to determine if beetles showed a preference for the species of host plant from which they were collected. Volatile collections revealed variability in volatile production within and between host plant species. Behavioral trials were highly variable and preference results were not obtained. AFLP analyses revealed variation that did not correspond to host plant commonality. Overall, the study revealed the dynamic nature and a high level of uncertainty surrounding the fundamental knowledge of this biological system. No evidence was found for host race existence in *B. pulicarius*. Alternative explanations for the observed variabilities are discussed.

CHAPTER 1

INTRODUCTION

Weeds

General Characteristics

Dalmatian and yellow toadflax are two common weeds in Montana and throughout the Western US and Canada (Coombs et al. 1996, Vujnovic and Wein 1996). Weeds, such as toadflaxes, are commonly defined as "any plant growing where it is not wanted (Hill 1977)" or an "unwanted or undesirable plant which interferes with the utilization of land and water resources and thus adversely affects human welfare (Rao 2000)." This broad definition allows for the classification of any plant as a weed under the particular circumstances that it is growing "out of place." Legally, noxious weeds are defined as "any plant designated by a particular federal, state, or county government to be injurous to public health, agriculture, recreation, wildlife, or any public or private property (Sheley et al. 1999)." In the United States and Canada, legislation has designated over 500 species of plants as noxious weeds (Lacey and Olson 1991).

If one considers the ecological characteristics of commonly occurring weed species, it becomes evident that weedy plants tend to share a variety of traits. Weeds are often "pioneer species" that commonly first colonize disturbed habitats (Taylor 1990). Weedy species tend to have rapid plant growth, with seed production beginning relatively early in the life cycle. Seed production continues over the entire duration of plant

growth, resulting in a large total output of seeds (Hill 1977). Often seed germination can occur in a wide variety of ecological conditions, and seeds remain viable in the soil over a long period of time. Generally, weedy species tend to be strong competitors for water and/or nutrients. Frequently in the United States the most invasive weeds are natives of Europe or Asia. Many were introduced intentionally, often as ornamentals. Others were brought to North America accidentally (Taylor 1990).

Importance

Weeds negatively impact humans' activities in a variety of ways. Infestations of weeds have large impacts on agriculture. They can decrease crop yield, lower land value, limit a producers' choices of which crops to grow, and decrease the quality of agricultural products. Weeds can also increase producers' control costs for pests that utilize the plants as secondary hosts. Weed infestations can clog waterways (Rao 2000). Some weeds are poisonous or harmful to livestock, or may simply taste bad, causing them to rarely be consumed. Often, weeds have undesirable physical traits – such as the existence of spines or thorns (Hill 1977). Due to all of their negative attributes, weed infestations can reduce the grazing capacity of rangeland by up to 75% (Sheley et al. 1999). Of total annual losses in agricultural production, weeds account for the largest percentage - roughly 45%, while insects account for 30%, diseases 20%, and other pests for the remaining 5% (Rao 2000).

Weed infestations also impact humans in other ways. Some plants cause allergies and can be poisonous if consumed (Rao 2000). Weeds have ecological impacts, often excluding native plants (Taylor 1990) and decreasing biodiversity (Lacey and Olson

1991). Invasive weed species can alter hydrologic, fire, and nutrient cycles, increase run-off and sedimentation, and change soil chemistry as well as displace important forages for wildlife (Cronk and Fuler 1995).

Through their many negative impacts, weedy species depreciate wildlife habitat, cropland, and rangeland. The combined effects of weed infestations are estimated to cost the United States at least $20 billion annually (Rao 2000).

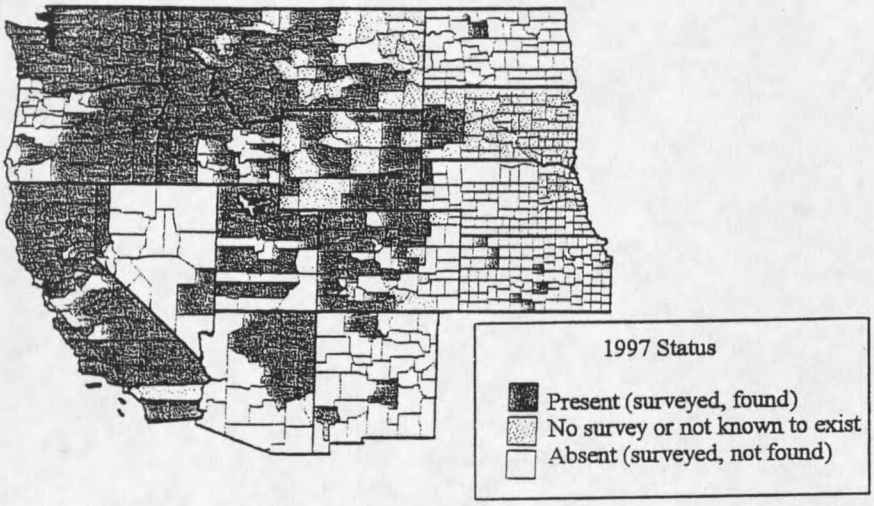
Dalmatian Toadflax

History & Biology

Linaria genistifolia ssp. *dalmatica* (L.) Maire & Petitmengin, commonly called Dalmatian toadflax or broad-leaved toadflax is considered an invasive weed in North America. The species is native to Mediterranean Europe and western Asia and has been cultivated there for over 400 years (Lajeunesse et al. 1993). Dalmatian toadflax originally came to North America in the late 1800's as an ornamental (Alex 1962, Nowierski 1996a, Lajeunesse 1999). However, over time the plant escaped cultivation and began to demonstrate its weedy characteristics throughout Canada and the United States (Alex 1962). As of the 1960's, Dalmatian toadflax was present in at least six Canadian provinces and 15 states in the US (Alex 1962). Currently, the heaviest infestations occur in the northwestern United States (Figure 1) and Canada (Lajeunesse 1999). In the provinces of Alberta and British Columbia, Dalmatian toadflax is considered a noxious weed (Vujnovic and Wein 1996). In the United States, Dalmatian

toadflax occurs much more commonly than its close relative, yellow toadflax, *Linaria vulgaris* Mill. (Lajeunesse 1999).

Figure 1. Distribution of *L. genistifolia* ssp. *dalmatica* in the Western US (from Lajeunesse 1999).



Dalmatian toadflax (Figure 2) is a "robust perennial herb with attractive yellow flowers and glaucous green foliage (Alex 1962)." It is a member of the family Scrophulariacae. Stems grow to 0.6-0.9 meters (2-3 feet) or taller (Lajeunesse 1999). Leaves are broad and heart-shaped and tend to wrap around the stem (Nowierski 1996a). The weed tolerates a variety of climatic ranges and soil types (Vujnovic and Wein 1996), but is usually found growing in xeric, open sites with sandy or rocky soils. It can also grow in loam soils and has even been observed growing, under cultivation, in heavy clay soil (Alex 1962). Dalmatian toadflax reproduces both by seed, with a single plant producing up to 400-500,000 seeds in a single growing season, and by vegetative root buds (Lange 1958, Nowierski 1996a). Flowers are bright yellow with an orange center, and are often referred to as resembling the blossoms of snapdragons (Lajeunesse 1999).

The blossoms are produced from May or June through September or October, with seed dispersal beginning as early as June and lasting through the winter (Lajeunesse et al. 1993).

Figure 2. *Linaria genistifolia* ssp. *dalmatica* (L.) Maire & Petitmengin. 1. Habit; 2. flower; 3. capsule; 4. seeds (from Vujnovic & Wein 1996).

Impacts

While many types of land are impacted by Dalmatian toadflax, the weed has its greatest impacts on rangeland and wildlife habitat (Lajeunesse 1999). Seedlings are poor competitors for water and nutrients, but once the plant is established it successfully outcompetes native plants and other more desirable forages (Nowierski 1996a). This often results in the loss of that forage's associated animal life as well (Lajeunesse 1999). Infestations of Dalmatian toadflax grow slowly but steadily over time (Lange 1958) and a single patch can easily persist up to 13 years (Lajeunesse et al. 1993). The plant contains chemicals including a glucoside antirrhinoside, a quinoline alkaloid, and penganin that reportedly make it toxic to livestock (Nowierski 1996a) and most grazing animals do not readily consume Dalmatian toadflax (Lange 1958). Infestations of the weed reduce the cattle carrying capacity of rangeland (Lajeunesse 1999) and decrease its overall productivity. An extensive root system and waxy leaves result in inconsistent efficacy of herbicide treatments (Lange 1958), making chemical control of Dalmatian toadflax very difficult (Nowierski 1996a).

Yellow Toadflax

History & Biology

Linaria vulgaris Mill., another member of the family Scrophulariacae, is commonly called yellow toadflax, common toadflax, or butter-and-eggs (McClay 1992, Nowierski 1996b). The plant is native to south central Eurasia and was introduced into New England in the late 1600's as an ornamental and folk remedy (Lajeunesse 1999). By

the late 1700's it was already being referred to as a weed by settlers (Mitich 1993). Yellow toadflax (Figure 3) is a herbaceous perennial and is widely dispersed in North America (McClay 1992). It occurs most commonly in the northeastern United States and southeastern Canada, and is localized in other parts of North America – especially Western Canada (Figure 4) (Lajeunesse 1999). It is said to now occur "throughout the continental United States" and in every Canadian province and territory (Saner et al. 1995). Because of its attractive snapdragon-like yellow and orange flowers yellow toadflax is still sold as an ornamental throughout the U.S. by gardening companies (Lajeunesse et al. 1993). The biology of yellow toadflax is similar to that of Dalmatian toadflax. Its morphology is different, though, with leaves that are narrow, pale green, alternating, and pointed at the end. Yellow toadflax has bright yellow and orange flowers, like those produced by Dalmatian toadflax (Lajeunesse 1999). Plants generally produce them from May until October (Lajeunesse et al. 1993). Yellow toadflax can occur in a wide variety of habitats and plant communities, but prefers mesic sites (Saner et al. 1995, Nowierski 1996b). The weed tends to have a very well-developed root system (Nowierski 1996b) and reproduces both by seed and vegetatively (McClay 1992, Nadeau et al. 1992). Seed production is highly variable (Saner et al. 1995), with a single plant producing up to 35,000 seeds per season (Nowierski 1996b). Germination rates, however, can be quite low (Nadeau and King 1991), often with rates less than 10% (Saner et al. 1995). Seedlings are considered to be poor competitors (Lajeunesse et al. 1993, Saner et al. 1995). Because of these factors, vegetative propagation is considered a key factor in yellow toadflax's ability to persist and spread locally (Bakshi and Coupland

1960, Arnold 1982, Saner et al. 1995). However, seeds are clearly important in its ability to infest new areas.

Figure 3. *Linaria vulgaris* Mill. A. Whole plant; B. flower; C. flowering-fruiting spike; D. seeds. Seedlings are similar to the root system regrowth shown in A (from Saner et al. (1995).

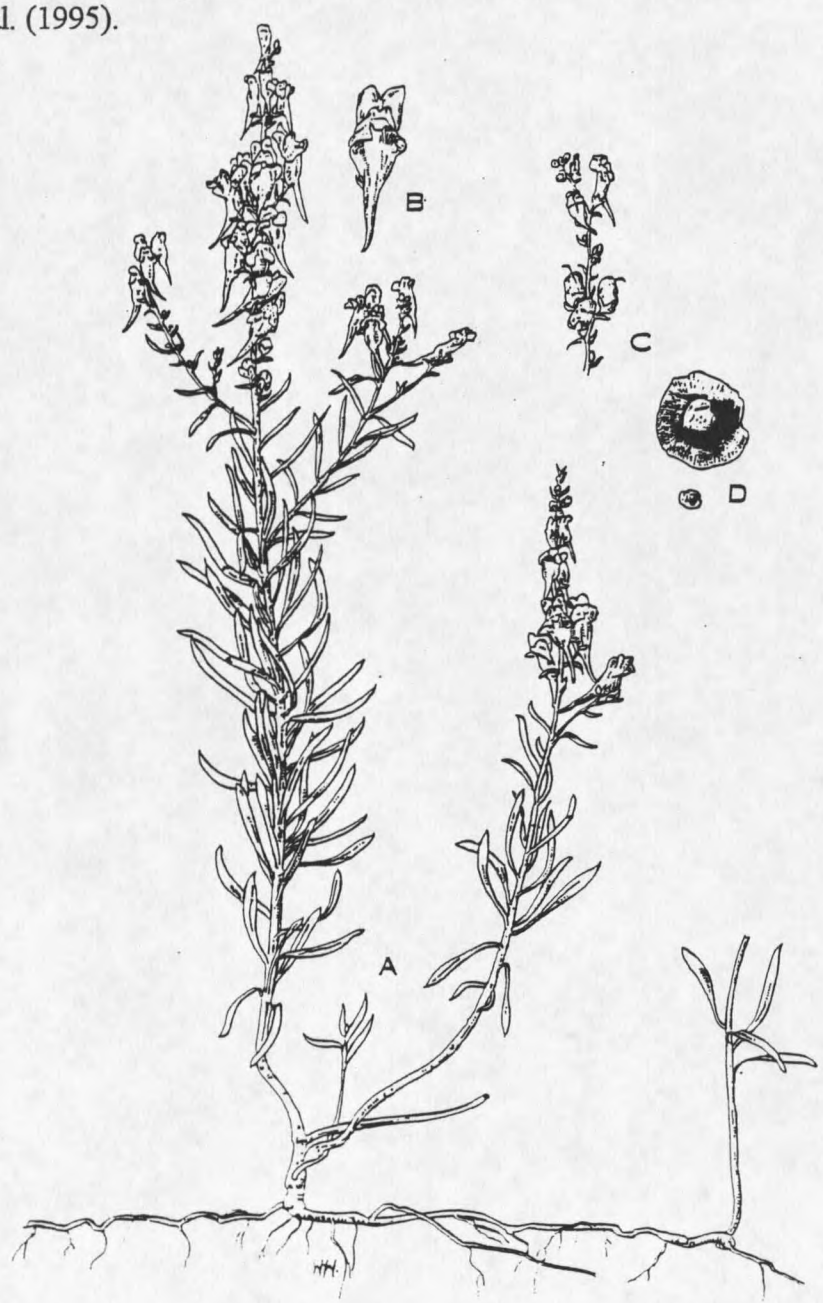
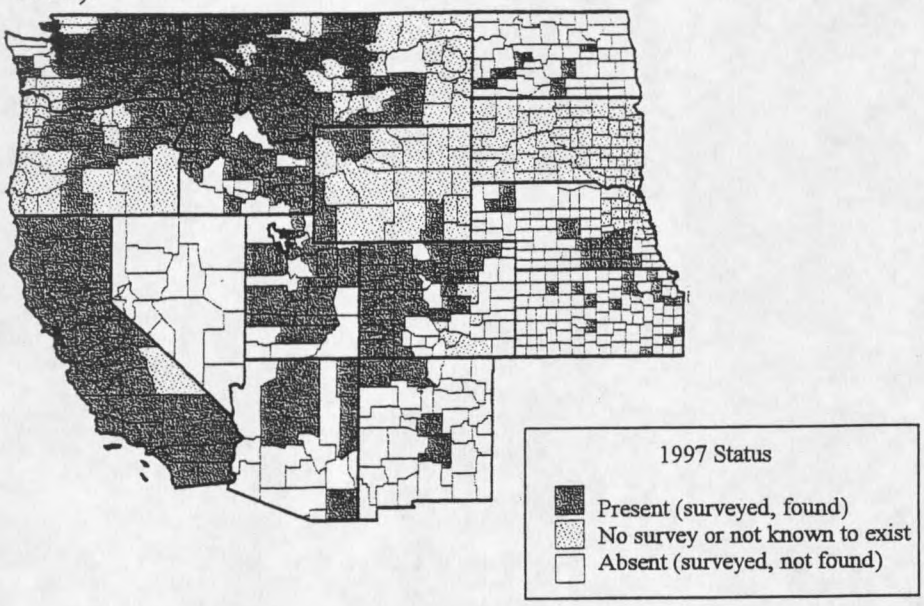


Figure 4. Distribution of *Linaria vulgaris* Mill. in the Western US (from Lajeunesse 1999).



Impacts

As is the case with Dalmatian toadflax, livestock do not readily utilize yellow toadflax as a forage (Nowierski 1996b). Occasional browsing of the weed by livestock and wildlife does occur but may actually facilitate its spread as seeds are not readily digested (Robocker 1970). Once established, the weed aggressively displaces desirable rangeland grasses (Nowierski 1996b). While both toadflax species negatively impact rangeland, yellow toadflax also has economic impacts in cultivated areas – especially when no-till or reduced tillage methods are utilized (Lajeunesse 1999). Yellow toadflax causes ecological damage by out-competing native plants (Nowierski 1996b). Additionally, the root system of the weed has been discovered to serve as an over-wintering site for cucumber mosaic virus and broad bean wilt virus – two economically important crop pests (Rist and Lorbeer 1989). Once this plant is established it is extremely hard to eradicate (Lajeunesse et al. 1993).

Weed Control

Methods

One of the best methods for controlling both Dalmatian and yellow toadflax is prevention. Good range management, including timing of grazing and encouraging competitive, desirable species can prevent toadflax seedlings from becoming established (Lajeunesse 1999). Controlling established toadflax infestations is very difficult. For even small infestations, hand-pulling of plants must be repeated for five or six years to deplete the root reserves. Seedlings can continue to sprout for ten to fifteen years, so each year the site must be re-visited and seedlings must be pulled annually. Using cultivation as a control is effective, but requires repetitions every seven to ten days for at least two years (Lajeunesse et al. 1993). Mowing and burning are ineffective on established toadflax stands (Lajeunesse 1999). The effectiveness of chemical control of Dalmatian and yellow toadflax is highly variable (Lajeunesse et al. 1993), and may not be economically feasible on lower economic value or "marginal" lands. After the weed has been effectively controlled, it is necessary to re-seed or otherwise re-vegetate to prevent re-infestation by toadflax or another undesirable species (Lajeunesse 1999).

Biological Control

As discussed previously, most terrestrial noxious weeds found in the United States originated in Europe or Asia. Frequently, when they were introduced to the U.S. they came without their natural enemies (Lacey and Olson 1991). This lack of pressure from such natural controls is thought to allow weeds to out-compete native plants

(Wilson and McCaffrey 1999). Biological control, or biocontrol, is "the deliberate introduction or manipulation of a pest's natural enemies, with the goal of suppressing the pest population" (Wilson and McCaffrey 1999). Biocontrol utilizes natural enemies including insects, nematodes, mites, plant pathogens, and vertebrates (Rees et al. 1996), and has been used against invertebrates, vertebrates, plant pathogens, and weeds (Wilson and McCaffrey 1999). The goal of biological control is not eradication of a pest species. Rather, bio-control attempts to introduce a new pressure on the pest that will effectively reduce its dominance in the ecosystem (Wilson and McCaffrey 1999). Weed bio-control has many advantages over more conventional weed control techniques. Once established, biological control is "self-perpetuating" and therefore more cost-effective than chemical controls. Also, biological methods are often considered more ecologically sound and more well-suited for integration with other weed control methods than more conventional controls (Wilson and McCaffrey 1999). However, there are also limits to the success of biological control efforts. The amount of damage (and therefore control) depends on the population density of the agents, as well as the condition of the plant and its relative ability to compete in its environment (Rao 2000). In addition, the long-term effects of biological control require patience, and more immediate control may be desired or necessary (Wilson and McCaffrey 1999). The greatest risks involved in using biological control concern the introduced agents' potential to use host plants that are not the desired target. Much past and current discussion has focused on these potential non-target effects. Well-known examples of detrimental non-target effects include the use of native *Cirsium* species by the introduced weevil, *Rhinocyllus conicus* (Unruh and Goeden

1987, Louda et al. 1997, Strong 1997), as well as others. In the current regulatory landscape, such potential for non-target effects is minimized through especially stringent host-specificity testing, and through cautious selection and continued monitoring of organisms being considered as biological control agents (Wilson and McCaffrey 1999).

Brachypterolus pulicarius

History

Brachypterolus pulicarius (L.) (Figure 5) is an ovary-feeding beetle in the family Nitidulidae and is native to Europe (Hervey 1927). It is a natural enemy of both species of toadflax (Coombs et al. 1996). The beetle arrived in North America accidentally, having been transported along with its host plants. The species was first described by Linneaus in 1758 as *Dermestis pulicarius*. In 1788 he changed the generic name to Silpha. Audisio (1993) provides a current, complete list of synonymy for the species. *B. pulicarius* was first recorded in the U.S. around 1919 in New York (Hervey 1927, Coombs et al. 1996) and in Canada in 1953 (Vujnovic and Wein 1996). The beetle is well established on yellow toadflax infestations throughout North America and also appears, although less frequently, on Dalmatian toadflax (Coombs et al. 1996). Hervey (1927) reported that *B. pulicarius* was known to reproduce only on *L. vulgaris* but observed individual beetles on the blossoms of strawberry (*Frangaria x ananassa* Duchesne), dandelion (*Taraxacum* Weber sect. *ruderalia* sp. Kirschner), wild mustard (*Sinapis arvensis* L. Brassica Kaber (D.C.) L.C. Wheeler var. *pinnatifida* (Stokes) L.C. Wheeler), clover (*Trifolium* sp.), apple (*Malus sylvestris* (L.) Mill.), and dogwood

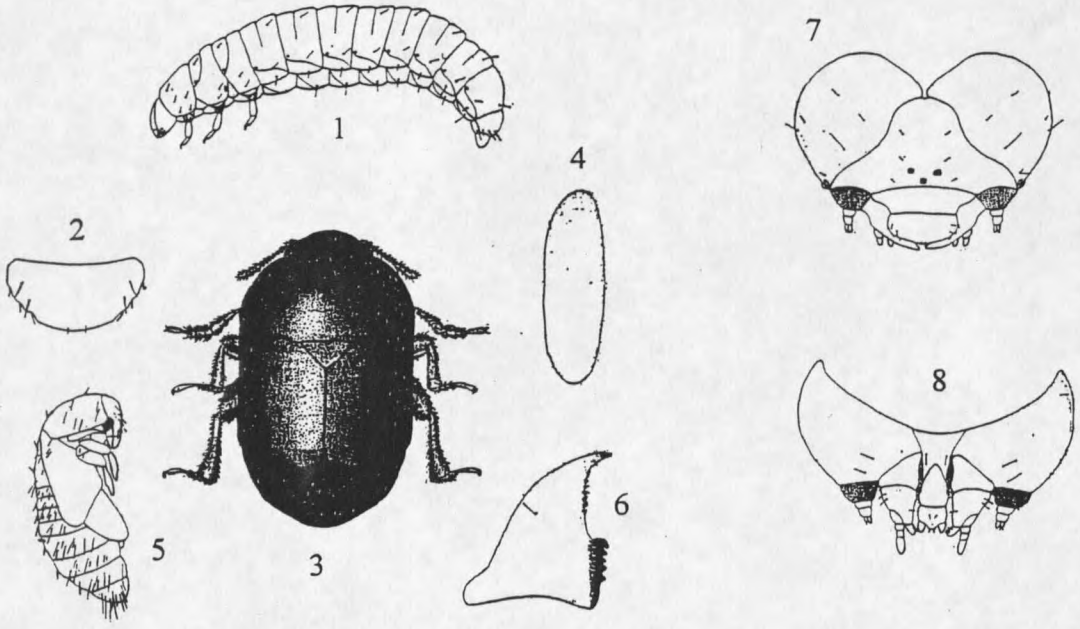
(*Cornus* sp.). *B. pulicarius* has also been collected from the blossoms of *Linaria supina*, *L. striata*, *Galium molugo*, and *Spireae ulmaria* in Europe (Hervey 1927). In 1959, Smith reported that the beetles were found developing only on *L. vulgaris* Mill. in a garden in Alberta containing other Scrophulariaceae including *L. genistifolia* ssp. *dalmatica* (L.) Maire & Petitmengin. He also reports, however, that in Saskatchewan *B. pulicarius* were found reproducing on Dalmatian toadflax in an area where it was well isolated from yellow toadflax (Smith 1959)

Biology

Hervey gave the following description of *B. pulicarius* adults (1927). "Form oval, strongly convex and sparsely clothed with brownish hairs. Color black and partly shining above; legs and antenna rufous; first segment of antenna darker than remainder; posterior legs usually somewhat darker than the others. Dorsal surface of body deeply and thickly punctate; punctures on head and dorsal surface of abdominal segments somewhat finer. Posterior half of sides of scutellum smooth shining and impunctate. Head about half as wide as thorax. Antenna sub-capitate; club elongate; joints one and two subequal, globular; three elongate and subequal to four and five. Thorax convex, about two thirds wider than long; sides parallel at base, strongly arcuate towards apex; apex strongly emarginate, angles acute; posterior angles rectangular; base trisinuate. Elytra one-third longer than thorax; apices rounded and separated. Two abdominal segments exposed dorsally, female; three, male. Abdominal segments two and three, ventrally, very short, not equal to fourth; fourth longest. Middle and posterior legs flattened; tibiae dilated at tip and crowned with a row of equal spines; outer margin of

tibiae of anterior and middle legs with a row of spinules. Length 2.2 – 2.6 mm. Width 1.0-1.2 mm."

Figure 5. *Brachypterolus pulicarius*. 1. Larva, 2. labrum of larva, 3. adult, 4. egg, 5. pupa, 6. mandible of larva, 7. dorsal aspect of head of larva, 8. ventral aspect of head of larva (from Hervey 1927).



Adult beetles feed on the growing shoots of the plant, and eggs are laid in the developing flower heads. Larvae feed within the flower heads on pollen, anthers, ovaries, and older larvae consume the maturing seeds (Harris 1961, Coombs et al. 1996). A single larva can easily move from one flower to another, destroying several during its development (Harris 1961). Harris stated that adult beetles disappear in early August in southern Ontario but are said to be present in the prairie provinces through the fall until freeze-up. He states that "no satisfactory explanation for the difference in the life cycles in southern Ontario and the prairies has been found (Harris 1961)." Pupation occurs in

the soil, and most of the literature indicates that the pupa is the over-wintering stage (Harris 1961, Coombs et al. 1996, Grubb et al. 2002). Because at the completion of his study most of the remaining immatures (68%) were pupae, Harris (1961) that the pupa was likely the over-wintering stage. Hervey (1927), however, commented that historical accounts of the species by Kaltenbach in 1874 indicated that the beetles "transform in the soil around the plant and emerge as adults in September." He also stated that in New York, "during September adults, presumably of the new generation were very numerous in blossoms," suggesting that *B. pulicarius* individuals over-winter as adults. Field observations and attempts to locate pupae in the soil near the plants in the early spring were unsuccessful, further supporting the contention that over-wintering may occur in the adult stage (personal observation). Pupae and pre-pupae were located in the top 50-75 cm. (2-3 inches) of soil at the end of August, 2002. However, as no comprehensive life history studies have been completed for the species the over-wintering form cannot be definitively stated. Also, confusion over species names makes historical accounts questionable. Both *Brachypterolus cinereus* (Heer) and *B. linariae* (Stephens) were at one time considered sub-species of *B. pulicarius* (Audisio 1993). Four other species in the genus, *B. antirrhini* (Murray) , *B. longulus* (Reitter), *B. cinereus* (Heer), and *B. linariae* (Stephens), have also been reported on *Linaria* sp., further increasing the potential for confusion in relating historical biological observations to the current species (Audisio 1993).

Effects/Importance

In 1961 in Saskatchewan, yellow toadflax produced an estimated average 5,584 seeds per flowering stem (Harris 1961). *Brachypterolus pulicarius* was considered a primary herbivore of yellow toadflax in Canada, and since 1953 the beetle had been present in every province (Harris 1961). The extent of impact by *B. pulicarius* on infestations of yellow toadflax is not fully understood. Smith (1959) reported that in area where the beetle was very common, the weed continued to expand and cause economic damage. Darwent et al (1975) also noted an increase in toadflax density in the presence of the *B. pulicarius*, but did report a decrease in per plant seed production. According to Coombs (1996) *B. pulicarius* causes increased secondary branching in its hosts. In a controlled greenhouse experiment the insect delayed and suppressed early season flowering, and reduced seed number, size (weight), and viability in yellow toadflax (McClay 1992). According to Harris (1961), infestations of *B. pulicarius* had the effect of "greatly reducing seed production and decreasing the vigor of the plants," allowing more effective control by competition from introduced grasses and by tillage techniques. In a study involving Dalmatian toadflax, the beetles had the effect of reducing plant height, increasing primary and secondary branching, causing fewer flowers to be produced, and reducing seed production (Grubb et al. 2002). However in all experiments, trials were terminated at the beginning of September, while it is known that in the field toadflax will continue to produce seed through mid-September (Robocker 1970) or October (Smith 1959). It is possible that once the beetles have completed their life cycle, the plants may be able to compensate for the observed effects of herbivory.

Because of this potential compensation, the conclusions made in studies with early fall termination dates may not provide an understanding of the late-season and overall effects of beetle infestation.

Phenology

As discussed previously, yellow and Dalmatian toadflax exhibit very similar biologies. This similarity in biology may be an important explanation for why *B. pulicarius* individuals are able to utilize the flowers of both hosts for reproduction. Across the whole range of toadflax infestations the flowering periods of the two species overlap. The ability of both toadflaxes to tolerate a wide variety of soil, climate, and plant community conditions (Vujnovic and Wein 1996, Saner et al. 1995) and to infest locations from sea level up to 2800 meters in elevation (Alex 1962, Saner et al. 1995) can help to explain the overall variability in flowering period. The literature often includes a large range of time over which flowering occurs for each species. For Dalmatian toadflax this range has been stated as from June through late fall in Washington (Vujnovic and Wein 1996), and midsummer through late fall (Lajeunesse et al. 1993) or June through September or October for Montana (Lajeunesse 1999). Lajeunesse et al. (1993) and Lajeunesse (1999) mention that flowering may begin sooner if the weather is warm. For yellow toadflax, the flowering is said to be highly variable depending on environmental conditions (Saner et al. 1995). The literature gives a range of time similar to that of Dalmatian toadflax lasting from May until October in Montana (Lajeunesse et al. 1993) (Lajeunesse 1999), or beginning in mid July with peak at the end of the month and lasting up through October in Canada and Germany (Saner et al. 1995). Plants are

not completely synchronized at a site, a trait which may have developed as a means to avoid complete loss of seeds to herbivores (Saner et al. 1995). Because of this, at any given time during the growing season plants in all stages from pre- to post-flowering can usually be observed at a site (personal observation). During the beetle collection period lasting from July 21-29, 1999 yellow toadflax was observed, throughout Montana, in various stages including not yet flowering to having nearly completed its flowering. Most plants at the sites were just beginning to flower and had not yet reached peak bloom. Dalmatian plants showed a similar range of phenology but most plants at each site were either at peak flowering or had nearly finished producing flowers (personal observations). These observations show a trend during late July of yellow toadflax generally being much less advanced in its flower production stage than Dalmatian toadflax. Overall, though, all stages of flowering were observed for both species during this time period. This observation is consistent with the time range for flowering provided (over a large spatial and temporal scale) in the literature.

On a smaller spatial/temporal scale, however, the periodicity of phenological changes related to flowering in the two species can be more distinct. While yellow toadflax tolerates a variety of habitats, it is generally limited to more moist conditions (Saner et al. 1995). Dalmatian toadflax, in contrast, tends to grown in open, sunny, dry sites (Vujnovic and Wein 1996). Because of this difference in site preference, it is rare to find the two species growing together, or sympatrically. This makes phenological comparisons more difficult. In a survey of the study area, over 40 toadflax sites were visited, of which only three sites were located where the two species were growing

sympatrically. These locations were near Boulder, MT (not included in analyses), near Townsend, MT (sites #1 and #2 in analyses), and in Yellowstone National Park, WY (not included in analyses). At the Boulder and Townsend locations, yellow toadflax was found growing in a clearly more shaded and potentially more damp portion of the site (personal observation). At Yellowstone, no significant small-scale difference was observed for the two species' locations at the site. The sites at Boulder and Townsend were visited two years in a row (between July 21 and July 23) and during all four visits Dalmatian toadflax was at a much more advanced stage of flowering than yellow toadflax (personal observation). At Yellowstone Park only an early season visit in 2002 (July 9) showed a difference in flowering stage, with yellow toadflax having not yet begun to flower while Dalmatian was at full-flowering stage. During later season visits (July 25, 2000 and August 26, 1999) both species were at similar stages of full or late flowering (personal observation). For the Boulder and Townsend sites the micro-climate difference provided by increased shade and water availability may have accounted for the observed phenology difference for the two toadflax species. This difference in flowering periodicity may prove to be an important factor in host plant use by *B. pulicarius*. During most visits to sympatric sites beetles were found on both host species. However, in the first visit to the Boulder site (July, 22 1999) yellow toadflax was not yet flowering and the Dalmatian toadflax had nearly completed its flowering. During this visit beetles were only found on the yellow toadflax. The cause of this apparent preference difference is not known, but it is possible that advanced Dalmatian toadflax plants do not provide adequate food and/or oviposition sites for the beetles. Further studies into host use by the

beetles may reveal seasonal differences in the relative suitability of the two host plant species. This host suitability difference may, potentially, provide selection pressure for synchronizing emergence and maturation timing of *B. pulicarius* individuals to their host plants' most suitable stages. This difference in emergence phenology could result in reproductive isolation (Craig et al. 1993), an important step in the development of host races. For *B. pulicarius*, a group of beetles' movement toward synchronization with one toadflax species' phenology could result in reproductive isolation from beetles more synchronized to the other host species. Such a scenario could potentially lead to host race development in *B. pulicarius*.

Host Races

Definition

Many definitions of a host race exist. According to Narang (1994) "A race is composed of groups of individuals or populations which differ from other groups within formally recognizable subspecies by virtue of distinct allozyme frequencies, features of chromosome structure, or some biological characteristics. A host race shows a preference for a specific host plant...that differs from other races." Craig et al. (1993) stated that host races are incompletely reproductively isolated. They argue that the definition of a host race is often too narrow. Diehl and Bush (1984) give a broader definition of a host race as "a population of a species that is partially reproductively isolated from other conspecific populations as a direct consequence of adaptation to a specific host." They argue that a population's possession of a trait such as emergence

timing corresponding with the phenology of its host, as discussed above, is sufficient to characterize that population as a host race (Diehl and Bush 1984, Craig et al. 1993). For the purpose of this study host races are defined as populations that posses a trait or traits that adapt them to, or are maintained as a result of adaptation to, a particular host species. Host races are potentially detectable through studies of the patterns of their genetic variability and/or behavioral preferences for a host plant.

Because *B. pulicarius* is an accidentally-introduced biological control agent, it has not undergone host-specificity testing. As discussed previously, even the basic biology is not clearly understood. Because of this, host range, suitability, and preferences are not known. Hervey (1927) stated that the beetle only reproduces on *L. vulgaris* while Smith (1959) reported it reproducing on Dalmatian toadflax in the absence of yellow toadflax. Because the beetle reproduces on two separate host plant species, researchers have hypothesized that the species actually consists of two distinct host races (Grubb 1998). Some observations tend to support the contention of a host species preference and potential existence of host races in *B. pulicarius*. Particularly, Smith (1959) observed that the species reproduced only on yellow toadflax in a garden also containing Dalmatian toadflax perhaps indicating a preference for yellow toadflax. The possible preference for one host plant over the other can be an important step in the formation of host races (Bush and Smith 1998). The observation that *B. pulicarius* tends to occur more commonly and in greater densities on yellow toadflax than on Dalmatian toadflax (Coombs et al. 1996), however, could be argued to contradict the idea of host races. Instead, it could suggest that Dalmatian toadflax is perhaps a secondary or less suitable

host, the use of which results in the observed relatively lower success of resident *B. pulicarius* populations.

Importance

The existence of host races has important implications for bio-control programs. Where host races are present, the matching of correct species of a biological control agent to its correct host species may not ensure success. That is, if host races are present in *B. pulicarius*, simply collecting the beetles from one host plant for control of either toadflax species may not be effective. Rather, biocontrol researchers must seek to match the correct host race of that species to its host (Narang et al. 1994). In this system, beetles collected from one species of toadflax might not be adapted to, and therefore would not prove to be effective control agents for the other species of toadflax. Beyond efficacy, host races have implications for biocontrol safety. Researchers may not be able to assume all members of a species of insect will constitute host-specific biocontrol agents if only a single host race has been tested during host-specificity testing. Failure to recognize this important issue in biological control can have serious negative consequences. The weevil, *Rhinocillus conicus* is an intentionally introduced biocontrol agent for the control of non-native thistles. Since release, however, its attack of native thistles has been the focus of much concern. Unruh and Goeden (1987) discuss how only a single host race of the weevil, has shifted from its desired host. Had only a single host race been utilized, non-target impacts could have been minimized. They argue that this fact "supports the contention that host races are of practical importance in biological control."

Molecular Genetics

Previous studies

In 1998, Grubb carried out isozyme analyses of individuals from nine North American populations of *B. pulicarius.* When isozyme results were analyzed via cluster analysis of genetic distance measures, populations collected from Dalmatian toadflax consistently grouped together (Grubb 1998). Yellow toadflax populations formed a second cluster, with the exception of one site that clustered nearest to the Dalmatian sites. The observed differences led Grubb (1998) to suggest that host races may exist in *B. pulicarius.* However, the analyses of the isozyme data did not take into account geographic distance between sites. Because all Dalmatian toadflax sites were collected from the Kamloops, British Columbia area while the yellow toadflax sites were from various parts of Montana, it is easy to conceive of a situation where geographic isolation could play a major role in the observed genetic distance and clustering of the nine toadflax sites. Nonetheless, Grubb's (1998) study supports the contention that host races exist in *B. pulicarius* and encourages further analyses of the variation in the species.

Amplified Fragment Length Polymorphisms

DNA extraction

Genetic data can reveal levels of divergence between potential host races. The first step in any genetic analysis is to determine a suitable technique for the extraction of DNA. According to Reineke et al. (1998), "the suitability of a DNA isolation method

depends on the DNA source because of differences in interfering substances present in biological material," and the resulting quality of the DNA obtained will depend on the extraction protocol that is used. In their study, Reineke et al. (1998) compared six different extraction techniques and analyzed them for quantity and quality of DNA. They used these measurements to determine the relative suitability of the various techniques for amplified fragment length polymorphism (AFLP) analysis. They established that three of the techniques yielded DNA of sufficient quantity and quality for AFLP analysis. In a later study, Reineke et al. (1999) utilized one of these techniques to extract DNA from the gypsy moth *Lymantria dispar* for AFLP analysis. A technique similar to one of the preferred methods was developed for use with small arthropods by Brian Farrell at Harvard University (Farrell 1999). This extraction protocol holds promise for use with the AFLP technique to examine patters of polymorphism in *B. pulicarius.*

AFLPs

The AFLP protocol was developed by Vos et al. (1995). The technique is based on "the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al. 1995)." According to Suazo and Hall (1999), the technique has grown in popularity because it detects a high amount of polymorphism and is easily reproduced. Also, AFLPs are "relatively cheap, easy, fast, and reliable (Mueller and Wolfenbarger 1999)," and the analysis does not require the researcher to have previous knowledge of DNA sequences (Reineke et al. 1999). In addition, the AFLP technique requires a very small amount of template DNA, allowing analysis of very small individuals (Mueller and Wolfenbarger 1999, Katiyar et al. 2000). Thus far, AFLPs have

been utilized in the analysis of bacteria (Janssen et al. 1996), fungi (Boucias et al. 2000, Cilliers et al. 2000, Vandemark et al. 2000), nematodes (Sharma et al. 1996), vertebrates (Herbergs et al. 1999, Knorr et al. 1999), plants (Cho et al. 1996, Mueller and Wolfenbarger 1999, Keiper and McConchie 2000, Shim and Jorgensen 2000, Zhang et al. 2000, Jakse et al. 2001, Koopman et al. 2001) and arthropods, including members of the Lepidoptera (McMichael and Pahley Prowell 1999, Reineke et al. 1999, Tan et al. 2001), Diptera (Yan et al. 1999, Katiyar et al. 2000), Hymenoptera (Suazo and Hall 1999), Homoptera (Forneck et al. 2000), Hemiptera (Cervera et al. 2000), Odonata (Wong et al. 2001), and Coleoptera (Hawthorne 2001). The AFLP technique is considered a "robust and reliable (Reineke and Karlovsky 2000)," "established protocol (Suazo and Hall 1999)." AFLP markers are useful for genetic "fingerprinting, mapping, and studying genetic relationships among organisms (Suazo and Hall 1999)." They have been most widely used to examine genetic variability within species, and are frequently utilized for investigations of population genetics (Mueller and Wolfenbarger 1999). Because of this, the AFLP technique holds promise for the investigation of host races in *B. pulicarius*.

Behavioral Assays

Previous Studies

In an experiment by Gotoh et al. (1993), spider mite host races were demonstrated using both genetic and behavioral evidence. According to the researchers, behavioral data alone provided "the first evidence" for host races, and together with electrophoretic data, their study showed that host races exist in the spider mite.

Volatile Collections

In order for *B. pulicarius* individuals to selectively orient toward one host plant versus the other, the two host plant species must have some sort of biological difference that the beetles can utilize in order to distinguish between them. Many different insects, including nitidulid beetles in the genus *Glishrochilus*, have been shown to orient to their hosts using olfactory cues (Bernays and Chapman 1994). Plants produce a range of volatile compounds, mostly as the result of the breakdown of leaf lipids (Bernays and Chapman 1994). These volatile compounds are often called green leaf chemicals, green leaf volatiles, or green odor volatiles (Bernays and Chapman 1994). The emission of these compounds is generally highly variable and depends on a number of factors, including temporal, seasonal, environmental, and genetic variation (Bernays and Chapman 1994). "No two plants are chemically identical (Bernays and Chapman 1994)," and it has been shown that some plants produce species-specific blends of green leaf volatiles (Bernays and Chapman 1994). If olfactory cues are utilized by *B .pulicarius* individuals in their effort to locate a preferred host plant, it would be necessary for the two host plant species to emit species-specific olfactory clues. Such a species-specific difference in plant volatiles and the resulting insect preference for one host over another are important steps toward the development of host races (Bernays and Chapman 1994). The determination of whether yellow and Dalmatian toadflax produce species-specific volatile blends is, therefore, an important factor in the investigation of host races in *B. pulicarius*.

Y-tube Olfactometry

All behavior is the "result of the interaction of external (stimulus) and internal (physiological state) factors (Baker and Carde 1984)." Because of this, the behavioral reaction of an insect is variable (Bernays and Chapman 1994) and at any given moment will depend on a series of factors, including the insect's life stage and experience, time since feeding and/or mating, environmental factors and genetic variability (Borden 1977, Schoonhoven 1977, Opp and Prokopy 1986, Bernays and Chapman 1994). Some insects are known to respond to host odors with certain volatiles acting as "attractants" which induce the insect to move toward their source and others acting as "repellents," causing the insect to move away from the source (Dethier et al. 1960, Bernays and Chapman 1994). Insects have also been shown to move up-wind, a task known as "anemotaxis," or, when an attractant is present upwind, "odor-induced anemotaxis" (Bernays and Chapman 1994). The tendency of insects to carry out odor-induced anemotaxis can be exploited by researchers utilizing a Y-tube olfactometer. The Y-tube olfactometer was described by Geier & Boeckh (1999) for bioassays with mosquitoes. The system provides an arena for conducting behavioral assays in which individuals are exposed to odors up-wind and allowed to react. Because of the Y-shape of the glassware, it is possible to expose an insect to two stimuli, one in each arm, to examine their reaction to, and possible preference for, one versus the other. In such experiments, this response is usually defined as a "choice" (Ignacimuthu et al. 2000). By exposing individual *B.*

pulicarius beetles to both host plant species in the Y-tube apparatus, the possibility of host preference can be examined.

Wind Tunnel

Another way of examining host plant preference involves the use of a wind tunnel apparatus. The wind tunnel has been described by Baker and Lian (1984) and has been utilized recently by Yamanaka et al. (2001). The apparatus consists of a large plexiglass box on a table top, called an arena. Air is pulled through the system at a constant flow rate. As with Y-tube olfactometry, wind tunnel experiments involve presenting insects with a stimulus and examining their response, generally taxes toward or away from the stimulus (Borden 1977). Insects are introduced, in groups, into the apparatus down-wind of the stimulus material. Tests can be conducted with either a single stimulus or two or more stimuli that the insects much choose among. For the experiments, a specific, discrete response must be clearly defined ahead of time (Baker and Carde 1984, Opp and Prokopy 1986). Response classifications should be mutually exclusive (Matthews and Matthews 1982). For studies of *B. pulicarius* the response was defined as an insect actually landing on the stimulus. Wind tunnel trials are run with a set amount of time and the frequency, or number of responses to the stimulus are recorded (Baker and Carde 1984). Similar to analyses of y-tube olfactometry results, a Chi-squared test can then be utilized to determine if the response frequency is significantly different from random (Matthews and Matthews 1982).

Importance

According to Narang et al. (1994), many researchers contend that the individuals of a population of insects must actually prefer their host in order to constitute a host race. In order for a population to be accepted as a host race under this more strict definition, showing that individuals actually have a preference for one host over another is necessary. Also, because host races may be incompletely reproductively isolated, the amount of molecular divergence between them can vary greatly (Narang et al. 1994). Often, if interbreeding occurs, the only consistent genetic difference between two host races may be only at the locus or loci specifically related to host preference (Bush and Smith 1998). In genetic techniques, like AFLPs, where only a portion of the genome is being sampled, the potential exists for failing to detect such small differences between races. Alternatively, because AFLPs involve random sampling of the genome (Vos et al. 1995) even if genetic differences are detected, their biological importance cannot necessarily be inferred. Because of this, a secondary technique such as a behavioral assay designed to demonstrate host preference can be invaluable in an investigation of host races. When host-related genetic differences are detected, behavioral preference data can provide secondary support for the findings. If no genetic differences are discovered, behavioral evidence can serve as an indicator of whether further genetic sampling might uncover host race differences. The results of behavioral and genetic data are, therefore, not mutually exclusive. Rather they can serve as two separate pieces of evidence in the investigation of host races in *B. pulicarius*.

Statement of Hypotheses

Hypotheses

The species *Brachypterolus pulicarius*, an ovary-feeding nitidulid, consists of two genetically distinct host races – one found on *Linaria genistifolia* ssp. *dalmatica* (Dalmatian toadflax) and the other on *L. vulgaris* (yellow toadflax). The two specific null hypotheses being tested are: 1) there is no significant genetic difference between *B. pulicarius* individuals collected from *L. vulgaris* and those collected from *L. genistifolia* ssp. *dalmatica*; and 2) there is no significant behavioral difference between beetles collected from the two host plant species.

CHAPTER 2

MATERIALS AND METHODS

Site Selection

Research sites (Table 1) were selected based on two major criteria. The first consideration was to simply locate sites where the beetles were present. *B. pulicarius* has been widely redistributed throughout North America as a biological control agent for toadflax. Once sites containing beetles were identified, an effort was made to limit collection to only those locations where no known releases have occurred. This was done in an attempt to collect beetles from the host plant species with which they would naturally be associated. The weed population at most of the selected collection sites was composed of a single species of toadflax. Separate sites with only one host species present, such as these, are termed allopatric sites. Sympatric sites, as discussed previously, are sites where both host plant species occur together. For *B. pulicarius* collections, only sites 1 and 2 were truly sympatric. At this location both host species are present growing on a hillside, with the yellow toadflax growing lower down on the hillside and to the west of the Dalmatian toadflax.

At each selected location, observations of general site characteristics were made. First the species of toadflax growing at the site and the general density of *B. pulicarius* were recorded. Observations were made about the presence and abundance of other toadflax natural enemy populations including the curculionids *Gymnetron antirrhini*

(Payk.), *G. netum* (Germ.), and *Mecinus janthinus* (Germ.), as well as the noctuid *Calophasia lunula* (Hufn.). The general stages of the toadflax plants at the site were recorded. Notes on time of day and weather conditions during collection were made. Finally, the GPS coordinates were recorded at each location. Plant collections were also made at each location for possible future genetic analyses.

Table 1. *Brachypterolus pulicarius* collection and site information for insects used in AFLP molecular analyses (n=sample size)

Site #	Collection Date	Location	Coordinates	Host Species	n	% Female
1	7/21/99	Townsend, MT	N46°18.990' W111°41.376'	*L. vulgaris*	41	59
2	7/21/99	Townsend, MT	N46°18.990' W111°41.376'	*L. genistifolia* ssp. *dalmatica*	39	54
3	8/26/99	Yellowstone Park	N45°02.399' W111°07.348'	*L. vulgaris*	29	45
4	6/21/99	Barriere, B.C.	N51°02.345' W120°13.401'	*L. vulgaris*	32	38
5	6/21/99	Peachland, B.C.	N49°47.667' W119°42.472'	*L. genistifolia* ssp. *dalmatica*	40	55
6	7/22/99	Boulder, MT	N46°13.500' W112°12.918'	*L. vulgaris*	38	39
7	7/23/99	Livingston, MT	N45°29.074' W110°37.292'	*L. genistifolia* ssp. *dalmatica*	35	40
8	7/27/99	Quake Lake, MT	N44°51.146' W111°32.283'	*L. genistifolia* ssp. *dalmatica*	39	49
9	7/29/99	Hebgen Lake, MT	N44°40.864' W111°11.252'	*L. vulgaris*	34	57
10	7/22/99	Boulder, MT	N46°17.013' W112°14.755'	*L. genistifolia* ssp. *dalmatica*	34	43
11	7/22/98	Macedonia	[a]	*L. genistifolia* ssp. *dalmatica*	25	40
12	7/14/99	Germany	[a]	*L. vulgaris*	25	28

[a] coordinate information not available

Collections

Once a site was selected, beetles were collected via aspiration. At some sites beetles were first collected with sweep nets. However, at most locations, insects were

simply hand-aspirated from the plants. A total of 50 individuals, or as many as could be found if less than 50, were collected at each site for genetic analysis. At some sites additional beetles were collected for behavioral analysis.

When collecting for genetic research, the live insects were placed into 95% ethyl alcohol for preservation. The insects were stored in the alcohol, in vials labeled with the site number, for future use in genetic studies. Later, beetles were removed from alcohol and placed into individual micro-centrifuge tubes for storage at –80° C.

Insects collected for behavioral research were placed into plastic canisters and brought back to the laboratory. Each individual was than placed into a glass vial or micro-centrifuge tube, and labeled with a number for identification. These tubes were placed at room temperature in a window sill (not in direct sun) so that insects would experience normal photoperiod. Individuals beetles were tested in the behavioral apparatus no sooner than the second day after collection. During bioassays observations and results were recorded, as discussed later. Beetles were then frozen for potential use in future genetic analyses.

Identification

General identification of the insects was made on site. Additionally, beetle samples were sent to Dr. Roger Williams, an entomologist who has worked with nitidulids at Ohio State University, for positive identification of the species. He confirmed that the collected beetles were members of the species *Brachypterolus pulicarius*.

Molecular Genetics

DNA extraction

Several extraction protocols were tested for use with *B. pulicarius* AFLP analysis. During protocol screening, extracted DNA was visualized on a 1% agarose gel, and resulting bands were compared for visible quantity of DNA. Based on visible quantity, repeatability, and simplicity, the extraction protocol was chosen. The selected protocol for DNA extraction is a revised version of the protocol developed by Brian Farrell (Farrell 1999), and modified by Bryan FitzGerald (B.C. FitzGerald unpublished protocol 1999). Individual insects were removed from the freezer. Sex was determined by examination of external morphology under a dissecting microscope, with males having an additional abdominal tergite visible (Hervey 1927). Next the abdomen was removed in an effort to minimize the influence of gut contents on DNA quality. The head and thorax of each beetle was then placed into a labeled 1.5 ml micro-centrifuge tube and put into liquid nitrogen for several minutes. Next, the tube was removed and the insect was ground by hand using a disposable micro-centrifuge pestle until it was ground to a fine, still frozen powder. Then 750 µl of Lefton buffer (Farrell 1999) containing 0.2 M sucrose 0.05 M EDTA, 0.1 M Tris (pH 9.0), 0.5% SDS (weight/volume), and 1.2 µl of 254 mg/ml Proteinase K (Sigma catalog # P2308) were added to the tube, it was vortexed briefly, and was placed into a heat block at 55°C. The process was then repeated for the remaining individuals. After incubation for 2 hours at 55°C, 50 µl of 8 M potassium acetate was added to each tube. Tubes were again vortexed and placed on ice for 20 minutes. Next, tubes were centrifuged for 15 minutes at 14,000 rpm, after which the

supernatant was decanted into a new 1.5 ml tube. The remaining pellet was discarded. Then, 50 µl of 3 M sodium acetate and 800 µl of cold (stored at 20°C) 95% ethyl alcohol were added to each tube, and they were placed into the freezer at 20°C for one hour. Tubes were then centrifuged for 30 minutes at 16,000 xg (14,000 rpm), and the supernatant was discarded. The remaining pellet, which may or may not have been readily visible, was washed with 70% ethanol and then allowed to air dry. The products were then re-suspended in 50 µl of 1X TE (Fischer Scientific product number BP1338-1, Tris-EDTA buffer, 100X diluted 1:100). Ten microliters of each sample could then be electrophoresed for 2 hours at 80 volts on a 1% agarose gel with 1X TBE buffer (54 g Tris base, 27.5 g Boric acid, 40 ml 0.25 M EDTA - diluted 1:5). Products were stained with ethidium bromide for 20 minutes and were visualized using the BioRad Flour-S multi-imager to verify extraction results. Remaining products were stored short-term at 4°C, or frozen at -80°C for long-term storage.

AFLP's

The AFLP analysis was carried out according to the general protocol designed by Vos et al. (1995), and using a portion of the protocol for the AFLP Plant Mapping Kit from Applied Biosystems (ABS, Foster City, CA). A 40 µl reaction containing 10 µl of extracted DNA was utilized for the digestion step. Each sample, along with 20 µl of HPLC (high pressure liguid chromatography) purified water was placed into a 500 µl micro-centrifuge tube. Then, a master mix containing 1 µl each of EcoR1 enzyme (20 units) and Mse1 enzyme (4 units), 3 µl of New England Biolabs (Beverly, MA) #4 buffer

(product number B7004S), 0.3 µl of 10 mg/ml BSA (20 mM KPO_4, 50 nM NaCl, 0.1 mM EDTA, 5% glycerol (pH 7.0 at 25°C), and 4.7 µl of water per reaction was prepared. Ten microliters of the master mix was then added to each reaction, and the tube was vortexed briefly. All tubes were then incubated at 37°C for three hours. Enzymes were purchased from New England BioLabs. All oligonucleotides (Table 2) were synthesized by Operon Technologies, Inc (Alameda, CA) for initial analyses and were later supplied with the AFLP kit (Applied Biosystems (ABS), Foster City, CA).

For the ligation step, 50 µl reactions were used, each containing the 40 µl reaction from the digestion step. A master mix was prepared, with 1 µl (10pm) EcoR1 Adapter (sequence Table 2), 1 µl (100pm) Mse1 Adapter, 1 µl (400,000 units/ml) T4 DNA Ligase (NEB product # M0202S), 1 µl 10X Ligation Buffer (50mM Tris-HCl (ph 7.5), 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP, 25 mg/ml bovine serum albumin), and 5 µl of water per sample, and 10 µl of master mix were added to each reaction. Each tube was then agitated with the pipet and then incubated at 22°C for three and a half hours. Reactions were then stored at 4°C overnight. For the Vos et al. (1995) protocol, adapters were synthesized and T4 DNA Ligase and buffer were purchased from New England BioLabs. Kit adapters were supplied with the AFLP Kit Pre-selective amplification module (ABS, Foster City, CA).

For the pre-amplification, the plant mapping kit (ABS, Foster City, CA) was utilized. 20 µl reactions were utilized. First, 1 µl of product from the ligation step was placed into a 500 µl HotStart storage and reaction tube and 19µl of the supplied Core

Mix, which included pre-primers, was added to each. PCR was run using a PTC-100 Programmable Thermal Controller according to the Vos et al. (1995) protocol (Table 3).

Table 2. Sequences of oligonucleotides used in AFLP analyses of *Brachypterolus pulicarius*

Adapter Sequences:	
EcoR1 Adapter 1: CTCGTAGACTGCGTACC EcoR1 Adapter 2: AATTGGTACGCAGTCTAC	Double Stranded: 5' CTCGTAGACTGCGTACC 3' 3' CATCTGACGCATGGTTAA 5'
Mse1 Adapter 1: GACGATGAGTCCTGAG Mse1 Adapter 2: TACTCAGGACTCAT	Double Stranded: 5' GACGATGAGTCCTGAG 3' 3' TACTCAGGACTCAT 5'
Primer Sequences:	
EcoR1 : GACTGCGTACCAATTC Mse1 : GATGAGTCCTGAGTAA	
Additional Selective Nucleotides:	
Pre-primer labels:	**Nucleotide:**
EcoR1 PP	+A
Mse1 PP	+C
Primer labels:	**ABS-provided fluorescent labels:**
EcoR1 TRY	+ACG
EcoR1 P1	+ACA FAM
EcoR1 P2	+AGG JOE
Mse1 P1	+CAT
Mse1 P2	+CTG

Table 3. PCR profile for the pre-amplification step of the AFLP process (20 cycles).

HOLD	20 CYCLES – EACH			HOLD	HOLD
72°C – 2 min.	94°C – 20 sec.	56°C – 30 sec.	72°C – 2 min.	60°C – 30 min.	4°C

The final amplification step was completed as specified by Vos et al. (1995) but with 20 µl reactions being used. Four microliters of product from the pre-amplification were placed into a 500 µl tube. The master mix was prepared to contain 1 µl of EcoR1

primer (50 ng) and 1.2 µl of Mse1 primer (100 ng), 0.75 µl each 10 mM dNTPs, 3 µl 10X PCR buffer, 2 µl 25 mM MgCl2, 0.2 µl Taq polymerase, and 15.68 µl of water per reaction. Twenty-six microliters of master mix was added to each tube, and they were placed into the thermocycler. PCR was again run according to the protocol (Table 4).

Table 4. PCR profile for the final amplification step of the AFLP process.

HOLD	CYCLE			Number of Cycles
94°C – 2 min.	94°C – 20 sec.	66°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	65°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	64°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	63°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	62°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	61°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	60°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	59°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	58°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	57°C – 30 sec.	72°C – 2 min.	1
	94°C – 20 sec.	56°C – 20 sec.	72°C – 2 min.	20
60°C – 30 min.				1
4°C				indefinitely

AFLP products were run on a 6% polyacrylamide gel for with 1X TBE electrophoresis buffer. They were initially visualized by staining with ethidium bromide. Bands examined were in the range from 300bp to 50bp. These preliminary runs helped with technique development and primer selection. A literature review of studies utilizing AFLP analyses for insects yielded a list of the most commonly used primer combinations. From this list, six primer sets were screened for suitability and four were selected for further screening (Table 5). For final results, products were visualized by Iowa State University's GeneScan service at the DNA Sequencing Facility. Products were run on the ABI Prism377 with fluorescent labels. The four selected primer sets were further

screened using the GeneScan service and primer set 2 (Table 5), consisting of EcoR1-AGG+JOE, and Mse1-CTG was selected for use in actual molecular genetic comparisons of *B. pulicarius*. Scored gel images were downloaded electronically from Iowa State and were scored utilizing Genographer software (Benham 2001). Using the Genographer software, for each gel, cut-offs were set by setting the intensity level to 10 and normalizing the signal on partial signal. Bands were then scored automatically by the software and were then adjusted manually, with all visible bands being scored based the series of assumptions that follow below. Bands appearing in separate individuals at the same locus were scored as homologous fragments and the absence of a fragment was assumed to represent the homozygous recessive. Quantity differences were not considered, with only a band's presence or absence in the gel being scored. The resulting data consisted of a list of 1's and 0's for each locus with 1 representing the presence of a scored band and 0 representing its absence for each individual. This data was imported into a text editor for modification into matrix format. The resulting matrix of 1's and 0's could then be imported, with minor format adjustments, into analytical software for statistical analyses (Reineke et al. 1999).

Table 5. Primer set pairs tested for AFLP analyses of *Brachypterolus pulicarius*.

Primer Set Number	EcoR1 Primer	Mse1 Primer:
Primer Set 1	EcoR1 P1	Mse1 P1
Primer Set 2	EcoR1 P2	Mse1 P2
Primer Set 3	EcoR1 P1	Mse1 P2
Primer Set 4	EcoR1 P2	Mse1 P1
Primer Set 5	EcoR1 TRY	Mse1 P1
Primer Set 6	EcoR1 TRY	Mse1 P2

Statistical Analysis

The AFLP technique provides researchers with information about genetic polymorphisms. One drawback of the analysis of the AFLP process and most genetic techniques is that certain unknown characteristics of the data must be assumed, and that any deviation from these assumptions could potentially decrease the accuracy of results of the analysis (Yan et al. 1999, Excoffier 2001). All analyses of AFLP data rely on a series of underlying assumptions. It is assumed that the markers are completely dominant (Mueller and Wolfenbarger 1999, Yan et al. 1999), an assumption which is generally well-accepted but has been recently challenged by researchers indicating that the markers may be co-dominant (Piepho and Koch 2000, Wong et al. 2001). Also, all fragments appearing in a gel at the same base pair size are assumed to be homologous fragments (Yan et al. 1999), an assumption supported by Waugh et al. (1997) but questioned for similar techniques by Black (1993). In order to do analyses, it must also be assumed that only two alternate alleles are present at each locus and that Hardy-Weinberg equilibrium and Mendelian segregation are in effect (Yan et al. 1999, Wong et al. 2001).

In the investigation of host races, several statistical analyses were utilized for the generated AFLP data. First, however, an effort was made to simply quantify the amount of genetic variability present in the species. Descriptive statistics were calculated using the Tools for Population Genetic Analysis (TFPGA) software (Miller 1997) to determine average heterozygosity and percent polymorphism for each population as described by Weir (1990). Average heterozygosity measures represent the frequency of heterozygotes for each locus, averaged over all the loci for a population. These values were also

averaged across all North American and European populations and across host plants. Heterozygosity values were first calculated by a direct calculation from the estimated frequency of the recessive homozygote. Secondly, average heterozygosity was calculated using Nei's less biased estimator for small sample sizes (Nei 1978). Percent polymorphism represents the percentage of loci that are polymorphic for each population. For descriptive statistics percent polymorphism was calculated using two different cut-off values. With a 99% criterion, all loci for which at least 1% of individuals possessed an alternate allele were counted as polymorphic. The calculation was repeated using a 95% criterion where only loci for which at least five percent of individuals possessed an alternate allele were included. The percent polymorphisms were also averaged for North American and European sites and across host plants.

Individuals were grouped by population and allele frequencies were estimated with TFPGA (Miller 1997) utilizing Lynch and Milligan's (1994) Taylor expansion, a less-biased estimator of allele frequencies which takes into account the variability in the data by incorporating the sample variance into the calculation. Using the estimated allele frequencies, genetic distances were calculated using Nei's 1978 (Nei 1978) formula.

In order to actually examine how the populations are related to one another cluster analyses were run. Once the matrix of all pairwise genetic distances was calculated, a cluster analysis using the unweighted pair group method using arithmetic averages (UPGMA) was run (Swofford and Olson 1990). Specifically, the goal was to determine if beetles collected from sites with the same host plant might be more similar to one another than to those collected at sites from the other species of host plant. That is, if

populations from yellow toadflax sites and Dalmatian toadflax sites would cluster together or if clustering might correspond to some commonality other than host plant. Clustering by host plant could be evidence of host race differences. The UPGMA analysis of genetic distance data was previously utilized in AFLP studies of heart-of-palm (Cardoso et al. 2000), fall armyworm (McMichael and Pahley Prowell 1999), and carrot (Shim and Jorgensen 2000) and with the similarly-analyzed random amplified polymorphic DNA (RAPD) technique for mosquito (Kambhampati et al. 1992). The TFPGA software produced a dendrogram and allowed bootstrapping to determine the reliability of each given branch in the cluster analysis. Additionally, all populations were then separated into male and female groups, and the analysis was re-run to examine the reliability of the 'forced' population groupings by looking for possible sexual dimorphism. Such sexual dimorphism could be represented by the clustering of same sex populations from different locations, rather than clustering with their corresponding population of the opposite sex. An investigation into sexual dimorphism was suggested by Katiyar et al. (2000) who discovered marker sexual dimorphism in AFLP analysis of the Asian rice gall midge (Katiyar et al. 2000).

Another way to examine the relationships is to examine the beetles' similarity at the individual level. In order to analyze AFLP data for gypsy moth (Reineke et al. 1999), Asian rice gall midge (Katiyar et al. 2000), fungi (Cilliers et al. 2000, Gagne et al. 2000), whiteflies (Cervera et al. 2000), and various crop plants (Sharma et al. 1996, Barrett and Kidwell 1998, Mace et al. 1999, Seefelder et al. 2000, Jakse et al. 2001, Sawkins et al. 2001) researchers used coefficients to determine genetic similarity for all individuals.

For analysis of *B. pulicarius*, similarities were determined with Dice's coefficient of genetic similarity (GS) (Equation 1) for all individuals in the data set.

Equation 1. Dice's Coefficient (Sneath and Sokal 1973).

$$GS(ij)=2a/(2a+b+c)$$

Where GS(ij) is genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i (Sneath and Sokal 1973).

Using the coefficient, a matrix of genetic similarities between all individuals was created and UPGMA analysis was run. This analysis sought to determine if individuals grouped by host plant or another commonalty, such as site, sex, or geographic area. Consistent grouping by host plant could serve as evidence for the existence of host races in *B. pulicarius*.

In order to quantify the observed population groupings and to determine exactly how variation was partitioned, an analysis of molecular variance (AMOVA) as described by Excoffier (2001) was run. The AMOVA has been frequently utilized to determine genetic variability partitioning within and among populations and among groups of populations (Excoffier et al. 1992, Huff et al. 1993, Huff 1997, Barrett and Kidwell 1998, Cardoso et al. 2000, Carelli et al. 2000, Keiper and McConchie 2000, Purwantara et al. 2000, Shim and Jorgensen 2000, Sawkins et al. 2001). This analysis can provide information about the presence of significant partitioning of variation by host plant,

population, or at the individual level. For the AMOVA, a comparison of covariance components was made similar to a standard analysis of variance (ANOVA) in which means are compared by examining variance components (Excoffier 2001). Like the previously-discussed UPGMA by distance matrix, the AMOVA again relies upon the estimation of allele frequencies by Lynch and Milligan's Taylor expansion. Data was first imported into AMOVA-PREP (Miller 1998) in order to format it for use with the Arlequin software (Schneider et al. 2000). Allele estimates were carried out using the Arlequin program (Schneider et al. 2000) and variance components and overall fixation indices were computed. Significance was tested by means of a series of random permutations to determine the probability of obtaining random fixation index values that were more extreme than the calculated value.

Because the small but significant amount of variation partitioned between populations was not well-explained by host plant, an alternative explanation, that geographic divergence might be important was examined using a Mantel test. The Mantel test, described by Sokal & Rohlf (1995) and utilized by Jaske et al. (2001) was run to compare and quantify the amount of correlation between genetic distance and geographic distance matrices. Here, a comparison between genetic distance and geographic distance were made to determine if larger genetic distances were correlated with larger geographic distances. The null hypothesis for the analysis stated that there was no correlation between elements of one matrix and the elements of the other matrix. The Mantel statistic (Z) was calculated by finding the sum of the products of corresponding elements in the two matrices. A larger than average Z value would

indicate that higher genetic distances are correlated with higher geographic distances. To test for significance, a randomization test was again run by a permutation test where the values of one of the matrices were randomly rearranged and resampled. Then, a probability of obtaining the observed value could be found by comparing its position within all of the randomly-generated outcomes. The TFPGA software was used to estimate allele frequencies (as discussed previously) and to automatically generate the first matrix of genetic distances (Nei's 1978) (Figure 6). The geographic distance matrix was calculated by entering coordinate data from collection sites into the Great Circle Calculator (Williams 2002) which generated the straight-line distance between two points (Figure 7). The data from the two matrices was formatted into a new matrix consisting of row number, column number, and corresponding genetic and geographic distances for each location. The analysis consisted of 999 permutations, and a scatterplot, Z-statistic, and p-values were generated. The test was run for only North American sites as no coordinate data was obtained for European collection locations. A second test was run for U.S. populations only, omitting the British Columbia sites from each of the matrices.

Figure 6. Genetic distance matrix of all North American Sites based on Nei's unbiased (1978) distance utilized in the Mantel test.

Site#	1	2	3	4	5	6	7	8	9	10
1	0	-	-	-	-	-	-	-	-	-
2	0.0243	0	-	-	-	-	-	-	-	-
3	0.0353	0.0289	0	-	-	-	-	-	-	-
4	0.0412	0.0393	0.0415	0	-	-	-	-	-	-
5	0.0282	0.0257	0.0343	0.0251	0	-	-	-	-	-
6	0.0338	0.0311	0.0292	0.0246	0.0254	0	-	-	-	-
7	0.0459	0.0405	0.0478	0.0429	0.0373	0.0390	0	-	-	-
8	0.0791	0.0818	0.0650	0.0398	0.0610	0.0297	0.0817	0	-	-
9	0.0699	0.0795	0.0640	0.0376	0.0587	0.0262	0.0754	0.0138	0	-
10	0.0650	0.0702	0.0617	0.0360	0.0484	0.0226	0.0715	0.0122	0.0077	0

Figure 7. Geographic distance matrix for all North American Sites (distances in kilometers) utilized in the Mantel test.

Site#	1	2	3	4	5	6	7	8	9	10
1	0	-	-	-	-	-	-	-	-	-
2	0	0	-	-	-	-	-	-	-	-
3	148.60	148.60	0	-	-	-	-	-	-	-
4	818.07	818.07	950.25	0	-	-	-	-	-	-
5	711.87	711.87	835.24	143.21	0	-	-	-	-	-
6	41.78	41.78	156.87	796.04	685.06	0	-	-	-	-
7	124.17	124.17	63.14	941.88	833.22	148.64	0	-	-	-
8	163.15	163.15	38.86	944.35	825.77	161.46	100.63	0	-	-
9	185.95	185.95	40.22	976.82	858.91	189.49	99.80	33.66	0	-
10	43.02	43.02	163.61	789.77	679.20	6.92	154.22	168.38	196.38	0

Behavioral Studies

Behavioral research was completed in an effort to lend support to and/or indicate deficiencies in the molecular genetic data. As discussed previously, molecular findings had the potential of failing to detect actual differences. They also could, potentially, discover differences that might not have any biological significance. Behavioral data was obtained to examine the potential for and existence of host plant preference. While not considered a necessary factor in determining the existence of host races, the presence of a biological basis for and/or the actual existence of host preference can be important steps in their formation.

Volatile Collections

The volatile collection system (VCS) was utilized to examine the potential for host preference in *B. pulicarius*. The system was similar to that described by Heath and Manukian (1992, 1994). Volatiles were collected from both Dalmatian and yellow toadflax for species-to-species comparisons in an effort to identify species-specific

differences. Such differences could serve as a biological basis for host preference. Additionally, efforts were made to characterize the variability in volatile production. Plants were collected from two allopatric sites (#7 and #9), the two sympatric sites (#1 and #2), and an additional location (Yellowstone National Park) where the plants grow sympatrically in order to make comparisons. By running multiple plants simultaneously, individual variability could be examined. Trials were repeated three times during the season to examine temporal differences in plant volatile emissions. Two collections were made of pre-flowering plants and one was made of flowering plants to examine phenological variation in volatile production. Collected plants were carefully removed from the soil, their roots were wrapped in wet paper towels, and they were placed in a cooler for transport back to the laboratory.. The root/cut ends of plants in the sample runs were wrapped first in wet cotton and then aluminum foil. The blanks in the trials contained only the foil-wrapped wet cotton as a control. Air was filtered and purified and pumped through the system at a constant flow rate of 1.0 Liter/min, maintained by a vacuum pump. This clean air passed into 31-mm diameter X 450-cm long Pyrex glass volatile collection chambers (VCC) containing either plant material or a blank. The VCC's were fitted at one end with an air diffuser inlet cap and sealed at both ends with No. 7 ChemThread inlets (1/4" ID) using rubber O-rings (Analytic Research Systems, Inc., Gainesville, Florida). The apparatus consisted of eight VCC's capable of holding sample material, and each trial consisted of six plant material runs and two blanks. The air passed over the plant chamber and through the volatile collector trap (VCT), a .635 cm (.25 inch) OD X 7.62 cm (3 inch) long glass tube with 30 mg of Super Q (Alltech

Associates, Inc, Deerfield, IL) absorbent inserted into one end. Each run lasted three hours. Compounds trapped in the Super Q were later eluded from each VCT with 225µl hexane into an auto-sampler vial with a concentration insert. Seven nanograms of decane was added to each as an internal standard. The vial was then automatically loaded into the coupled gas chromatography-mass spectrometry (GC-MS) apparatus for analysis. The GC was an Agilent Technologies (Palo Alto, CA) 6890 instrument fitted with a 30-m DB-1MS capillary column (.25-mm-ID, 0.25 µm film thickness; J & W Scientific, Folsom, CA). The temperature program was held for 50°C for 3 minutes and increased to 280°C at 5°C/min. The MS instrument was an Agilent Technogies 5973. The accompanying software was utilized to identify isolated peaks representing the presence of chemical compounds. These peaks were then compared to the known chemical compounds in the software's library/database so that identification and quantification of each chemical could be made. The resulting identifications could be utilized to determine the volatile composition of each plant's emissions. Differences in emissions were quantified for individual compounds by comparing the mean quantity from each sampling run. Using an analysis of variance (ANOVA) these means were compared by examining their variance components. Using the ANOVA, temporal, spatial, and species-to-species comparisons were made for both more concentrated, or major compounds and those present only in small quantities, or minor compounds. These comparisons allowed determination of whether the observed variability in volatile emission represented a statistically significant difference. The presence of significant differences in the volatile composition emitted by each of the toadflax species could then

be compared to determine the overall variability and the potential for host preference in *B. pulcarius.*

Y-Tube Olfactometer

The Y-tube olfactometer was utilized to examine whether beetles showed a preference for their host plant. The system functioned by pumping filtered, humidified air into a Y-shaped glass tube. The air was released into the tube at a constant flow rate, passed over stimuli in both branches of the Y, and then moved down the tube and out through the straight end (Janssen et al. 1990, Geier and Boeckh 1999, Ignacimuthu et al. 2000). In trials with *B. pulicarius*, a single individual was introduced at the downwind/open end of the tube and allowed to proceed upwind to the branch of the Y. There, the beetle had to make a decision, or "choice" as to whether to proceed up the left side or the right, thereby deciding to preferentially proceed toward either the stimulus on the left or that on the right. Stimuli included a control, or blank, or various parts of individual plants. In all cases, the wounded end of the plant material was wrapped in wet cotton to minimize the effects of localized wound response volatiles and the blank contained a similar amount of wet cotton. Insects were collected from various study sites throughout the summer and kept alive, in individual vials, at room temperature until behavioral trials could be run. Insects were not given food or water and generally lived for about 1-2 weeks.

Before Y-tube bioassays could be completed, much effort was focused on technique development and attempting to minimize the effects of confounding factors. Three areas of potential complication were identified: beetle/environmental factors, plant

variability, and problems with the apparatus. Preliminary trials were run in an attempt to identify and remedy specific concerns. Initial attempts were made to control for all apparatus factors. Results of preliminary trials indicated that an individual beetle would follow the apparent scent trail of another beetle if the same glassware was utilized for multiple trials, so clean tubes were required for each trial. A smoke test (Janssen et al. 1990, Koschier et al. 2000, Doromont and Roques 2001) showed that an initial flow rate of 0.6 L/min allowed back-mixing of odors in the tube, so a flow of 0.8 L/min was selected. In trials with the glassware lying horizontally on the table, beetles crawled along the top of the inside of the tube where potentially little or no odor source was flowing, so a frame was built so that the glassware could be suspended vertically (Ho and Millar 2001). With the vertical apparatus, smoke tests indicated that odors still flowed with gravity, bouncing off the glassware if it was off-center and causing back-mixing. To remedy this, the tube was balanced in the vertical plane. If stimuli were allowed to rest at the bottom of extender tubes in the apparatus air flow disruption occurred, resulting in back-mixing. To avoid this, plant and blank materials were affixed to the top of wire rods and then inserted into the extender tubes.

Attempts were also made to control for beetle factors. Time since last feeding was examined in preliminary trials and revealed that if insects were starved for 24 hours or longer they showed a more immediate response to plant material. In light versus dark trials (where one side of the apparatus was wrapped in black fabric), 12 out of 12 beetles went to the light side of the tube, indicating the importance of balancing light levels between sides of the tube. Temperature factors were important, with beetles reacting

quickly and sporadically at high room temperatures (>30°C) while at very low temperatures (<20°C) they did not respond. A potential dose-response to plant material, as previously noted by Doromont and Roques (2001) and Koschier et al. (2000), was observed with very large quantities of plant material actually acting as a repellent.

Plant physiology factors were also a concern. In 2000, plants were harvested early in the season from Ovando, MT for rearing in the greenhouse. However, a severe infestation of thrips and aphids required two treatments with insecticidal soap. The effect of these treatments on *B. pulicarius* behavior is unknown. In order to avoid similar problems in 2001 plant material was harvested from a local infestation the morning of each trial. In 2002 plants were harvested from site #7, #9, and the Yellowstone National Park location and placed in pots outdoors. Sprigs of plant material were cut immediately before use. Because a large mixture of volatiles is present outdoors and potentially on the surface of a newly-harvested plant, each plant was placed into the apparatus with the flow running for ten minutes before assays were begun. Early season results indicated a potential drop-off in beetle reactivity after a plant had been in the apparatus for more than an hour as might be expected with a decrease or cessation in volatile production. To minimize this effect, a new plant was utilized after each set of ten trials. The effect of hot weather on volatile production by the plants was unknown, but decreased beetle reactivity in the afternoon on very hot days (>30°C) indicated a potential decrease in volatile production. Because of this, attempts were made to run trials before 1:00 pm.

The preferred technique utilized a single beetle for an individual trial. Trials were run between 7am and 1pm. Between trials, the stimuli were switched and a new Y-tube

was supplied. By switching stimuli, two treatments were used, one with a particular stimulus on the left and a second treatment with that same stimulus on the right. Each tube was washed with soapy water, was then rinsed with acetone, then with hexane, and was then allowed to fully dry before being re-used. A room temperature of around 25°C was maintained. Room lights were turned off, and only a single bulb, suspended between the branches of the Y-shaped tube was utilized. The apparatus was suspended from the vertical frame, and attempts were made to balance the tube vertically. Stimulus and blank materials were affixed to the top of a metal rod within the extender tube and air-flow was run over them for ten minutes prior to the first assay. Each stimulus was utilized for a set of ten individual trials at which time a new stimulus was harvested. A single beetle was introduced at the bottom of the Y-tube, and allowed to proceed up the tube toward the stimuli. Each individual trial lasted five minutes maximum, with most beetles immediately proceeding up the tube to the stimulus. Beetles were observed to perform two general types of behavior in the apparatus which are being termed "directed" and "less-directed" or "un-directed." Directed behaviors included crawling straight up one side of the tube to a stimulus, making a distinct cross-over from one side to the other at the fork of the Y-tube, and zig-zagging along the center of the tube. These behaviors were commonly observed when beetles were responding more consistently to the plant material and were, therefore, considered to represent olfactory orientation within the apparatus. Less- or un-directed behavior generally consisted of beetles moving up the tube in a spiral motion. This behavior was observed in the absence of stimulus material and often occurred during trials when inconsistent results were obtained. Generally, less-

or un-directed behavior is considered to represent a random or indifferent response to stimulus material. While the general trends in behavior discussed above were observed for some trials, overall the observed behavior varied with individual, stimulus, and trial. Because of the high amount of variability in behavioral response, a choice was defined, regardless of behavior type, as a beetle moving past the fork of the Y and up the straight of one of the branches. The number of individuals choosing a particular side for each treatment was recorded, and a Chi squared test, adjusted for small sample size (Equation 2), was utilized for analysis of results. This test of significance was used to determine if the orientation within the apparatus was significantly different from random. Consistent random orientation by beetles within the apparatus would indicate a lack of preference for one stimulus versus the other. In a plant versus blank trial such a result would indicate a lack of response to the plant stimulus material. In a plant versus plant trial a random result indicates a lack of preferential orientation toward one plant. The expected random value for a given trial, verified by the results of preliminary blank versus blank trials, was a value of 0.5 for either side of the tube. A significant deviation from this result would therefore indicate preferential orientation.

Equation 2. Chi-squared test from Sokal and Rohlf (1995).

$$X^2 = [|obsX_i - expX_i| - 0.05]^2/expX_i + [|obsX_j - expX_j| - 0.05]^2/expX_j$$

Where: $obsX_i$ = the observed occurrence (X) of outcome i, $expX_i$ = the expected occurrence of outcome i, $obsX_j$ = the observed occurrence of outcome j, $expX_j$ = the expected occurrence of outcome j, and df = 1-number of choices.

Individuals utilized in behavioral assays were sexed after the trials. This was done to examine the possibility of sexual differences in responsiveness that might conceal actual preferential orientation. As with the insects utilized in genetic analyses, sex ratios of beetles collected for behavioral assays were not different from zero. Overall, no consistent differences in response for males and females were found. Responsiveness of the two sexes was not significantly different.

Wind Tunnel

Wind tunnel experiments also were used to examine host plant preference. In preliminary trials insects of a single population were assayed together in the apparatus. The stimulus material was introduced upwind in the tunnel. The specific response recorded was whether or not individuals actually landed on a host plant during the given time period. The number of responders and non-responders could then be recorded and a test of significance could be made. However, during the several preliminary assays run in June of 2001 a lack of suitability of the apparatus for use with *B. pulicarius* was indicated. Beetles tended to either not move at all within the apparatus or to congregate on the brightest outside wall. Attempts were made to balance and adjust light levels to limit their influence on behavior. These efforts only resulted in a greater number of non-responders and further attempts to utilize the wind tunnel for the investigation of host races in *B. pulicarius* were abandoned so that behavioral efforts could be concentrated on Y-tube olfactometry.

CHAPTER 3

RESULTS

Molecular Genetics

The specific null hypothesis for the molecular genetic results was that there is no genetic difference between beetles collected from yellow toadflax and those collected from Dalmatian toadflax.

The AFLP technique was easy-to-use and results were highly reproducible. A total of 411 individuals were analyzed via the AFLP technique, and a significant number of fragments were generated. In order to make comparisons, only bands in the range from 50 to 256 base pairs were scored. Within this range, 137 loci were recorded as present or absent (Table 6). Frequencies of band presence were calculated for each population at each locus and are provided in the Appendix. An effort was made to identify potential fingerprints corresponding to each host plant. Such fingerprints would contain bands only found, or found in significantly different frequency in individuals collected from one host plant than for individuals collected from the other host plant. However, average frequencies for each host plant did not yield any obvious band presence frequency differences between yellow toadflax and Dalmatian toadflax populations. Because of this, host-plant specific fingerprints could not be identified and analyses were run for all scored loci.

Table 6. Loci generated by AFLP analysis of *Brachypterolus pulicarius*.

Locus	Base Pair Range	Locus	Base Pair Range	Locus	Base Pair Range	Locus	Base Pair Range
1	50.65 +/- 0.5	36	96.72 +/- 0.5	71	141.44 +/- 0.4	106	183.68 +/- 0.5
2	51.75 +/- 0.5	37	97.86 +/- 0.5	72	142.46 +/- 0.5	107	184.97 +/- 0.5
3	52.65 +/- 0.4	38	98.99 +/- 0.5	73	143.62 +/- 0.5	108	186.32 +/- 0.5
4	53.63 +/- 0.5	39	101.54 +/- 0.5	74	144.6 +/- 0.4	109	187.47 +/- 0.5
5	54.79 +/- 0.5	40	102.64 +/- 0.5	75	145.5 +/- 0.4	110	188.58 +/- 0.5
6	55.99 +/- 0.5	41	103.84 +/- 0.5	76	146.42 +/- 0.5	111	189.71 +/- 0.5
7	57.09 +/- 0.5	42	105.21 +/- 0.5	77	147.55 +/- 0.5	112	190.84 +/- 0.5
8	58.19 +/- 0.5	43	106.53 +/- 0.5	78	148.62 +/- 0.5	113	192.05 +/- 0.5
9	59.65 +/- 0.5	44	107.61 +/- 0.5	79	149.79 +/- 0.5	114	193.42 +/- 0.5
10	60.88 +/- 0.5	45	108.96 +/- 0.5	80	150.9 +/- 0.5	115	194.87 +/- 0.5
11	61.92 +/- 0.4	46	110.36 +/- 0.5	81	152.93 +/- 0.5	116	196.14 +/- 0.5
12	62.78 +/- 0.4	47	112.04 +/- 0.5	82	154.0 +/- 0.5	117	198.14 +/- 0.5
13	63.89 +/- 0.5	48	113.34 +/- 0.5	83	155.14 +/- 0.5	118	199.3 +/- 0.5
14	65.07 +/- 0.5	49	114.57 +/- 0.5	84	156.66 +/- 0.5	119	201.32 +/- 0.5
15	66.75 +/- 0.5	50	115.64 +/- 0.5	85	158.58 +/- 0.5	120	202.93 +/- 0.5
16	67.77 +/- 0.45	51	118.0 +/- 0.5	86	159.66 +/- 0.5	121	204.05 +/- 0.5
17	70.06 +/- 0.5	52	119.13 +/- 0.5	87	160.7 +/- 0.5	122	205.18 +/- 0.5
18	71.23 +/- 0.5	53	120.18 +/- 0.5	88	161.82 +/- 0.5	123	206.3 +/- 0.5
19	72.19 +/- 0.5	54	121.38 +/- 0.5	89	162.94 +/- 0.5	124	207.42 +/- 0.4
20	73.16 +/- 0.5	55	122.32 +/- 0.5	90	164.06 +/- 0.5	125	208.38 +/- 0.5
21	74.17 +/- 0.5	56	123.46 +/- 0.5	91	165.87 +/- 0.5	126	209.5 +/- 0.5
22	75.43 +/- 0.5	57	124.53 +/- 0.5	92	167.19 +/- 0.5	127	210.62 +/- 0.4
23	76.64 +/- 0.5	58	127.11 +/- 0.5	93	168.33 +/- 0.5	128	211.61 +/- 0.5
24	78.26 +/- 0.5	59	128.11 +/- 0.5	94	169.43 +/- 0.5	129	212.74 +/- 0.5
25	81.14 +/- 0.5	60	129.24 +/- 0.5	95	170.55 +/- 0.5	130	213.85 +/- 0.5
26	82.21 +/- 0.5	61	130.7 +/- 0.5	96	171.67 +/- 0.5	131	214.98 +/- 0.5
27	83.98 +/- 0.5	62	132.1 +/- 0.5	97	172.84 +/- 0.5	132	220.64 +/- 0.4
28	85.31 +/- 0.5	63	133.21 +/- 0.5	98	173.9 +/- 0.5	133	225.71 +/- 0.5
29	86.48 +/- 0.5	64	134.33 +/- 0.5	99	175.06 +/- 0.5	134	226.81 +/- 0.5
30	87.45 +/- 0.5	65	135.47 +/- 0.5	100	176.18 +/- 0.5	135	227.89 +/- 0.5
31	90.59 +/- 0.5	66	136.52 +/- 0.5	101	177.62 +/- 0.5	136	243.84 +/- 0.5
32	91.78 +/- 0.5	67	137.7 +/- 0.5	102	178.82 +/- 0.5	137	255.92 +/- 0.5
33	92.82 +/- 0.4	68	138.54 +/- 0.4	103	179.95 +/- 0.5		
34	94.23 +/- 0.5	69	139.61 +/- 0.5	104	181.48 +/- 0.5		
35	95.54 +/- 0.5	70	140.61 +/- 0.4	105	182.58 +/- 0.5		

Descriptive statistics were calculated in order to characterize genetic diversity of *B. pulicarius*. From the estimated allele frequencies, average heterozygosity and percent polymorphism for each population were calculated (Table 7). These statistics were

averaged for all yellow toadflax and all Dalmatian toadflax sites in North America and for all North American and European populations to make general comparisons and conclusions about the genetic diversity of the species. North American populations generally exhibited consistent amounts of genetic diversity.

Table 7. Descriptive statistics calculated for *Brachypterolus pulicarius* AFLP markers.

Site Number	Sample Size	(AH) Average Heterozygosity	AH. (unbiased)	% Polymorphism (99% criterion)	% Polymorphism (95% criterion)
1	41	0.3443	0.3485	92.7007	91.2409
2	39	0.3740	0.3789	93.4307	93.4307
3	29	0.3722	0.3787	92.7007	92.7007
4	32	0.3295	0.3347	94.1606	90.5109
5	40	0.3351	0.3394	95.6204	91.2409
6	38	0.3262	0.3306	93.4307	89.7810
7	35	0.3416	0.3466	93.4307	90.5109
8	39	0.2709	0.2744	99.2701	89.0511
9	35	0.2412	0.2447	89.7810	69.3431
10	35	0.2497	0.2534	96.3504	70.8029
11	25	0.3899	0.3978	97.0803	96.3504
12	25	0.3330	0.3398	97.8102	97.8102
Overall Average					
Yellow Toadflax – N. America:		0.3227	0.3214	92.5547	86.7153
Dalmatian toadflax - N. America:		0.3143	0.3185	95.6205	87.0073
North America:		0.3184	0.3230	94.0886	86.8631
Europe:		0.3615	0.3688	97.4453	97.0803

To examine relatedness between populations and potential grouping by host plant, cluster analyses were run. In order to examine possible host-related population-level differences in allele frequencies, UPGMA analysis was conducted with a matrix of genetic distance for all 12 sites. The analysis yielded a dendrogram (Figure 8) resulting in four major groups (at a cut-off of distance = 0.04). One cluster consisted only of European populations. Each of the remaining clusters contained populations that did not consistently share commonalities related to host plant nor to geographic region. Most

sites were contained in one major cluster. One site (#7), from Paradise Valley, MT formed its own branch. Distances for each node are shown in Table 8. Bootstrapping was run in order to determine confidence of the clustering patterns obtained (Table 9). It revealed variability in the confidence levels for the branches. Overall, no indications of host plant-related differences were observed.

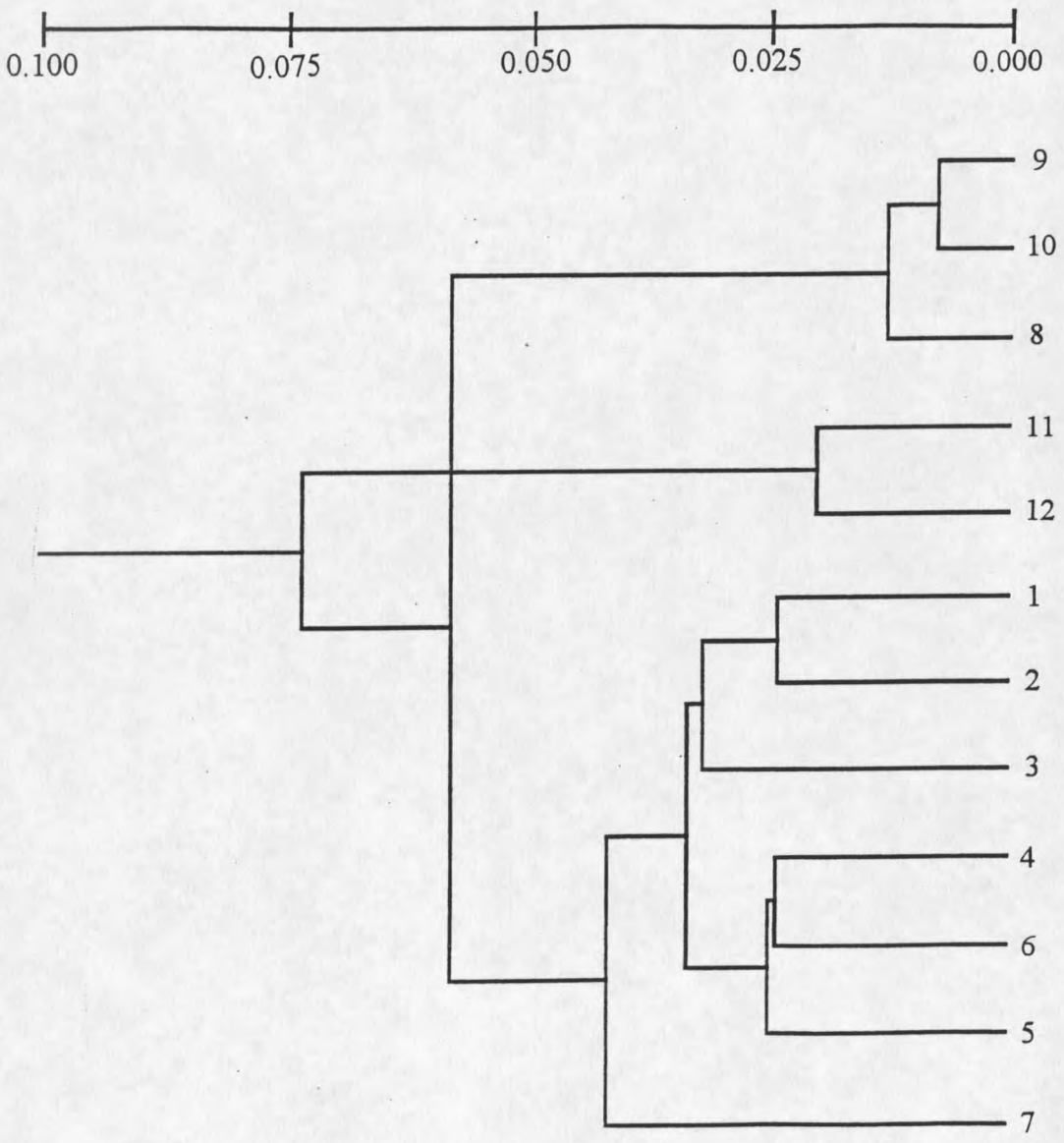
Table 8. Node information for UPGMA cluster analysis of all 12 *Brachypterolus pulicarius* populations.

Node	Distance	Includes Populations
1	0.0077	8 10
2	0.0130	8 9 10
3	0.0203	11 12
4	0.0243	1 2
5	0.0321	1 2 3
6	0.0246	4 6
7	0.0253	4 5 6
8	0.0338	1 2 3 4 5 6
9	0.0422	1 2 3 4 5 6 7
10	0.0583	1 2 3 4 5 6 7 8 9 10
11	0.0736	1 2 3 4 5 6 7 8 9 10 11 12

Table 9. Results of bootstrapping for UPGMA analysis of all 12 *Brachypterolus pulicarius* populations (1000 permutations).

Node	Proportion of similar replicates
1	0.9480
2	1.0000
3	1.0000
4	0.6130
5	0.2480
6	0.3050
7	0.3340
8	0.4080
9	0.5780
10	0.6160
11	1.0000

Figure 8. Cluster analysis (UPGMA) of Nei's (1978) distances for all 12 *Brachypterolus pulicarius* sites – output from TFPGA software (Miller 1997).



Sexual dimorphism in AFLP markers could potentially confuse the cluster analysis results. To examine if sexual differences might exist, each of the twelve sites

was next divided into one male and one female population (Table 10). Then the UPGMA analysis was run again with bootstrapping. Node information and distances are given in Table 11. In the resulting dendrogram (Figure 9) the males and females from each site clustered together for all American populations. For the two sites from Canada and the two from Europe, however, population clustering did not remain. The two female groups from the different sites in Canada branched together while for European populations the two male groups grouped out together. Bootstrapping again yielded variable confidence for the branches (Table 12). Overall no consistent sexual dimorphism was revealed.

Table 10. Population site labels when divided by sex for UPGMA analysis.

By-Sex Site Number	Overall Site Number	Location	Host Plant Species	Sex
1	1	Townsend	*Linaria vulgaris*	Male
2	1	Townsend	*Linaria vulgaris*	Female
3	2	Townsend	*Linaria genistifolia* ssp. *dalmatica*	Male
4	2	Townsend	*Linaria genistifolia* ssp. *dalmatica*	Female
5	3	Yellowstone	*Linaria vulgaris*	Male
6	3	Yellowstone	*Linaria vulgaris*	Female
7	4	British Columbia	*Linaria vulgaris*	Male
8	4	British Columbia	*Linaria vulgaris*	Female
9	5	British Columbia	*Linaria genistifolia* ssp. *dalmatica*	Male
10	5	British Columbia	*Linaria genistifolia* ssp. *dalmatica*	Female
11	6	Boulder	*Linaria vulgaris*	Male
12	6	Boulder	*Linaria vulgaris*	female
13	7	Paradise Valley	*Linaria genistifolia* ssp. *dalmatica*	male
14	7	Paradise Valley	*Linaria genistifolia* ssp. *dalmatica*	female
15	8	Hwy 287	*Linaria genistifolia* ssp. *dalmatica*	male
16	8	Hwy 287	*Linaria genistifolia* ssp. *dalmatica*	female
17	9	Hebgen Lake	*Linaria vulgaris*	male
18	9	Hebgen Lake	*Linaria vulgaris*	female
19	10	Boulder	*Linaria genistifolia* ssp. *dalmatica*	male
20	10	Boulder	*Linaria genistifolia* ssp. *dalmatica*	female
21	11	Macedonia	*Linaria genistifolia* ssp. *dalmatica*	male
22	11	Macedonia	*Linaria genistifolia* ssp. *dalmatica*	female
23	12	Germany	*Linaria vulgaris*	male
24	12	Germany	*Linaria vulgaris*	female

Figure 9. Cluster analysis (UPGMA) of Nei's (1978) genetic distances for all 12 populations divided by sex – output from TFPGA software (Miller 1997).

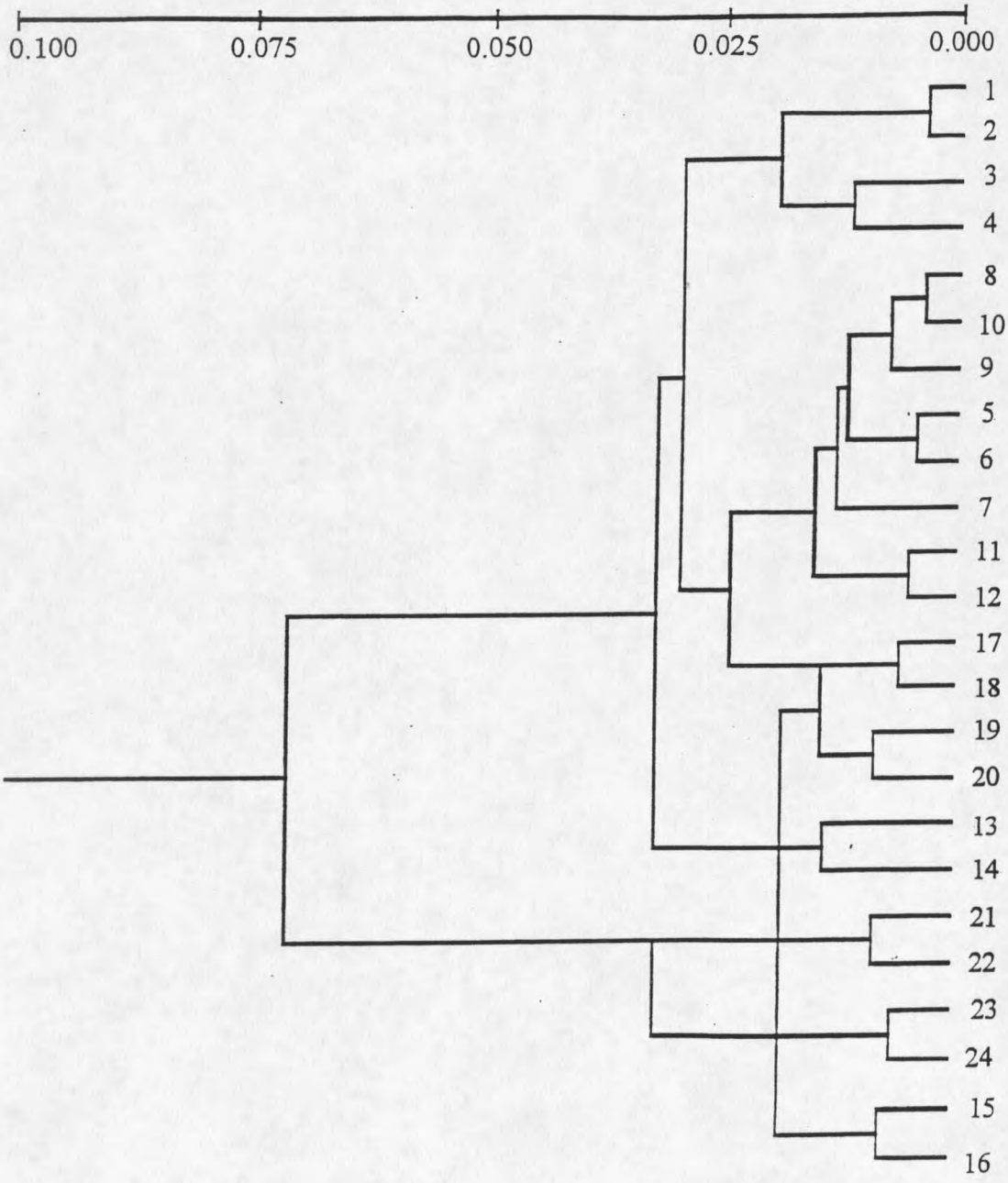


Table 11. Node information for UPGMA cluster analysis of all 12 *Brachypterolus pulicarius* populations divided by sex.

Node	Distance	Includes Populations
1	0.0035	1 2
2	0.0122	3 4
3	0.0197	1 2 3 4
4	0.0036	8 10
5	0.0077	8 9 10
6	0.0043	5 6
7	0.0126	5 6 8 9 10
8	0.0137	5 6 7 8 9 10
9	0.0049	11 12
10	0.0159	5 6 7 8 9 10 11 12
11	0.0062	17 18
12	0.0092	19 20
13	0.0243	5 6 7 8 9 10 11 12 17 18
14	0.0295	1 2 3 4 5 6 7 8 9 10 11 12 17 18
15	0.0148	13 14
16	0.0322	1 2 3 4 5 6 7 8 9 10 11 12 13 14 17 18
17	0.0091	21 22
18	0.0068	23 24
19	0.0711	1 2 3 4 5 6 7 8 9 10 11 12 13 14 17 18 21 22
20	0.0151	17 18 19 20
21	0.0080	15 16
22	0.0191	15 16 17 18 19 20
23	0.0321	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

In order to examine possible patterns of genetic variability at the individual level a third cluster analysis was run. This UPGMA analysis conducted with a similarity matrix for all 411 individuals using Dice's coefficient resulted in a dendrogram (back pocket) with individuals having high genetic similarities. Most individuals were more than 65% similar. In this very large dendrogram, no consistent commonalities between individuals in each cluster were found. In certain areas groups of individuals did cluster by host plant, site, and sex indicating some shared similarity between individuals.

However, individuals did not consistently cluster by host plant, overall. There was also no general clustering by geographic isolation, nor was consistent clustering by sex was found. These results did not suggest the presence of host races in *B. pulicarius.*

Table 12. Results of bootstrapping UPGMA analysis for all 12 Brachypterolus pulicarius populations divided by sex (1000 permutations).

Node	Proportion of similar replicates
1	0.9810
2	0.7840
3	0.3740
4	0.6580
5	0.5690
6	0.8510
7	0.1730
8	0.3560
9	0.9730
10	0.4060
11	0.9390
12	0.5830
13	0.0610
14	0.0590
15	0.6070
16	0.0390
17	0.7520
18	0.8680
19	0.0160
20	0.2330
21	0.8470
22	0.2200
23	1.0000

Because cluster analyses resulted in inconclusive results and some unexpected groupings, an AMOVA was run to determine if populations were significantly different and to quantify their differences. For the AMOVA, most of the variability was explained by within population variation. A small, but significant portion was contained among populations. A negative value was obtained with a non-significant *P*-value for the populations grouped by host plant (Table 13). The results indicate that nearly all the

variability in the studied *B. pulicarius* is accounted for by individual variation. A small, but significant portion of the variability is explained by population differences and none of the variation can be explained by the host plant differences.

Table 13. AMOVA results table for all North American *Brachypterolus pulicarius* populations divided into groups by host plant.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of variation
Among groups	1	57.141	-0.45347 Va	-1.74
Among populations	8	1101.353	3.17287 Vb	12.15
Within populations	351	8212.564	23.39762 Vc	89.59
Total:	360	9371.058	26.11712	
Fixation Indices	F_{SC}:	0.11941		
	F_{ST}:	0.10412		
	F_{CT}:	-0.01736		

Significance Tests (1023 Permutations)

Vc and F_{ST}:	P(random value > observed value) = 0.00000
	P(random value = observed value) = 0.00000
	P(random value >= observed value) = 0.00000 +/- 0.00000
Vb and F_{SC}:	P(random value > observed value) = 0.00000
	P(random value = observed value) = 0.00000
	P(random value >= observed value) = 0.00000 +/- 0.00000
Va and F_{CT}:	P(random value > observed value) = 0.96188
	P(random value = observed value) = 0.01075
	P(random value >= observed value) = 0.97263 +/-0.00532

Thus far none of the observed patterns of variability in *B. pulicarius* could be attributed to host plant differences. Because of this, a Mantel test was run to examine geographic isolation, another potential explanation for the observed relationships. For the Mantel test, the null hypothesis stated that the two matrices were not correlated, or that there was no relationship between the genetic and geographic distances for the sites.

For all North American sites (Figure 14) the *P*-values of *P*=0.8340 for the significance tests was not significant. The r-value of r = -0.1630 for the scatterplot (Figure 10) of the scatter plot indicates a poor fit of the regression line. Both results indicate failure to reject the null hypothesis, or that there is no significant correlation between genetic and geographic distance for the collected *Brachypterolus pulicarius* populations. For the second analysis with only North American populations (Table 15, Figure 11) the results were similar, with non-significant *P*-values and a poor fit of the scatter plot, again indicating failure to reject the null hypothesis that the two matrices were not correlated. Overall, geographic isolation did not have strong correlation with the observed patterns of genetic variability.

Table 14. Mantel test results for comparisons of genetic distance and geographic distances matrices (Figures 6 and 7) for all North American populations of *Brachypterolus pulicarius*.

Comparison of two 10 x 10 matrices	
Correlation of two matrices:	r = -.1630
Z from original data:	Z = 672.9197
Avg Z after 999 permutations:	Z_{ave} = 723.8281
Significance Test – 999 Permutations:	
[a] Upper tail probability: *P* = 0.8340	
[b] Lower tail probability: *P* = 0.1670	

[a] 833 out of the 999 permutated data sets had Z-scores greater than or equal to the original Z-score
[b] 166 out of the 999 permuted data sets had Z-scores less than or equal to the original Z-score

Figure 10. Scatterplot of Mantel test for all North American populations of *Brachypterolus pulicarius* – output from TFPGA software (Miller 1997).

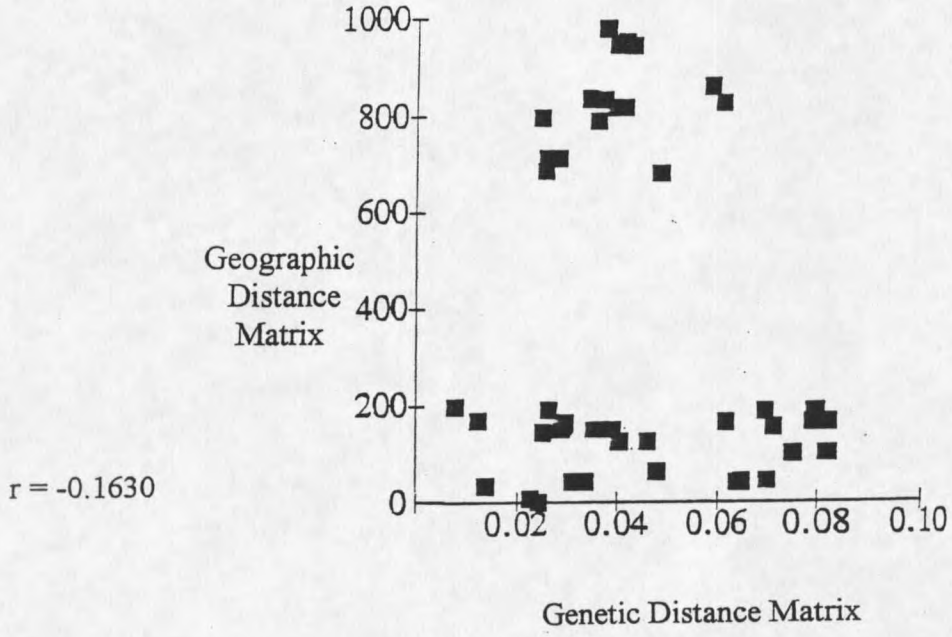
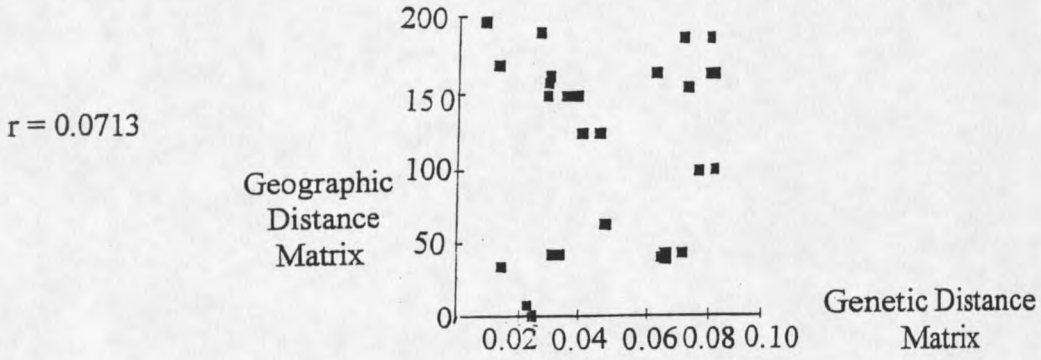


Table 15. Mantel test results for comparison of genetic and geographic distance matrices for all 10 U.S. populations of *Brachypterolus pulicarius*.

Comparison of two 8 x 8 matrices	
Correlation of two matrices:	r = 0.0713
Z from original data:	Z = 152.1392
Avg. Z after 999 permutations:	Z_{ave} = 149.1929
Significance Test – 999 Permutations:	
[a] Upper tail probability:	P = 0.2180
[b] Lower tail probability:	P = 0.7830

[a] 217 out of the 999 permutated data sets had Z-scores greater than or equal to the original Z-score.
[b] 782 out of the 999 permuted data sets had Z-scores less than or equal to the original Z-score.

Figure 11. Scatterplot of Mantel test for all U.S. populations of *Brachypterolus pulicarius* – output from TFPGA software (Miller 1997).



Behavioral Assays

The specific null hypothesis for the behavioral assays is that there is no behavioral difference between beetles collected from *Linaria vulgaris* (yellow toadflax) and those collected from *L. genistifolia* ssp. *dalmatica* (Dalmatian toadflax).

Volatile Collections

Volatile collections were run to determine variability in host plant production and to examine the potential for host preference in *B. pulicarius*. The results of plant volatile collections are presented in Tables 16-24. For all tables R.T. represents retention time from GC-MS analysis. Quantities of collected compounds were normalized by plant weight to determine the given production rate in nanograms of volatile produced per gram of plant material (weighed at the conclusion of the VCS trial) per hour (ng/g/hr). Sometime during the spring of 2002, the populations at the sympatric sites near Townsend (Sites #1 and #2) were treated with an unidentified herbicide. While the effects of herbicide treatment are unknown, it is conceivable that they would have an

effect on plant physiology and potentially on volatile production. Because of this complication the lists of collected volatiles were included in the results but were not included in the statistical comparisons. Each replication represents an individual plant.

Table 16. Volatiles collected via VCS from *Linaria vulgaris* (not flowering) June 5, 2002 from Site #9 (ng/g/hr)

R.T.	Tentative ID	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Mean	St. Error
6.93	3 – Hexanol	1.01	0.83	0.5	0.37	0.79	0.93	2.84	1.04	0.83
7.31	2 – Hexenol	0	0	0	0	0	0	0	0.00	0.00
12.55	3 – Octanol	1.36	4.03	2.22	0.38	1.05	1.76	1.37	1.74	1.16
13.10	3 - Hexenol acetate	11.5	5.96	1.69	1.17	3.86	2.39	38.23	9.26	13.26
13.40	Hexyl acetate	0.88	1.75	0	0.33	0.15	0.79	2.32	0.89	0.86
13.54	2 - Hexanol acetate	1.55	5.11	0.90	1.05	1.64	1.62	4.70	2.37	1.76
14.06	2 – Ethyl - 1 -Hexanol	1.95	3.35	1.32	3.62	1.82	0.31	1.42	1.97	1.16
14.48	1R - α - Pinene	0.38	0.42	0	0	0.62	0.35	1.69	0.49	0.57
14.96	3 – careen	2.47	1.49	0	0.42	2.85	0.93	6.16	2.05	2.09
20.66	Ethyl Benzoate	1.37	2.77	0.83	0.13	0.12	0.11	1.68	1.00	1.01
21.72	Methyl Salicylate	3.55	1.16	1.18	1.56	2.44	1.73	2.18	1.97	0.84
31.68	Caryophylene	0.14	0	0	0.44	0.18	0	0.34	0.16	0.18
34.23	β - cubebene	1.47	1.88	0.41	0.18	0.86	0.7	2.22	1.10	0.77

Table 17. Volatiles collected via VCS from *Linaria vulgaris* (not flowering) June 11, 2002 at Site #9 (ng/g/hr)

R.T.	Tentative ID	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Mean	St. Error
6.93	3 – Hexanol	0.73	0.67	0.76	0.30	0.43	0.41	0.55	0.19
7.31	2 – Hexenol	0.22	0.11	0.21	0.16	0.14	0.15	0.17	0.04
12.55	3 – Octanol	0.07	0.09	0.06	0.08	0.10	0.08	0.08	0.01
13.10	3 - Hexenol acetate	3.85	4.20	1.30	2.00	3.11	3.06	2.92	1.10
13.40	Hexyl acetate	0.19	0.12	0.11	0.13	0.15	0.14	0.14	0.03
13.54	2 - Hexanol acetate	0.51	0.26	0.34	0.24	0.27	0.29	0.32	0.10
14.06	2 - Ethyl – 1 -Hexanol	0.06	0.10	0.05	0.12	0.17	0.14	0.11	0.05
14.48	1R - α - Pinene	0.10	0.09	0.08	0.04	0.14	0.11	0.09	0.03
14.96	3 – careen	0.24	0.18	0.20	0.07	0.27	0.16	0.19	0.07
20.66	Ethyl Benzoate	0.08	0.07	0.09	0.15	0.14	0.11	0.11	0.03
21.72	Methyl Salicylate	0.31	0.21	0.28	0.21	0.28	0.23	0.25	0.04
31.68	Caryophylene	0.11	0.05	0.05	0.06	0.08	0.07	0.07	0.02
34.23	β - cubebene	0.16	0.08	0.13	0.07	0.09	0.10	0.11	0.03

Table 18. Volatiles collected via VCS from *Linaria genistifolia* ssp. *dalmatica* (not flowering) June 6, 2002 at Site #7 (ng/g/hr)

R.T.	Tentative ID	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Mean	St. Error
6.93	3 - Hexanol	0.08	0.17	0.11	0.16	0.32	0.49	0.22	0.16
12.55	3 - Octanol	0.01	0.04	0.08	0.08	0.09	0.09	0.07	0.03
13.1	3 - Hexenol acetate	0.96	1.69	0.71	1.32	3.28	3.49	1.91	1.19
13.4	Hexyl acetate	0.05	0.10	0.06	0.04	0.13	0.17	0.09	0.05
13.54	2 - Hexanol acetate	0.09	0.26	0.18	0.15	0.19	0.39	0.21	0.10
14.06	2 - Ethyl - 1 -Hexanol	0.12	0.11	0.17	0.16	0.15	0.16	0.15	0.02
14.48	1R - α - Pinene	0.07	0.09	0.06	0.13	0.12	0.17	0.11	0.04
14.96	3 - carene	0.04	0.26	0.22	0.14	0.41	0.76	0.31	0.25
20.66	Ethyl Benzoate	0.21	0.45	0.45	0.15	0.21	0.14	0.27	0.14
21.72	Methyl Salicylate	0.03	0.02	0.03	0.01	0.01	0.03	0.02	0.01
31.68	Caryophylene	0.32	3.59	0.40	1.13	0.38	0.61	1.07	1.27
34.23	β - cubebene	0.31	1.97	0.30	0.51	0.30	0.47	0.64	0.66

Table 19. Volatiles collected via VCS from *Linaria genistifolia* ssp. *dalmatica* (not flowering) June 12, 2002 at Site #7 (ng/g/hr)

R.T.	Tentative ID	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Mean	St. Error
6.93	3 - Hexanol	0.07	0.001	0.16	0.29	0.18	0.73	0.10	0.22	0.24
12.55	3 - Octanol	0	0	0	0	0	0	0	0.00	0.00
13.1	3 - Hexenol acetate	0.38	1.41	1.76	3.28	0.72	5.93	0.74	2.03	1.97
13.4	Hexyl acetate	0	0.14	0.20	0	0	0.22	0.001	0.08	0.10
13.54	2 - Hexanol acetate	0.05	0.18	0.40	0.28	0.10	0.33	0.08	0.20	0.14
14.06	2 - Ethyl - 1 -Hexanol	1.59	0	0.43	0.51	0.40	0.18	0.12	0.46	0.53
14.48	1R - α - Pinene	0	0.2	0.55	0.42	0.39	0.6	0.10	0.32	0.23
14.96	3 - carene	0.17	0.46	3.09	0.83	1.39	2.09	0.25	1.18	1.08
20.66	Ethyl Benzoate	0.26	1.00	1.04	0.19	0.41	0.86	0.25	0.57	0.38
21.72	Methyl Salicylate	0	0	0	0	0	0	0	0.00	0.00
31.68	Caryophylene	0	0.17	0.2	3.81	2.69	1.67	0.61	1.31	1.47
34.23	β - cubebene	0	0.31	0.73	2.05	2.41	3.14	0.36	1.29	1.23

Table 20. Volatiles collected via VCS from *Linaria vulgaris* (not flowering) June 4, 2002 at Site #1 (ng/g/hr) – reduced number of replications due contamination

R.T.	Tentative ID	Rep 1	Rep 2	Rep 3	Rep 4	Mean	St. Error
6.93	3 - Hexenol	1.3	0.84	0.63	0.49	0.82	0.35
[a]	6-methyl-5-hepten-2-one	0.97	0.76	1.15	0.55	0.86	0.26
[a]	b - terpinene	0	0	0	1.64	0.41	0.82
12.55	3 - Octanol	0.11	0.06	2.16	0.52	0.71	0.99
13.1	3 - Hexenol acetate	24.94	12.99	22.38	20.09	20.10	5.14
13.4	Hexyl acetate	1.21	0.47	1.03	0.81	0.88	0.32
13.54	2 - Hexenol acetate	1.31	0.51	1.05	0.87	0.94	0.34
14.48	1R - a - Pinene	1.73	0.69	1.08	0.8	1.08	0.47
14.96	3 - carene	9.52	3.02	6.54	4.77	5.96	2.77
31.68	Caryophylene	3.87	1.42	2.72	4.22	3.06	1.27
34.23	b - cubebene	4.28	1.61	3.85	5.73	3.87	1.71

a – missing data

Table 21. Volatiles collected via VCS from *Linaria genistifolia* ssp. *dalmatica* (not flowering) June 4, 2002 at Site #2 (ng/g/hr) – reduced number of replications due to contamination

R.T.	Tentative ID	Rep 1
6.93	3 – Hexenol	2.32
[a]	6-methyl-5-hepten-2-one	1.36
[a]	β - terpinene	0
12.55	3 – Octanol	0
13.1	3 - Hexenol acetate	27.00
13.4	Hexyl acetate	3.34
13.54	2 - Hexenol acetate	11.19
14.48	1R.-α - Pinene	0.36
14.96	3 – carene	16.48
31.68	Caryophylene	1.29
34.23	β - cubebene	7.26

a – missing data

Table 22. Volatiles collected via VCS from *Linaria vulgaris* (flowering) August 22, 2002 at Yellowstone National Park (ng/g/hr) – reduced number of replications due to contamination.

R.T.	I.D.	Rep 1	Rep 2	Rep 3	Mean	St. Error
6.93	3-Hexenol	1.44	1.05	1.11	1.20	0.16
8.7	β-Pinene	4.83	1.10	0.39	2.11	1.82
8.78	1-Octen-3-ol	2.34	1.62	3.13	2.36	0.51
10.16	6-methyl-5-hepten-2-one	3.67	7.56	5.14	5.46	1.40
12.55	3-Octanol	0.55	1.32	3.53	1.80	1.15
13.1	3-Hexenol actetate	3.82	3.62	14.28	7.24	4.69
14.48	1R-α-Pinene	1.77	1.58	2.85	2.07	0.52
14.96	3-carene	1.92	0.06	0.09	0.69	0.82
15.05	Ocimene	9.96	5.69	16.38	10.68	3.80
15.25	Acetophenone	0.69	61.32	1.43	21.15	26.78
31.68	Caryophylene	2.30	8.78	2.48	4.52	2.84
33.91	Geranyl acetate	2.89	10.47	4.57	5.98	3.00

Table 23. Volatiles collected via VCS from *Linaria vulgaris* (flowering) August 22, 2002 at Site #9 (ng/g/hr) – reduced number of replications due to contamination

R.T.	I.D.	Rep 1	Rep 2	Rep 3	Mean	St. Error
6.93	3-Hexenol	1.39	5.73	3.62	3.58	1.46
8.7	β-Pinene	1.36	1.04	1.11	1.17	0.13
8.78	1-Octen-3-ol	1.01	0.61	1.23	0.95	0.23
10.16	6-methyl-5-hepten-2-one	4.44	2.84	2.09	3.12	0.88
12.55	3-Octanol	0.98	2.04	1.93	1.65	0.45
13.1	3-Hexenol actetate	6.68	10.96	10.25	9.30	1.74
14.48	1R-α-Pinene	0.08	0.02	0.09	0.06	0.03
14.96	3-carene	0.77	0.31	0.82	0.63	0.22
15.05	Ocimene	3.03	0.95	1.00	1.66	0.91
15.25	Acetophenone	2.96	6.91	11.76	7.21	3.03
31.68	Caryophylene	12.62	4.45	10.98	9.35	3.27
33.91	Geranyl acetate	8.50	2.22	3.37	4.70	2.54

Table 24. Volatiles collected via VCS from *Linaria genistifolia* ssp. *dalmatica* (flowering) August 22, 2002 at Yellowstone National Park (ng/g/hr)

R.T.	I.D.	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Mean	St.Error
6.93	3-Hexenol	0.72	0.56	0.23	0.96	0.38	1.02	0.645	0.26
8.70	β-Pinene	0.62	0.31	0.51	0.91	13.2	4.11	3.28	3.59
8.78	1-Octen-3-ol	0.09	0.11	0.06	0.03	0.59	0.21	0.182	0.15
10.16	6-methyl-5-hepten-2-one	2.97	2.48	1.42	2.91	5.18	4.06	3.17	0.97
12.55	3-Octanol	0.03	0.11	0.21	0.07	0.45	0.51	0.23	0.17
13.10	3-Hexenol actetate	2.89	2.13	1.96	2.03	3.66	3.02	2.615	0.58
14.25	D-Limonene	2.11	1.55	64.17	4.02	31.62	23.11	21.1	18.54
14.48	1R-α-Pinene	0.36	0.27	1.41	0.31	1.11	0.86	0.72	0.41
14.96	3-carene	0.47	0.63	0.81	0.91	0.41	0.77	0.67	0.16
15.05	Ocimene	1.12	0.66	1.33	1.09	1.09	2.02	1.22	0.30
15.25	Acetophenone	0.44	0.32	0.94	0.55	0.51	0.36	0.52	0.15
31.68	Caryophylene	0.63	0.51	2.34	0.98	1.11	1.26	1.14	0.44
33.91	Geranyl acetate	2.93	2.06	1.89	1.06	2.09	2.61	2.11	0.44

In comparisons from the remaining sites, the volatile composition of both species of toadflax was generally dominated by one compound, 3-Hexenol acetate. Analyses of variance (ANOVAs) were run to compare means for individual compounds to examine temporal, spatial, and species to species differences. Table 25 lists the samples used and the questions being examined as well as the resulting p-values for each of the AMOVA comparisons. Their significance are summarized in Table 26. Overall, significance varied with the major compound (3-Hexenol acetate) not being significantly different for the two species. Some significant temporal and/or spatial as well as species to species differences were obtained for minor compounds but their biological importance is questionable. One compound, 2-Hexenol was novel to yellow toadflax collected from

Table 25. *P*-values for ANOVA comparisons of *Linaria* sp. volatile collections.

Compound	#1[a]	#2[b]	#3[c]	#4[d]	#5[e]	#6[f]	#7[g]	#8[h]	#9[i]
3 - Hexanol	0.0000	0.9801	0.0371	0.0214	0.0298	0.1317	0.0035	0.0107	0.0032
2 - Hexenol	0.0000	-[j]	-[j]	0.0000	-[j]	-[j]	0.0000	-[j]	0.0000
3 - Octanol	0.0000	0.0003	0.0049	0.0000	0.0340	0.8827	0.0251	0.0077	0.0071
3 - Hexenol acetate	0.1752	0.1957	0.2058	0.3493	0.0887	0.6136	0.5885	0.6623	0.4239
Hexyl acetate	0.0020	0.8080	0.0459	0.1955	-[j]	-[j]	0.0458	0.0680	0.0537
2 - Hexanol acetate	0.0000	0.9183	0.0126	0.1136	-[j]	-[j]	0.0074	0.0031	0.0114
2 – Ethyl - 1 -Hexanol	0.0000	0.1750	0.0029	0.1331	-[j]	-[j]	0.0007	0.0564	0.0014
1R.-α - Pinene	0.0000	0.0436	0.1290	0.0334	0.0102	0.0072	0.0001	0.0093	0.1518
3 - carene	0.0668	0.0801	0.0687	0.0472	0.9572	0.9333	0.1363	0.0987	0.0883
Ethyl Benzoate	0.0060	0.0911	0.1067	0.0123	-[j]	-[j]	0.0499	0.0026	0.0548
Methyl Salicylate	0.0000	0.0001	0.0002	0.0000	-[j]	-[j]	0.0000	0.0000	0.0001
Caryophylene	0.0000	0.7655	0.0841	0.0657	0.0523	0.2150	0.0000	0.9356	0.0000
β - cubebene	0.0062	0.2770	0.2753	0.0393	-[j]	-[j]	0.0038	0.0441	0.0061
β - Pinene	0.0720	-[j]	-[j]	-[j]	0.7220	0.5347	0.0102	0.0942	0.0000
1-Octen-3-ol	0.0000	-[j]	-[j]	-[j]	0.0002	0.0402	0.0000	0.0212	0.0000
6-methyl-5-hepten-2-one	0.0000	-[j]	-[j]	-[j]	0.0719	0.1540	0.0000	0.0000	0.0000
Acetophenone	0.0307	-[j]	-[j]	-[j]	0.1608	0.5291	0.1223	0.0000	0.0001
D-Limonene	0.0054	-[j]	-[j]	-[j]	0.1930	-[j]	-[j]	0.0224	-[j]
Ocimene	0.0000	-[j]	-[j]	-[j]	0.0025	0.0471	0.0000	0.0000	0.0004
Geranyl acetate	0.0000	-[a]	-[a]	-[a]	0.0415	0.6917	0.0004	0.0000	0.0003

[a] 1 = comparison of all samples to examine overall variability
[b] 2 = comparison of all Site #7 samples to examine temporal variability
[c] 3 = comparison of samples from Site #7 (6/5/02) to Site #9 (6/6/02) to examine spatial/species-to-species variability
[d] 4 = comparison of samples from Site #7 (6/11/02) to Site #9 (6/12/02) to examine spatial/species-to-species variability
[e] 5 = comparison of YNP site *Linaria vulgaris* to *L. genistifolia* ssp. *dalmatica* samples to examine species-to-species variability
[f] 6 = comparison of YNP *L. vulgaris* samples to Site #9 late season samples to examine spatial variability within *L. vulgaris* samples
[g] 7 = comparison of all *L. vulgaris* samples to examine within species temporal/spatial variability
[h] 8 = comparison of *L. genistifolia* ssp. *dalmatica* samples to examine temporal/spatial variability
[i] 9 = comparison of all samples from Site #9 to examine overall variability at this site
[j] missing data

site #9 on 6/11/02. Another compound, D limonene appeared only in Dalmatian toadflax samples collected from the Yellowstone site on 8/22/02. Its production was highly variable and appeared in very large quantities in some samples. Overall, however, its

presence was not significant when compared to samples in which it was lacking. These results indicate that volatile production is highly variable within a species, throughout the season, and within a site. Overall, however, both species are generally dominated by one compound and the other species-to-species differences may not be significant enough to serve as a mechanism for host plant selection.

Table 26. Summary of significant *P*-values from ANOVA comparisons of *Linaria* sp. volatile collections. * indicates a significant value, n/s indicates a lack of significance

Compound	#1	#2	#3	#4	#5	#6	#7	#8	#9
3 – Hexanol	*	n/s	*	*	*	n/s	*	*	*
2 – Hexenol	*	-[a]	-[a]	*	-[a]	-[a]	*	-[a]	*
3 – Octanol	*	*	*	*	*	n/s	*	*	*
3 - Hexenol acetate	n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s
Hexyl acetate	*	n/s	*	n/s	-[a]	-[a]	*	n/s	n/s
2 - Hexanol acetate	*	n/s	*	n/s	-[a]	-[a]	*	*	*
2 - Ethyl - 1 -Hexanol	*	n/s	*	n/s	-[a]	-[a]	*	n/s	*
1R.-α - Pinene	*	*	n/s	*	*	*	*	*	n/s
3 – careen	n/s	n/s	n/s	*	n/s	n/s	n/s	n/s	n/s
Ethyl Benzoate	*	n/s	n/s	*	-[a]	-[a]	*	*	n/s
Methyl Salicylate	*	*	*	*	-[a]	-[a]	*	*	*
Caryophylene	*	n/s	n/s	n/s	n/s	n/s	*	n/s	*
β - cubebene	*	n/s	n/s	*	-[a]	-[a]	*	*	*
β - Pinene	n/s	-[a]	-[a]	-[a]	n/s	n/s	*	n/s	*
1-Octen-3-ol	*	-[a]	-[a]	-[a]	*	*	*	*	*
6-methyl-5-hepten-2-one	*	-[a]	-[a]	-[a]	n/s	n/s	*	*	*
Acetophenone	*	-[a]	-[a]	-[a]	n/s	n/s	n/s	*	*
D-Limonene	*	-[a]	-[a]	-[a]	n/s	-[a]	-[a]	*	-[a]
Ocimene	*	-[a]	-[a]	-[a]	*	*	*	*	*
Geranyl acetate	*	-[a]	-[a]	-[a]	*	n/s	*	*	*

[a] - comparison of zeros only due to lack of compound in samples being compared

Y-Tube Olfactometry

The Y-tube olfactometer was utilized to examine possible host plant preference in *B. pulicarius*. During the course of the study, over 1000 Y-tube trials were run. However, due to continual discovery of confounding variables, as discussed previously, most trials were considered 'iterations' of technique development. During 2001, nearly 200 plant versus blank trials were run (Table 27). Overall, results were not significant, with individuals randomly orienting within the apparatus. However, trials at the end of the season produced significant Chi-squared values, indicating non-random selection of the host plant stimulus. These trials were done with a small amount of plant material and it is possible that the shift in response was due to a dose response and/or that beetle responsiveness had changed in some way at the end of the season. Some very preliminary plant versus plant trials were completed (Table 28). These trials did not produce significant results.

During the 2002 season of behavioral work, 698 plant versus blank trials were run, with only about 337 using the final preferred technique. The results of these trials are shown in Table 29. For Dalmatian toadflax trials a highly significant Chi-squared value of 12.9643 was obtained, indicating significant orientation away from the host plant. The cause of this repellency is not known. Overall, for yellow toadflax trials, as indicated by the lack of significant Chi-squared values, consistent orientation to the plant was never

Table 27. *Brachypterolus pulicarius* y-tube olfactometry trials with plant (*Linaria* sp.) versus blank stimuli (2001).

Trial Number	Number Run	Host Plant Species	# Oriented To Plant	Chi-Square Value	Significance
1	7	*Linaria vulgaris*	3	0	$P > 0.05$
2	7	*L. vulgaris*	2	0.5714	$P > 0.05$
3	25	*L. vulgaris*	9	1.4400	$P > 0.05$
4	14	*L. vulgaris*	6	0.0714	$P > 0.05$
5	14	*L. genisifolia* ssp. *dalmatica*	4	1.7857	$P > 0.05$
6	11	*L. genisifolia* ssp. *dalmatica*	7	0.3636	$P > 0.05$
7	24	*L. vulgaris*	13	0.0417	$P > 0.05$
8	23	*L. vulgaris*	9	0.0696	$P > 0.05$
9	24	*L. vulgaris*	11	0.0417	$P > 0.05$
Subtotal:	149	n/a	64	2.6846	$P > 0.05$
10[a]	23	*L. vulgaris*	19	8.5217	$P < 0.01$*
11[a]	25	*L. vulgaris*	17	2.5600	$P > 0.05$
Subtotal:	48	n/a	36	11.0200	$P < 0.01$*
L. vulgaris Subtotal:	172	*L. vulgaris*	89	0.1453	$P > 0.05$
L. genistifolia ssp. *dalmatica* Subtotal:	25	*L. genisifolia* ssp. *dalmatica*	11	0.1600	$P > 0.05$
Total:	197	n/a	100	0.0203	$P > 0.05$

[a] Began using a small sprig of flowers wrapped with cotton as plant stimulus.

Table 28. Preliminary results of y-tube olfactometry trials of *Brachypterolus pulicarius* with *Linaria genistifolia* ssp. *dalmatica* (D) versus *L. vulgaris* (y) as stimuli (2001)

Trial Number	Number Run	Trial	Response	Responders	Chi-Squared Value	Significance	Collection Location
1	2	D vs. y	to y	1	0.50	$P > 0.05$	Belgrade
2	14	D vs. y	to y	11	3.5	$P > 0.05$	Belgrade
3	13	D vs. y	to y	6	0	$P > 0.05$	Belgrade
4	12	D vs. y	to y	6	0.0833	$P > 0.05$	Belgrade
5	13	D vs. y	to y	4	1.2308	$P > 0.05$	Belgrade
Total:	54	D vs. y	to y	28	0.0185	$P > 0.05$	Belgrade
6	5	D vs. y	to y	3	0	$P > 0.05$	Mallards' rest
7	11	D vs. y	to y	5	0	$P > 0.05$	Mallards' rest
8	13	D vs. y	to y	7	0	$P > 0.05$	Mallards' rest
9	14	D vs. y	to y	3	3.5	$P > 0.05$	Mallards' rest
10	21	D vs. y	to y	13	0.7619	$P > 0.05$	Mallards' rest
Total:	64	D vs. y	to y	31	0.0156	$P > 0.05$	Mallards' rest

achieved. However, during individual days and during certain periods of the summer significant trials were conducted. During the period of trials 16-19, 90 trials were run and 59 of the beetles were attracted to the plant. The resulting Chi-squared value for these trials is 8.1 which is highly significant. Here, apparent strong attraction to the host plant was occurring. However, during this time period, individual plant attractiveness appeared highly variable. During trial 17, the first two plants resulted in 14 of 19 individuals orienting toward the plant. The last plant only yielded 5 out of 10 positive responders. For trial 18 the first two plants again yielded 14 of 19 positive responses. The final plant resulted in only 3 of 6 positive responses. For trial 19, a total 6 of 8 individuals responded positively to the plant. For the early morning-only runs during this time period the result is a total of 34 of 46 individuals orienting to the host plant. The Chi-squared value here is a highly significant 9.587. During trial 6, apparent attraction occurred for all but one plant. For the first several plants the result was 22 of 27 individuals orienting toward the plant (Chi-squared value = 9.481). Addition of the final plant resulted in a response of 24 out of 34 individuals to the plant and a lower, but still significant overall Chi-squared value (4.9706). These values indicate the high level of response variability. Because of the overall inconsistency and inability to obtain a significant Chi-squared value, actual plant versus plant trials were not run to examine host plant species preference in *B. pulicarius*.

Table 29. *Brachypterolus pulicarius* y-tube olfactometry trials with plant (*Linaria* sp.) versus blank stimuli (2002).

Trial Number	Number Run	Host Plant	# Oriented To Plant	Chi-Squared Value	Significance
1	52	*L. vulgaris*	24	0.1731	$P > 0.05$
2	38	*L. vulgaris*	22	0.6579	$P > 0.05$
3	45	*L. vulgaris*	23	0	$P > 0.05$
4	21	*L. vulgaris*	10	0	$P > 0.05$
5	11	*L. vulgaris*	5	0	$P > 0.05$
6	34	*L. vulgaris*	24	4.9706	$P < 0.05$*
7	42	*L. genisifolia* ssp. *dalmatica*	10	10.5	$P < 0.01$*
8	43	*L. vulgaris*	17	1.4884	$P > 0.05$
9	33	*L. vulgaris*	17	0	$P > 0.05$
10	42	*L. genisifolia* ssp. *dalmatica*	15	2.8810	$P > 0.05$
L. genisifolia ssp. *Dalmatica* Subtotal	84	*L. genisifolia* ssp. *dalmatica*	25	12.9643	$P < 0.01$*
L. vulgaris Subtotal:	277	n/a	142	0.1769	$P > 0.05$
11[a]	30	*L. vulgaris*	15	0.0333	$P > 0.05$
12[a]	16	*L. vulgaris*	10	0.5625	$P > 0.05$
13[a]	24	*L. vulgaris*	9	1.0417	$P > 0.05$
14[a b]	38	*L. vulgaris*	13	3.1842	$P > 0.05$
15[a b]	28	*L. vulgaris*	11	0.8929	$P > 0.05$
16[a b]	28	*L. vulgaris*	17	0.8929	$P > 0.05$
17[a b]	29	*L. vulgaris*	19	2.2069	$P > 0.05$
18[a b]	25	*L. vulgaris*	17	2.56	$P > 0.05$
19[a b]	8	*L. vulgaris*	6	1.125	$P > 0.05$
20[a b]	30	*L. vulgaris*	15	0.0333	$P > 0.05$
21[a b]	9	*L. vulgaris*	4	0	$P > 0.05$
22[a b]	13	*L. vulgaris*	6	0	$P > 0.05$
23[a b]	24	*L. vulgaris*	7	3.375	$P > 0.05$
24[a b]	15	*L. vulgaris*	8	0	$P > 0.05$
25[a b]	5	*L. vulgaris*	2	0	$P > 0.05$
26[a b]	15	*L. vulgaris*	7	0	$P > 0.05$
Subtotal:	337	n/a	166	0.0742	$P > 0.05$

[a] Used a metal rod to support plant material.
[b] Trials occurred in the morning only.

CHAPTER 4

DISCUSSION

Background Material

Brachypterolus pulicarius were collected from a limited number of sites in the US and from even fewer sites in Canada and Europe. The beetles utilized for genetic analyses were all collected during a single collection trip, providing only a 'snap-shot' of each population. Collection methods for behavioral and genetic techniques involved collecting the beetles that were contacted while at the site. The potential exists for bias resulting from only collecting those beetles that tend to rest high on the plants or in the blossoms. Additionally, because the biology of the beetles is not well known, the age and life stage of the collected beetles cannot be stated. Plants used for behavioral assays were variable and it was not possible to control for this diversity since its mechanisms are not known. The results of genetics and behavioral trials may, therefore, be influenced by the chosen sampling techniques and the resulting material.

Molecular Genetics

All molecular genetic analyses were run for AFLP results from only one primer set. Six primer sets were screened for general amount of polymorphism and the set with the largest amount of readily detectable polymorphism was selected for AFLP analysis. However, it is unknown how well the chosen primers and the AFLP technique represent

the actual genetic variability of the individuals and populations. Because of this uncertainty, the results of these analyses can only be considered, with confidence, to represent the conditions observed with this single primer set. Further studies with additional primer sets will allow more confident inference about trends seen here.

Descriptive statistics were computed to characterize the amount of genetic diversity in the analyzed *B. pulicarius*. The resulting average heterozygosity values were variable across populations. No clear differences were observed between the overall average heterozygosity for beetle populations collected from yellow toadflax and those collected from Dalmatian. North American and European populations also exhibited similar average hetrozygosities. Percent polymorphism was variable site-to-site. The average percent polymorphism under the 95% criterion for N. American sites was about 10% less than for European sites. The cause of this apparent decrease in polymorphism and whether it has significant implications for North American populations is unknown. It is possible, though, that such an increase in percent of monomorphic loci could be the result of a bottleneck resulting from a limited number of founding individuals or original introduction events. The resulting genetic drift, or "founder effect" (Hartl and Clark 1997) could lead to fixation of what were formerly rare alleles. A founder effect would lead to a relative decrease in percent polymorphism in the emigrant populations. Such an effect, however, would also be expected to result in lower average heterozygosity, which is not readily visible overall for the North American populations. The potential existence of decreased diversity in North American populations, however, encourages expansion of this study to include more European populations so that more effective comparisons can

be made. If decreased genetic diversity is a real phenomenon, it may have detrimental effects on the success of North American populations of *B. pulicarius* (Hartl and Clark 1997). In such a scenario, efforts to increase the diversity of North American populations may become of interest to biocontrol practitioners.

A UPGMA cluster analysis was run to determine how individual beetles were related. When all individuals were clustered by genetic similarity using Dice's coefficient, the resulting dendrogram showed no consistent, overall trends in commonality of individuals clustered together. In certain areas, groups of individuals from one population did cluster out, and in other areas, groups of beetles from the same host plant species clustered together. In some areas, beetles grouped by sex. However, overall, all individuals were quite similar and no consistent trends were seen. Such results suggest that the populations consist of individuals that are variable but are overall somewhat homogeneous in their variability.

Cluster analysis among populations was utilized to reveal population groupings with Nei's genetic distance. No obvious commonalities were observed for the populations that clustered together. Because of this, the four major clusters cannot be easily explained. The exception was that the sites from Europe grouped together, and separate from the North American sites. This is most likely a result of their geographic isolation. One unexpected result was that the distance between the two sites was fairly small (0.02), which is lower than for most of the North American populations. Further analysis with more European populations will determine if this small distance represents a genetic homogeneity that is consistent with the origin populations. Within the largest

cluster of North American sites, the two sympatric sites from Townsend clustered closely together, as would be expected in a situation where they are likely not completely reproductively isolated. The rest of the North American sites, however, did not separate corresponding to geographic isolation, nor did they group by host plant. The branching of site #7 separate from the other North American sites was also unexpected. This site is a fairly isolated patch of Dalmatian toadflax along the Yellowstone River. Most of the site is within the floodplain and is prone to complete inundation during flood events. At one time, high densities of *B. pulicarius* were present there (Bryan FitzGerald - personal communication), but the site has since undergone major flooding and a sharp decrease in population density of both beetles and their host plants. Descriptive statistics did not show a significant difference in heterozygosity or percent polymorphism at this site as might be expected with a limited number of individuals surviving flood events. However, the effects of population fluctuation and potential inbreeding are not known, but may account, to some degree, for the individual branching of this site. Overall, results for the cluster analysis of all sites did not indicate host race differences.

General groupings obtained in the population-level analysis held for the UPGMA clustering by sex. The clustering indicated a lack of consistent sexual dimorphism, with males and females from one site having a smaller genetic distance between them than between groups of beetles of the same sex from other locations. For sites from Europe and Canada, however, members of the same sex from separate sites grouped together, indicating potential sexual dimorphism. Because only two sites were sampled from each location, the extent of this apparent dimorphism is unknown. If this dimorphism is

consistent for Canadian and European populations the causes of its apparent loss in U.S. populations is also unknown. However, it is conceivable that a loss in overall genetic diversity due to a founder effect could result in the loss of a sex-specific marker.

The AMOVA was run to quantify relationships in the cluster analysis and to characterize the partitioning of variability in *B. pulicarius*. The analysis resulted in partitioning of most of the variability within populations ($P = 0.0000$). A small, but significant portion of variability was accounted for among populations ($P = 0.0000$). A non-significant amount of variability was explained by the host plant groups ($P = 0.97263$). This indicates that populations are distinctly different, but that most of the variability in the species as a whole is accounted for by individual genetic variation. It also shows that host plant differences do not account for any significant variability which questions the presence of host races. This would tend to suggest that other factors must be contributing to most of the genetic variability among populations.

The Mantel test was run to further examine one potential contributing factor, geographic isolation. As discussed previously, neither of the UPGMA analyses provided clustering corresponding to geographic distance. The Mantel test results also indicated a significant lack of correlation between geographic and genetic distance matrices, further supporting the conclusion that geographic isolation is not a key element in the population similarities and differences (all $P > 0.1$). If geographic distance between sites was the primary factor affecting genetic distances the two matrices would be highly correlated. Further, if all of the studied populations were founded from a single location and then spread outward, becoming more separated, a strong correlation would be expected (Sokal

and Rohlf 1995). However, a large amount of migration between sites might decrease the genetic distance between them. Little is known about the introduction history of *B. pulicarius* in the Western US and Canada. It is possible that the multiple small introductions occurred and the Mantel test results indicate that the studied populations did not all originate from a single founder event. However, the lack of correlation may also indicate significant migration between populations. The migration abilities and patterns of the species are unknown.

Overall, results of genetic analyses indicate that host plant is not an important factor in explaining AFLP patterns. Analyses also indicate that geographic isolation does not appear to be a helpful factor in explaining the observed levels of genetic variation. Because of the lack of knowledge concerning the exact history and biology of *B. pulicarius* and the fact that not much is understood about the genetic variability of its hosts, any number of other factors might be important. Potentially, the studied populations may represent a large group under the effect of small, or few founder events. The observed similarities between populations may be related only to the sharing of an ancestral founder population. Like for many invasive weeds, the spread of yellow and Dalmatian toadflax on a large scale has been mostly human-aided (Lajeunesse 1999). Populations of *B. pulicarius*, because of the beetle's propensity for inhabiting flowers during nearly all life stages, have often been transported along with their hosts. Because of this human help, the movement of founder populations may not directly correspond to expected geographic patterns, where all populations in one geographic area would be the descendents of a single founder group. Rather, dispersal through human-aided spread

creates the potential for a single founder population to be transported sporadically throughout the Western US and Canada. Such conditions could result, without knowledge of the corresponding hosts' dispersal history, in seemingly confusing patterns of genetic distance and similarity in populations of *B. pulicarius*.

Alternatively, population similarities may correspond more clearly to the migration patterns of *B. pulicarius*. Like much of the biology, migration abilities and patterns are unknown. However, it has been observed that individual *B. pulicarius* are capable of sustained, within-patch flight (personal observations). They are relatively small in size and may be able to take advantage of thermal updrafts and wind currents. If the beetles are capable of sustained flights over larger distances, migration between most or all of the study sites may be possible. The routes of migration could explain the observed patterns of genetic variability, with sites within a migration route interbreeding and becoming more similar to one another than to sites outside the migration route.

A third possibility is that host plant diversity is the driving factor in population divergence and similarity. Toadflaxes have high fecundity and are known to possess very high levels of morphological and genetic variability (Lajeunesse 1999). No quantitative research has been done on the exact levels and patterns of variability in either species of toadflax. Beyond the effects of human-aided dispersal, it is likely that the plants, as they adapt to new environments, provide a variable diet for *B. pulicarius* individuals. This variation in plant hosts could exert selection pressure on their herbivores, potentially causing divergence in the beetles. Similar selection pressure on the weeds in different locations could conceivably result in similar genetic changes in the beetles at those

locations, regardless of geographic isolation. Because of this, a study of the population genetics of the host plants may provide an explanation for the patterns of genetic variation in the studied beetle populations.

This study does not provide evidence to explain the genetic variability within and among *B. pulicarius* populations. Rather, the results are inconclusive and tend to raise more questions than to provide answers. It does, however, suggest the variables that may provide insight through their further examination. These factors, including introduction history of the beetles and their hosts, *B. pulicarius* biology, especially migration ability and routes, and patterns of diversity in host plant populations could allow a greater understanding of the genetic variation observed in populations of *B. pulicarius*.

Behavioral Assays

Volatile Collections

Volatile collections were completed to characterize variability in volatile production in the host plants and to examine the potential for host plant preference in *B. pulicarius*. The collections revealed that individual plants are highly variable in their chemical emissions. Comparisons were made of volatiles collected from plants harvested from the two allopatric sites and the additional Yellowstone Park location. However, as discussed previously, the phenology of yellow and Dalmatian toadflax are very different, making such site-to-site and species-to-species comparisons difficult. Because of the phenological differences, plants collected from the two sites on consecutive days will not necessarily represent identical life stages for each species. However, efforts were made

to collect plants that appeared to be at the same stage (i.e. no buds present or in full bloom) on each pair of dates. Because these efforts were made to control for phenological differences, basic comparisons can be made and trends discussed. Some obvious differences in the quantities of volatiles being produced were found between yellow and Dalmatian toadflax species. Overall, the trend for yellow toadflax was a decrease in volatile production from June 5 to June 11 while the overall trend for Dalmatian toadflax was an increase in volatile production. One volatile compound, 2-Hexenol was novel to yellow toadflax plants collected June 12. However, volatile compositions of both species were dominated by 3-Hexenol acetate. Resulting *P*-values from ANOVA comparisons for that compound were not significant, indicating no difference in the mean chemical emission of the compound for the two species. ANOVA results yielded some significant *P*-values for less concentrated compounds, but the biological significance of these differences in minor volatile compounds is questionable. The lack of profound differences in volatile production by the two species of toadflax may suggest that the beetles cannot differentiate between them. The absence of an obvious potential mechanism for olfactory identification of host plant species could be viewed as a major impediment to the host race hypothesis. However, the potential for the presence of host races separated only by emergence timing corresponding with one host plant's phenology (rather than actual host preference) still exists. Additionally, further studies with better synchrony of host plant phenology would allow more confident comparisons of the two plant species. Rearing the two species of toadflax under identical conditions prior to volatile collection may provide such plant similarity. Studies using

more identical life stages of the two plants might identify important differences that were not detected in this analysis. They could also provide important information on specific volatile production and blends. If differences are detected, such data could be useful for synthesis of an artificial blend of compounds to create a lure for each species of toadflax. Such a lure, with consistent volatile concentration would prove invaluable in future behavioral bioassays with *B. pulicarius* and, potentially, with other biological control agents of yellow and Dalmatian toadflax.

Y-Tube Olfactometry

The goal of Y-tube assays was to examine host plant preference in *B. pulicarius* individuals. However, as discussed previously, the response of individual beetles in plant versus blank Y-tube trials was highly variable. This variability in response could be due to a number of things including insect, plant, and apparatus factors. Bernays (1994) (Bernays and Chapman 1994) stated that "a host-specific odor...only elicits a behavioral response under certain circumstances. The response depends on integration of the information about the odor with all the other factors operating on and in the insect." Variation in the insects' reactivity could depend upon time of day, amount of time since last meal, wind velocity within the apparatus or any number of other factors (Bernays and Chapman 1994). Response inconsistencies could also be related to variability in the plant stimuli (Bernays and Chapman 1994). The result of these variations in the insects and plants is that "the behavior we observe will often not be that which we might have expected (Bernays and Chapman 1994)."

The timing of behavioral trials may be a key factor in the observed responsiveness of *B. pulicarius* individuals. Beetles are long-lived and are present in the field at sites, such as site #9, from early May through the late summer or fall. The plants at this site haven't begun to flower until mid- or late August (personal observation). Beetles are, therefore at the site up to three or four months before blossoms are present. A small amount of feeding on meristem tissue reportedly occurs during this period (Coombs et al. 1996). However, because the flower is the significant plant structure in the reproduction and life cycle of *B. pulicarius* the beetles' interest in and responsiveness to other parts of their host plant and even the plant itself prior to flowering are questionable. Achievement of consistent late summer trials in 2001 raised more questions about the beetles' responsiveness earlier in the season. Unfortunately, this consistency was not obtained when trials were repeated in 2002. Nonetheless, the potential for seasonal variation in beetle responsiveness is high. If it is a real phenomenon, a lack of early season responsiveness may account for the failure of wind tunnel assays that were all attempted in June.

While the causes and mechanisms of the potential seasonal fluctuations in responsiveness are unknown, a possible explanation for the suspected late season responsiveness relates back to the questions surrounding the biology of *B. pulicarius*. Hervey (1927) said that new adults emerged and were seen in blossoms in September. Consistent late season results could be explained if adults in Montana emerge in the late summer to feed prior to diapause and overwintering. Such new adults could be extremely interested in feeding on, and therefore responsive to, their host plant as they prepare for

overwintering. Because adults are strong fliers (personal observation), the potential location of these fall-emerging beetles' overwintering sites could be nearly limitless. Such a scenario could account for the fact that pupae were located in the soil in the late summer of 2002 but an earlier effort to find pupae in the soil near the base of their host plants in the early spring 2001 was unsuccessful. Strong fluctuations in beetle responsiveness could also explain the observed high level of behavioral variability in *B. pulicarius*. While questions about seasonal variation in responsiveness are purely speculation, they should be of major interest to future researchers hoping to study the behavior of *B. pulicarius* and, potentially, other insects. Quantification of such fluctuations could allow for more consistent assays with the Y-tube olfactometer and could provide renewed potential for the use of the wind tunnel for bioassays to examine the possibility of host preference.

Beyond beetle variability, inconsistent Y-tube results could also be the result of problems with the apparatus itself. Orientation is rarely only via olfaction and olfactory clues may only serve as an initial stimulus to initiate host seeking behavior (Bernays and Chapman 1994). Often, tactile and visual cues such as color, spatial orientation, and UV wavelength have important roles in an insect's ultimate location of a host (Kellogg et al. 1962, Harris and Rose 1990, Harris et al. 1993). The Y-tube olfactometer requires orientation in the absence of visual cues. Depending on the relative importance of visual cues in the host-finding behavior of *B. pulicarius* this could be a major flaw of the system. Also, Baker and Lian (1984) stated that "bioassays utilizing flight in wind are probably the most discriminating in pheromone research." Due, most likely, to the small

diameter of the glass tube, beetles never flew within the apparatus. Kellogg and Wright (1962) recommended that for behavioral observations, conditions should be "as nearly natural as possible." The restriction on the flying behavior of *B. pulicarius* individuals may have placed limits on their ability to orient toward the host plant stimulus in a 'natural' fashion. Because the wind tunnel apparatus provides both olfactory and visual stimulation and allows flight it may still prove important in future behavioral assays. Work done here with the Y-tube apparatus may reveal some of the important factors that will need to be considered to develop a wind tunnel assay technique for *B. pulicarius*. Because of the amount of technique development already completed for the Y-tube system, regardless of its limitations, the apparatus holds promise for future studies of host preference in *B. pulicarius*. The creation of an artificial lure, as discussed previously, could provide a consistent stimulus, eliminating all of the discussed plant physiology effects. Removing the plant variability from the potential confounding factors could facilitate effective future Y-tube bioassays with *B. pulicarius*.

The overall goal of running behavioral assays was to lend support to, and indicate deficiencies in the molecular genetic data. Because of the many unexpected confounding factors, plant preference was never examined and data was never obtained to help explain the inconclusive genetic data. Rather, behavioral trials had the result of demonstrating the inherent variability in this biological system. Because of its accidental introduction into North America, *B. pulicarius,* unlike most biological control agents, has never undergone host-specificity testing nor has a comprehensive study of its biology in the northwestern U.S. ever been completed. Some observations do indicate the potential for

host races in the species, and the question of their existence is valid. However, the realization of the many uncertainties discussed in this thesis and the fundamental lack of basic information about *B. pulicarius* suggests that the investigation of host races in the species may have been premature. More appropriate questions to ask might have been, what is the biology of the species in this geographic region? What level of genetic variability is present in the beetles? What is the level of genetic diversity in their hosts? Will *B. pulicarius* individuals respond to host plants in either of the available behavioral apparatus? Does the behavior suggest a host plant preference?

The high levels of individual genetic and overall behavioral variability in *B. pulicarius* will provide a challenge for future researchers. The results of this study suggest that the quantities and patterns of diversity of the two host species need to be fully examined and may prove necessary to the understanding of patterns of variability in the beetle. Additionally, a comprehensive study of the life history and biology of *B. pulicarius* in the northwestern U.S. and Canada should serve as an absolute prerequisite to future work with this species. Being able to answer the more basic questions, presented above, may allow future researchers to reach a more conclusive answer to the question of host races in *B. pulicarius*. This study's identification of several important areas of uncertainty and variability in this biological system may serve as the necessary groundwork for completion of such future research.

CHAPTER 5

CONCLUSION

This study found no evidence to support the hypothesis that the species *Brachypterolus pulicarius* consists of two distinct host races. This hypothesis, as previously discussed, has practical implications. The presence of host races in *B. pulicarius* would suggest to biocontrol practitioners that one group, or race, of individuals might be a more effective control agent for a particular host plant. The understanding of host races in this species, and others, could allow more efficient matching of the insects to their target species, thus enhancing their ability to control the target plant infestation. However, if host races are not present, practitioners could feel confident that they are not limiting their biological control program's potential efficacy by utilizing individuals collected from one host species to control the other toadflax species. While the genetic results did not find evidence to suggest that host races are present in *B. pulicarius*, the results of this study are inconclusive and neither definitively support nor refute the host race hypothesis. Because of this, biocontrol practitioners who wish to ensure correct matching of insects to their hosts should continue to relocate beetles from one host plant species onto the same toadflax species.

LITERATURE CITED

Alex, J. F. 1962. The taxonomy, history, and distribution of *Linaria dalmatica*. Can. J. Bot. 40: 295-307.

Arnold, R. M. 1982. Pollination, predation and seed set in *Linaria vulgaris* (Scrophulariaceae). Am. Midl. Nat. 107: 360-369.

Audisio, P. 1993. Coleoptera, Nitidulidae-Kateretidae. Calderini, Bologna.

Baker, T. C., and C. E. J. Lian. 1984. Wind tunnels in pheromone research, pp. 75-110. *In* H. E. Hummel and T. A. Miller [eds.], Techniques in pheromone research. Springer & Verlang, New York.

Baker, T. C., and R. T. Carde. 1984. Techinques in behavioral bioassays, pp. 45-73. *In* H. E. Hummel and T. A. Miller [eds.], Techniques in pheromone research. Springer-Verlang, New York.

Bakshi, T. S., and R. T. Coupland. 1960. Vegetative propagation in *Linaria vulgaris*. Can. J. Botany 38: 243-249.

Barrett, B. A., and K. K. Kidwell. 1998. AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. Crop Sci. 38: 1261-1271.

Benham, J. J. 2001. Genographer. Computer program, version 1.6.0. By J. J. Benham, Bozeman, MT.

Bernays, E. A., and R. F. Chapman. 1994. Host-plant selection by phytophagous insects. Chapman & Hall, New York.

Black, W. C. 1993. PCR with arbitraty primers: approach with care. Insect Mol. Biol. 2: 1-6.

Borden, J. H. 1977. Behavioral responses of Coleoptera to pheromones, allomones, and kairomones., pp. 169-198. *In* H. H. Shorey and J. J. J. McKelvey [eds.], Chemical control of insect behavior - theory and application. John Wiley & Sons, New York.

Boucias, D., C. Stokes, A. Suazo, and J. Funderburk. 2000. AFLP analysis of the entomopathogen *Nomuraea rileyi*. Mycologia 92: 638-648.

Bush, G. L., and J. J. Smith. 1998. The genetics and ecology of sympatric speciation: a case study. Res. Popul. Ecol. 10: 175-187

Cardoso, S. R. S., N. B. Eloy, J. Provan, M. A. Cardoso, and P. C. G. Ferreira. 2000. Genetic differentiation of *Euterpe edulis* Mart. populations estimated by AFLP analysis. Mol. Ecol. 9: 1753-1760.

Carelli, M., S. Gnocchi, S. Fancelli, M. Alessio, D. Paffetti, C. Scotti, and M. Bazzicalupo. 2000. Genetic diversity and dynamics of *Sinorhizobium meliloti* populations nodulating different alfalfa cultivars in Itialian soils. Appl. Environ. Micorbiol. 66: 4785-4789.

Cervera, M. T., J. A. Cabezas, B. Siman, J. M. Martinez-Zapater, F. Beitia, and J. L. Cenis. 2000. Genetic relationships among biotypes of *Bemisia tabaci* (Hempitera: Aleyrodidae) based on AFLP analysis. Bull. Entomol. Res. 90: 391-396.

Cho, Y. G., M. W. Blair, O. Panaud, and S. R. McCouch. 1996. Cloning and mapping of variety-specific rice genomic DNA sequences: amplified fragment length polymorphisms (AFLP) from silver-stained polyachrylamide gels. Genome 39: 373-378.

Cilliers, A. J., L. Herselman, and Z. A. Pretorius. 2000. Genetic variability within and among mycelial compatibility groups of *Sclerotium rolfsii* in South Africa. Phytopathology 90: 1026-1031.

Coombs, E., R. Nowierski, and G. Piper. 1996. *Brachypterolus pulicarius. In* N. E. Rees, P. C. J. Quimby, G. L. Piper, E. M. Coombs, C. E. Turner, N. R. Spencer and L. V. Knutson [eds.], Biological Control of Weeds in the West. Western Society of Weed Science, Bozeman, MT.

Craig, T. P., J. K. Itami, W. G. Abrahamson, and J. D. Horner. 1993. Behavioral evidence for host-race formation in *Eurosta solidaginis*. Evolution 47: 1696-1710.

Cronk, Q. C. B., and J. L. Fuler. 1995. Plant Invaders: The threat to natural ecosystems. Chapman & Hall, New York.

Darwent, A. L., W. Lobay, W. Yarish, and P. Harris. 1975. Distribution and importance in Northwestern Alberta of toadflax and its insect enemies. Can. J. Plant Sci. 55: 157-162.

Dethier, V. G., L. B. Browne, and C. N. Smith. 1960. The designation of chemicals in terms of the responses they elicit from insects. J. Econ. Entomol. 53: 134-136.

Diehl, T. P., and G. L. Bush. 1984. An evolutionary and applied perspective of insect biotypes. Annu. Rev. Entomol. 29: 471-504.

Doromont, L., and A. Roques. 2001. Why are seed cones of Swiss stone pine (*Pinus cebra*) not attacked by the specialized pine cone weevil, *Pissodes validirostris*? A case of host selection vs. host suitability. Entomol. Exp. Appl. 99: 157-163.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Aplication to human mitochondrial DNA restriction data. Genetics 131: 479-491.

Excoffier, L. 2001. Analysis of Population Subdivision, pp. 271-307. *In* D. J. Balding, M. Bishop and C. Cannings [eds.], Handbook of Statistical Genetics. John Wiley & Sons, New York.

Farrell, B. D. 1999. www.oeb.harvard.edu/faculty/farrell/research/extract.html.

FitzGerald, B. 1999. Revised DNA extraction protocol.

Forneck, A., M. A. Walker, and R. Blaich. 2000. Genetic structure of an introduced pest, grape phylloxera (*Daktulosphaira vitifoliae* Fitch), in Europe. Genome 43: 669-678.

Gagne, G., P. Roeckel-Drevet, B. Grezes-Besset, P. Shindrova, P. Ivanov, c. Grand-Ravel, F. Vear, G. Charmet, and P. Nicolas. 2000. Amplified fragment length polymorphism (AFLP) as suitable markers to study *Orobanche cumana* genetic diversity. J. Phytopath 148: 457-459.

Geier, M., and J. Boeckh. 1999. A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odours. Entomol. Exp. Appl. 92: 9-19.

Gotoh, T., J. Bruin, M. W. Sabelis, and S. B. J. Menken. 1993. Host race formation in *Tetranychus urticae:* genetic differentiation, host plant preference, and mate choice in a tomato and a cucumber strain. Entomol. Exp. Appl. 68: 171-178.

Grubb, R. T. 1998. Effects of *Brachypterolus pulicarius* on growth and seed production of Dalmatian toadflax. Entomology. Montana State University, Bozeman.

Grubb, R. T., R. M. Nowierski, and R. L. Sheley. 2002. Effects of B*rachypterolus pulicarius* (L.) (coleopteraL Nitidulidae) on growth and seed production of Dalmatian toadflax, *Linaria genistifolia* ssp. *dalmatica* (L.) Maire and Petitmengin (Scrophulariaceae). Biological Control 23: 107-114.

Harris, P. 1961. Control of toadflax by *Brachypterolus pulicarius* (L.) (Coleoptera: Nitidulidae) and *Gymnaetron antirrhini* (Payk.) (Coleoptera: Circulionidae) in Canada. Can. Entomol. 93: 977-981.

Harris, M. O., and S. Rose. 1990. Chemical, color, and tactile cues influencing oviposition behavior of the Hessian fly (Diptera: Cecidomyiidae). Environ. Entomol. 19: 303-308.

Harris, M. O., S. Rose, and P. Malsch. 1993. The role of vision in the host plant-finding behaviour of the Hessian fly. Physiol. Entomol. 18: 31-42.

Hartl, D. L., and A. G. Clark. 1997. Principles of Population Genetics. Sinauer Associates, Inc., Sunderland, Massachusetts.

Hawthorne, D. J. 2001. AFLP-based genetic linkage map of the Colorado potato beetle *Leptinotarsa decemlineata*: Sex chromosomes and a pyrethroid-resistance candidate gene. Genetics 158: 695-700.

Heath, R. R., and A. Manukian. 1992. Development and evaluation of systems to collect volatile semiochemicals from insects and plants using a charcoal-infused medium for air purification. J. Chem. Ecol. 18: 1209-1226.

Heath, R. R., and A. Manukian. 1994. An automated system for use in collecting volatile chemicals released from plants. J. Chem. Ecol. 20: 593-608.

Herbergs, J., M. Siwek, R. P. M. A. Crooijmans, J. J. Van der Poel, and M. A. M. Groenen. 1999. Multicolor flourescent detection and mapping of AFLP markers in chicken (*Gallus domesticus*). Animal Genetics 30: 274-285.

Hervey, G. E. R. 1927. A European nitidulid, *Brachypterolus pulicarius* (L.) (Coleoptera: Nitidulidae). J. Econ. Entomol. 20: 809-814.

Hill, T. A. 1977. The Biology of Weeds. The Camelot Press Ltd., Southampton.

Ho, H.-Y., and J. G. Millar. 2001. Identification and synthesis of a male-produced sex pheromone from the stink bug *Chlorochroa sayi*. J. Chem. Ecol. 27: 1177-1201.

Huff, D. R., R. Peakall, and P. E. Smouse. 1993. RAPD variation within and among natural populations of outcrossing buffalograss [*Buchloe dactyloides* (Nutt.) Englem.]. Theor. Appl. Genet. 86: 927-934.

Huff, D. R. 1997. RAPD characterization of heterogeneous perennial ryegrass cultivars. Crop Sci. 37: 557-564.

Ignacimuthu, S., F. L. Wachers, and S. Dorn. 2000. The role of chemical cues in host finding and acceptance by *Collosobruchus chinensis*. Entomol. Exp. Appl. 96: 213-219.

Jakse, J., K. Kindlhofer, and B. Jovernik. 2001. Assessment of genetic variation and differentiation of hop genotypes by microsatellite and AFLP markers. Genome 44: 773-782.

Janssen, A., C. D. Hofker, A. R. Braun, N. Mesa, M. W. Sabelis, and A. C. Bellotti. 1990. Preselecting predatory mites for biological control: the use of an olfactometer. Bull. Entomol. Res. 80: 177-181.

Janssen, P., T. Coopman, G. Huys, J. Swings, M. Bleeker, P. Vos, M. Zabeau, and K. Kersters. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. Microbiology 142: 1881-1893.

Kambhampati, S., W. C. I. Black, and K. S. Rai. 1992. Random amplified polymorphic DNA of mosquito species and populations (Diptera: Culicidae): Techniques, statistical analysis, and applications. J. Med. Entomol. 29: 939-945.

Katiyar, S. K., G. Chandal, Y. Tan, Y. Zhang, B. Huang, L. Nugaliyadde, K. Fernando, J. S. Dentur, S. Inthavong, S. Constantino, and J. Dennet. 2000. Biodiversity of Asian rice gall midge (*Orseolia oryzae* Wood Mason) from five countries examined by AFLP analysis. Genome 43: 322-332.

Keiper, F. J., and R. McConchie. 2000. An analysis of genetic variation in natural populations of *Sticherus flabellatus* [R. Br. (St. John)] using amplified fragment length polymorphism (AFLP) markers. Mol. Ecol. 9: 571-581.

Kellogg, F. E., and R. H. Wright. 1962. The olfactory guidance of flying insects. III. A technique for observing and recording flight paths. Can. Entomol. 94: 486-493.

Kellogg, F. E., D. E. Frizel, and R. H. Wright. 1962. The olfactory guidance of flying insects. IV. Drosophila. Can. Entomol. 94: 884-888.

Knorr, C., H. H. Cheng, and J. B. Dodgson. 1999. Application of AFLP markers to genome mapping in poultry. Animal Genetics 30: 28-36.

Koopman, W. J. M., M. J. Zevenbergen, and R. G. Van den Berg. 2001. Species relationships in *Lactuca* s.l. (Lactuceae, Asteraceae) inferred from AFLP fingerprints. Am. J. Botany 88: 1881-1887.

Koschier, E. H., W. Jan De Kogel, and J. H. Visser. 2000. Assessing the attractiveness of volatile compounds to western flower thrips *Frankliniella occidentalis*. J. Chem. Ecol. 26: 2643-2655.

Lacey, J. R., and B. E. Olson. 1991. Environmental and economic impacts of noxious range weeds, pp. 5-16. *In* L. F. James and J. O. Evans [eds.], Noxious Range Weeds. Westview Press, Boulder, Colorado.

Lajeunesse, S. E., P. K. Fay, D. Cooksey, J. R. Lacey, R. M. Nowierski, and D. Zamora. 1993. Dalmatian and yellow toadflax: weeds of pasture and rangeland., pp. 13, Extension publications office. Montana State University, Bozeman, Montana.

Lajeunesse, S. 1999. Dalmatian and yellow toadflax, pp. 202-216. *In* R. L. Sheley and J. K. Peroff [eds.], Biology and Management of Noxious Rangeland Weeds. Oregon State University Press, Corvalis.

Lange, A. W. 1958. Dalmatian toadflax - a possible rival of goatweed as a serious range weed. Weeds 6: 68-70.

Louda, S. M., D. Kendall, J. Connor, and D. Simberloff. 1997. Ecological effects of an insect introduced for the biological control of weeds. Science 277: 1088-1090.

Lynch, M., and B. G. Milligan. 1994. Analysis of populations genetic structure with RAPD markers. Mol. Ecol. 3: 91-99.

Mace, E. S., C. G. Gebhardt, and R. N. Lester. 1999. AFLP analysis of genetic relationships in the tribe Datureae (Solanaceae). Theor. Appl. Genet. 99: 634-641.

Matthews, J. R., and R. W. Matthews. 1982. Insect behavior - a source book of laboratory and field exercises. Westview Press, Boulder.

McClay, A. S. 1992. Effects of *Brachypterolus pulicarius* (L.) (Coleoptera: Nitidulidae) on flowering and seed production of common toadflax. Can. Entomol. 124: 631-636.

McMichael, N., and D. Pahley Prowell. 1999. Differences in amplified fragment-length polymorphisms in fall armyworm (Lepidoptera: Noctuidae) host strains. Ann. Entomol. Soc. Am. 92: 175-181.

Miller, M. P. 1997. Tools for population genetic analyses (TFPGA) 1.3: A Windows program for the analysis of allozyme and molecular population genetic data. Computer program, version 1.3. By M. P. Miller, Computer software distributed by author.

Miller, M. P. 1998. AMOVA-PREP computer program, version By Miller, M. P., Computer software distributed by author.

Mitich, L. W. 1993. Yellow toadflax. Weed Technol. 7: 791-793.

Mueller, U. G., and L. L. Wolfenbarger. 1999. AFLP genotyping and fingerprinting. TREE 14: 389-394.

Nadeau, L. B., and J. R. King. 1991. Seed dispersal and seedling establishment of *Linaria vulgaris* Mill. Can. J. Plant Sci. 71: 771-782.

Nadeau, L. B., J. R. King, and K. N. Harker. 1992. Comparison of growth of seedlings and plants grown from root pieces of yellow toadflax (*Linaria vulgaris*). Weed Sci. 40: 43-47.

Narang, S. K., W. J. Tabachnick, and R. M. Faust. 1994. Complexities of population genetic structure and implications for biological control programs., pp. 39-39. *In* S. K. Narang, A. C. Bartlett and R. M. Faust [eds.], Applications of Genetics to Arthropods of biological control significance. CRC Press, Ann Arbor.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 585-590.

Nowierski, R. M. 1996a. Yellow toadflax. *In* N. E. Rees, P. C. J. Quimby, G. L. Piper, E. M. Coombs, C. E. Turner, N. R. Spencer and L. V. Knutson [eds.], Biological Control of Weeds in the West. Western Society of Weed Science, Bozeman.

Nowierski, R. M. 1996b. Dalmatian toadflax. *In* N. E. Rees, P. C. J. Quimby, G. L. Piper, E. M. Coombs, C. E. Turner, N. R. Spencer and L. V. Knutson [eds.], Biological Control of Weeds in the West. Western Society of Weed Science, Bozeman.

Opp, S. B., and R. J. Prokopy. 1986. Approaches and methods for direct behavioral observation and analysis of plant-insect interactions, pp. 1-22. *In* J. R. Miller and T. A. Miller [eds.], Insect-plant interactions. Springer-Verlang, New York.

Piepho, H.-P., and G. Koch. 2000. Codominant analysis of banding data from a dominant marker system by normal mixtures. Genetics 155: 1459-1468.

Purwantara, A., J. M. Barrins, A. J. Cozijnsen, p. K. Ades, and B. J. Howle. 2000. Genetic diversity of isolates of the *Leptosphaeria maculans* species complex from Australia, Europe, and North America using amplified fragment length polymorphism analysis. Mycol. Res. 104: 772-781.

Rao, V. S. 2000. Principles of Weed Science. Science Publishers, Inc., Enfield, NH.

Rees, N. E., P. C. J. Quimby, and B. H. Mullin. 1996. Biological control of weeds, pp. 3. *In* N. E. Rees, P. C. J. Quimby, G. L. Piper, E. M. Coombs, C. E. Turner, N. R. Spencer and L. V. Knutson [eds.], Biological Control of Weeds in the West. Western Society of Weed Science, Bozeman.

Reineke, A., P. Karlovsky, and C. P. W. Zebitz. 1999. Amplified fragment length polymorphism analysis of different geographic populations of the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae). Bull. Entomol. Res. 89: 79-88.

Reineke, A., and P. Karlovsky. 2000. Simplified AFLP protocol: replacement of primer labeling by the incorporation of alpha-labeled nucleotides during PCR. BioTechniques 28: 622-623.

Rist, D. L., and J. W. Lorbeer. 1989. Ocurrence and overwintering of cucumber mosaic virus and broad bean wilt virus in weeds growing near commercial lettuce fields in New York. Phytopathology 79: 65-69.

Robocker, W. C. 1970. Seed characteristics and seedling emergence of Dalmatian toadflax. Weed Sci. 18: 720-725.

Saner, M. A., D. R. Clements, M. R. Hall, D. J. Doohan, and C. W. Crompton. 1995. The biology of Canadian weeds. 105. *Linaria vulgaris* Mill. Can. J. Plant Sci. 75: 525-537.

Sawkins, M. C., B. L. Maass, B. C. Pengelly, H. J. Newbury, and B. V. Ford-Lloyd. 2001. Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment length polymorphism. Mol. Ecol. 10: 1947-1958.

Schneider, S., D. Roessli, and L. Excoffier 2000. Arlequin: A software for population genetics data analysis. computer program, version 2.000. By S., D. Schneider, Roessli, and L. Excoffier, Geneva.

Schoonhoven, L. M. 1977. Insect chemosensory responses to plant & animal hosts. *In* H. H. Shorey [ed.], Chemical Control of Insect Behavior Theory and Application. John Wiley & Sons, New York.

Seefelder, S., H. Ehrmaier, G. Schweizer, and E. Seigner. 2000. Genetic diversity and phylogenetic relationships among accessions of hop, *Humulus lupulus*, as determined by amplified fragment length polymorphism fingerprinting compared with pedigree data. Plant Breeding 119: 257-263.

Sharma, S. K., M. R. Knox, and T. H. N. Ellis. 1996. AFLP analysis of the diversity and phylogeny of *Lens* and its comparison with RAPD analysis. Theor. Appl. Genet. 93: 751-758.

Sheley, R. L., J. K. Petroff, and M. M. Borman. 1999. Introduction, pp. 1-3. *In* R. L. Sheley and J. K. Petroff [eds.], Biology and Management of Noxious Rangeland Weeds. Oregon State University Press, Corvalis, OR.

Shields, E. J. 1989. Artificial light: Experimental problems with insects. Bull. Entomol. Soc. Am. 00: 40-44.

Shim, S. I., and R. B. Jorgensen. 2000. Genetic structure in cultivated and wild carrots (*Daucus carota* L.) revealed by AFLP analysis. Theor. Appl. Genet. 101: 227-233.

Smith, J. M. 1959. Notes on insects, especially *Gymnaetron* spp. (Coleoptera: Curculionidae), associtated with toadflax, *Linaria vulgaris* Mill. (Scrophulariaceae), in North America. Can. Entomol. 91: 115-121.

Sneath, P. H. A., and R. R. Sokal. 1973. Numerical Taxonomy. Freeman and Company, San Francisco.

Sokal, R. R., and F. J. Rohlf. 1995. Biometry: the Principles and Practices of Statistics in Biological Research. W. H. Freeman and Company, New York.

Strong, D. R. 1997. Fear no weevil? Science 277: 1058-1059.

Suazo, A., and H. G. Hall. 1999. Modification of the AFLP protocol applied to honey bee (*Apis mellifera* L.) DNA. BioTechniques 26: 704-709.

Swofford, D.L., and G. J. Olsen. 1990. Phylogeny reconstruction, pp. 411-501. *In* D. M. Hillis and C. Moritz [eds.], Molecular Systematics. Sinaur Associates, Sunderland, MA.

Tan, Y.-D., C. Wan, Y. Zhu, C. Lu, Z. Xiang, and H.-W. Deng. 2001. An amplified fragment length polymorphism map of the silkworm. Genetics 157: 1277-1284.

Taylor, R. J. 1990. Northwest weeds: the ugly and beautiful villians of fields, gardens, and roadsides. Mountain press publishing company, Missoula, MT.

Unruh, T. R., and R. D. Goeden. 1987. Electrophoresis helps to identify which race of the introduced weevil, *Rhinocyllus conicus* (Coleoptera: Circulionidae), has transferred to two native southern California thistles. Environ. Entomol. 16: 979-983.

Vandemark, G., O. Matinez, V. Pecina, and M. de Jesus Alvarado. 2000. Assessment of genetic relationships among isolates of *Macrophomina phaseolina* using a simplified AFLP techinque and two different methods of analysis. Mycologia 92: 656-664.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. 23: 4407-4414.

Vujnovic, K., and R. W. Wein. 1996. The biology of Canadian weeds. 106. *Linaria dalmatica* (L.) Mill. Can. J. Plant Sci. 77: 483-491.

Waugh, R., N. Bonar, E. Baird, B. Thomas, A. Graner, P. Hayes, and W. Powell. 1997. Homology of AFLP products in three mapping populations of barley. Mol. Gen. Genet. 255: 311-321.

Weir, B. S. 1990. Genetic Data Analysis. Sinauer Associates, Inc., Sunderland, Massachusetts.

Williams, E. 2002. Great Circle Calculator. http://williams.best.vwh.net/gccalc.htm

Wilson, L. M., and J. P. McCaffrey. 1999. Biological control of noxious rangeland weeds, pp. 97-115. *In* R. L. Sheley and J. K. Petroff [eds.], Biology and Management of Noxious Rangeland Weeds. Oregon State University Press, Corvalis.

Wong, A., M. R. Forbes, and M. L. Smith. 2001. Characterization of AFLP markers in damselflies: prevalence of codominant markers and implications for population genetic applications. Genome 44: 677-684.

Yamanaka, T., S. Tatsuki, and M. Shimada. 2001. Flight characteristics and dispersal patterns of fall webworm *(Lepidoptera: Arctiidae)* males. Environ. Entomol. 30: 1150-1157.

Yan, G., J. Romero-Severson, M. Walton, D. D. Chadee, and D. W. Severson. 1999. Population genetics of the yellow fever mosquito in Trinidad: comparisons of amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers. Mol. Ecol. 8: 951-963.

Zhang, D., M. A. Dirr, and R. A. Price. 2000. Discrimination and genetic diversity of *Cephalotaxus* accessions using AFLP markers. J. Amer. Soc. Hort. Sci. 125: 404-412.

APPENDIX

Table 30. Frequency of AFLP-generated band presence data

Table 30. Frequency of AFLP-generated band presence data.

Site	Locus:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Site #1	Frequency:	0.46	0.71	0.15	1	0.98	0.20	0.17	0.22	0.76	0.39	0.90	0.32	0.83	0.61	0.88	0.27	0.61	0.71	0.56
Site #2	Frequency:	0.46	0.87	0.51	1	0.85	0.41	0.44	0.44	0.62	0.62	1	0.38	0.79	0.54	0.82	0.46	0.87	0.69	0.46
Site #3	Frequency:	0.55	0.93	0.76	1	0.66	0.41	0.45	0.31	0.72	0.69	1	0.55	0.62	0.48	0.69	0.76	0.55	0.72	0.59
Site #4	Frequency:	0.34	0.47	0.25	1	0.28	0.53	0.44	0.53	0.78	0.56	0.88	0.41	0.78	0.53	0.75	0.31	0.66	0.91	0.50
Site #5	Frequency:	0.53	0.98	0.53	0.95	0.73	0.35	0.30	0.53	0.90	0.63	0.93	0.30	0.88	0.48	0.85	0.33	0.65	0.83	0.65
Site #6	Frequency:	0.32	0.97	0.66	1	0.37	0.18	0.55	0.39	0.82	0.74	0.97	0.53	0.66	0.63	0.71	0.39	0.34	0.71	0.55
Site #7	Frequency:	0.74	1.00	0.29	1	0.86	1	0.51	0.83	0.51	0.71	1	0.37	0.97	0.51	0.86	0.57	0.83	0.80	0.69
Site #8	Frequency:	0.21	0.87	0.26	0.95	0.18	0.31	0.49	0.05	0.67	0.44	0.79	0.15	0.46	0.62	0.51	0.13	0.33	0.38	0.41
Site #9	Frequency:	0.21	0.54	0.09	1	0.26	0.53	0.35	0.09	0.79	0.62	0.91	0.50	0.74	0.53	0.74	0.24	0.59	0.51	0.47
Site #10	Frequency:	0.29	0.88	0.15	1	0.06	0.18	0.21	0.26	0.65	0.47	0.97	0.32	0.85	0.38	0.79	0.26	0.44	0.50	0.35
Site #11	Frequency:	0.60	0.52	0.76	1	0.88	0.16	0.44	0.44	0.84	0.64	0.56	0.32	0.56	0.52	0.48	0.84	0.20	0.48	0.44
Site #12	Frequency:	0.52	0.76	0.48	0.92	0.92	0.40	0.72	0.48	0.76	0.64	0.68	0.44	0.92	0.64	0.36	0.88	0.40	0.48	0.60

Site	Locus:	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Site #1	Frequency:	0.24	0.80	1	0.63	0.63	0.71	1.00	0.32	0.68	0.27	0.37	0.54	1.00	0.80	0.51	0.66	0.37
Site #2	Frequency:	0.38	0.74	1	0.92	0.51	0.54	0.97	0.51	0.85	0.56	0.36	0.77	0.97	0.79	0.36	0.87	0.31
Site #3	Frequency:	0.45	0.83	1	1	0.79	0.66	0.86	0.17	0.79	0.48	0.52	0.76	1.00	0.83	0.79	0.83	0.31
Site #4	Frequency:	0.53	0.50	1	0.59	0.97	0.38	0.91	0.09	0.66	0.34	0.47	0.25	0.78	0.91	0.41	0.75	0.47
Site #5	Frequency:	0.35	0.40	1	0.85	0.48	0.15	0.88	0.13	0.90	0.40	0.15	0.53	0.98	0.85	0.58	0.75	0.33
Site #6	Frequency:	0.45	0.66	1	0.58	0.63	0.58	0.97	0.34	0.84	0.29	0.37	0.63	0.87	0.84	0.37	0.89	0.05
Site #7	Frequency:	0.77	0.54	1	0.71	0.80	0.83	0.89	0.29	0.91	0.37	0.46	0.40	0.69	0.91	0.37	0.77	0.37
Site #8	Frequency:	0.15	0.51	0.95	0.36	0.56	0.26	0.72	0.15	0.82	0.13	0.38	0.49	0.82	0.85	0.10	0.67	0.08
Site #9	Frequency:	0.26	0.74	0.97	0.62	0.62	0.38	0.91	0.24	0.79	0.24	0.18	0.24	0.97	0.74	0.15	0.94	0.26
Site #10	Frequency:	0.03	0.38	1	0.74	0.47	0.09	0.88	0.29	0.56	0.03	0.26	0.38	0.88	0.74	0.15	0.85	0.06
Site #11	Frequency:	0.44	0.60	1	0.44	0.36	0.76	1.00	0.24	0.68	0.56	0.76	0.60	0.76	0.76	0.32	0.72	0.48
Site #12	Frequency:	0.40	0.32	1	0.64	0.72	0.88	0.96	0.32	0.36	0.32	0.20	0.72	0.72	0.64	0.36	0.76	0.36

Site	Locus:	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
Site #1	Frequency:	0.37	1	1	0.39	0.34	0.07	0.83	0.29	0.41	0.27	0.85	0.27	0.41	0.95	0.61	0.61	0.39	1
Site #2	Frequency:	0.87	1	0.95	0.28	0.38	0.23	0.64	0.31	0.33	0.13	0.79	0.49	0.31	0.92	0.31	0.62	0.38	0.97
Site #3	Frequency:	0.55	1	0.90	0.52	0.24	0.28	0.55	0.31	0.28	0.24	0.72	0.55	0.31	1	0.59	0.72	0.76	1
Site #4	Frequency:	0.53	1	0.97	0.31	0.28	0.09	0.59	0.28	0.13	0.09	0.56	0.34	0.16	0.75	0.22	0.69	0.44	1
Site #5	Frequency:	0.65	1	0.95	0.53	0.20	0	0.75	0.38	0.20	0.10	0.93	0.28	0.23	0.80	0.38	0.60	0.63	1
Site #6	Frequency:	0.53	1	1	0.05	0.24	0	0.61	0.37	0.18	0.03	0.68	0.37	0.24	0.95	0.53	0.76	0.74	1
Site #7	Frequency:	0.71	1	0.97	0.43	0.29	0.11	0.74	0.46	0.46	0.23	0.94	0.60	0.31	0.97	0.37	0.77	0.60	1
Site #8	Frequency:	0.87	0.95	0.95	0.05	0.31	0.10	0.23	0.05	0.13	0.15	0.46	0.31	0.08	0.87	0.38	0.72	0.56	0.97
Site #9	Frequency:	0.44	1	0.97	0.24	0.44	0.09	0.44	0.09	0.21	0.15	0.59	0.32	0.18	0.97	0.32	0.88	0.41	0.97
Site #10	Frequency:	0.79	1	1	0.09	0.21	0	0.35	0.06	0.09	0	0.53	0.29	0.12	0.76	0.50	0.85	0.41	1
Site #11	Frequency:	0.24	0.40	0.84	0.64	0.36	0.32	0.44	0.28	0.72	0.32	0.80	0.44	0.32	0.56	0.56	0.56	0.72	1
Site #12	Frequency:	0.28	0.24	0.80	0.56	0.24	0.16	0.32	0.24	0.52	0.16	0.48	0.44	0.40	0.44	0.40	0.56	0.68	1

Site	Locus:	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
Site #1	Frequency:	0.90	0.29	0.56	0.27	0.24	0.66	0.98	0.61	0.44	0.22	0.34	0.73	0.29	0.29	0.71	0.46	0.88	0.73
Site #2	Frequency:	0.64	0.28	0.64	0.15	0.51	0.69	0.92	0.36	0.26	0.67	0.51	0.74	0.54	0.28	0.79	0.49	0.97	0.87
Site #3	Frequency:	0.62	0.62	0.52	0.21	0.34	0.83	0.66	0.45	0.31	0.48	0.21	0.72	0.52	0.31	0.86	0.62	0.83	0.76
Site #4	Frequency:	0.22	0.41	0.41	0.09	0.19	0.69	0.81	0.38	0.25	0.31	0.19	0.19	0.50	0.31	0.78	0.47	0.75	0.75
Site #5	Frequency:	0.45	0.28	0.33	0.05	0.05	0.68	0.78	0.25	0.05	0.08	0.08	0.70	0.28	0.03	0.58	0.50	0.73	0.75
Site #6	Frequency:	0.50	0.05	0.39	0.24	0.26	0.87	0.76	0.37	0.08	0.50	0.24	0.39	0.18	0.18	0.68	0.55	0.79	0.58
Site #7	Frequency:	0.37	0.37	0.51	0.14	0.37	0.86	0.91	0.43	0.17	0.14	0.17	0.80	0.23	0.11	0.69	0.51	0.80	0.89
Site #8	Frequency:	0.15	0.10	0.18	0.08	0.13	0.51	0.69	0.15	0.05	0.33	0.05	0.10	0.08	0.13	0.62	0.38	0.72	0.54
Site #9	Frequency:	0.35	0.15	0.35	0.27	0.18	0.82	0.77	0.29	0.12	0.18	0.09	0.56	0.12	0.12	0.68	0.41	0.82	0.56
Site #10	Frequency:	0.09	0.03	0.18	0.21	0.12	0.62	0.79	0.09	0.03	0.24	0.03	0.26	0.03	0.06	0.71	0.18	0.74	0.59
Site #11	Frequency:	0.44	0.48	0.48	0.48	0.72	0.64	0.72	0.52	0.60	0.40	0.48	0.56	0.40	0.32	0.48	0.28	0.52	0.56
Site #12	Frequency:	0.52	0.16	0.36	0.24	0.24	0.56	0.52	0.28	0.28	0.40	0.20	0.28	0.28	0.32	0.48	0.44	0.44	0.28

Site	Locus:	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Site #1	Frequency:	0.37	0.12	0.78	0.95	0.78	0.88	0.32	0.27	0.66	0.78	1	0.90	0.46	0.68	0.59	0.41	0.39	0.39
Site #2	Frequency:	0.54	0.23	0.74	1	0.92	1	0.56	0.49	0.46	0.72	1	0.95	0.54	0.64	0.46	0.56	0.26	0.23
Site #3	Frequency:	0.38	0.48	0.76	0.97	0.86	0.79	0.48	0.69	0.62	0.93	1	0.97	0.45	0.62	0.55	0.48	0.28	0.28
Site #4	Frequency:	0.38	0.28	0.72	0.97	0.88	1	0.72	0.66	0.47	0.41	1	0.94	0.41	0.63	0.31	0.25	0.25	0.28
Site #5	Frequency:	0.45	0.28	0.55	0.98	0.98	0.95	0.80	0.45	0.38	0.58	0.98	0.88	0.35	0.65	0.45	0.45	0.23	0.28
Site #6	Frequency:	0.26	0.00	0.74	0.95	0.82	0.82	0.16	0.34	0.66	0.42	1	0.97	0.50	0.26	0.32	0.34	0.13	0.18
Site #7	Frequency:	0.69	0.23	0.74	0.91	0.51	0.80	0.17	0.71	0.60	0.69	1	0.97	0.37	0.49	0.34	0.23	0.31	0.11
Site #8	Frequency:	0.28	0.10	0.62	0.85	0.62	0.67	0.10	0.10	0.54	0.54	0.95	0.92	0.46	0.36	0.28	0.13	0.10	0.10
Site #9	Frequency:	0.32	0.09	0.59	0.91	0.65	0.82	0.21	0.18	0.62	0.59	0.94	0.94	0.29	0.24	0.09	0.12	0.03	0.21
Site #10	Frequency:	0.03	0.03	0.53	0.88	0.71	0.71	0.12	0.09	0.56	0.50	0.97	0.50	0.35	0.18	0.12	0.06	0.06	0.06
Site #11	Frequency:	0.52	0.40	0.68	0.80	0.84	0.64	0.88	0.68	0.40	0.72	0.96	0.64	0.44	0.60	0.40	0.28	0.32	0.60
Site #12	Frequency:	0.40	0.40	0.52	0.56	0.72	0.44	0.88	0.44	0.32	0.60	1	0.80	0.24	0.32	0.20	0.12	0.32	0.28

Site	Locus:	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
Site #1	Frequency:	0.54	0.80	0.90	0.90	0.32	0.49	0.15	0.22	0.39	0.32	0.71	1	0.90	0.46	0.29	0.15	0.54	0.90
Site #2	Frequency:	0.46	0.79	0.79	0.87	0.62	0.33	0.39	0.44	0.54	0.28	0.74	0.97	0.62	0.90	0.28	0.51	0.62	0.87
Site #3	Frequency:	0.24	0.83	0.86	0.90	0.59	0.52	0.21	0.38	0.45	0.35	0.31	1	0.83	0.69	0.31	0.24	0.72	0.66
Site #4	Frequency:	0.22	0.56	0.66	0.78	0.38	0.31	0.31	0.16	0.34	0.28	0.41	1	0.50	0.56	0.38	0.38	0.31	0.41
Site #5	Frequency:	0.20	0.78	0.83	0.93	0.53	0.53	0.23	0.38	0.23	0.18	0.43	0.98	0.75	0.73	0.40	0.20	0.20	0.50
Site #6	Frequency:	0.24	0.76	0.58	0.87	0.42	0.18	0.11	0.21	0.18	0.11	0.58	0.90	0.37	0.55	0.24	0.21	0.42	0.63
Site #7	Frequency:	0.46	0.77	0.77	0.74	0.23	0.17	0.03	0.26	0.20	0.06	0.63	0.94	0.46	0.46	0.26	0.14	0.37	0.69
Site #8	Frequency:	0.03	0.46	0.56	0.59	0.15	0.23	0.08	0.05	0.13	0.03	0.33	0.85	0.13	0.77	0.13	0.15	0.49	0.23
Site #9	Frequency:	0.24	0.62	0.21	0.76	0.32	0.12	0.09	0.09	0.09	0.27	0.53	0.88	0.29	0.38	0.06	0.03	0.41	0.56
Site #10	Frequency:	0.12	0.68	0.53	0.59	0.09	0.03	0.03	0.03	0.06	0.09	0.38	0.82	0.12	0.56	0.12	0.09	0.29	0.59
Site #11	Frequency:	0.28	0.72	0.84	0.48	0.48	0.16	0.52	0.48	0.32	0.40	0.48	0.72	0.60	0.64	0.40	0.08	0.56	0.68
Site #12	Frequency:	0.28	0.52	0.52	0.36	0.36	0.16	0.40	0.44	0.40	0.36	0.28	0.36	0.36	0.48	0.16	0.28	0.32	0.40

Site	Locus:	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126
Site #1	Frequency:	0.71	0.42	0.54	0.37	0.32	0.49	0.63	0.78	0.93	0.76	1	0.78	0.66	0.20	0.10	0.15	0.37	0.39
Site #2	Frequency:	0.51	0.54	0.64	0.51	0.51	0.74	0.69	0.74	0.85	0.80	1	0.90	0.56	0.41	0.97	0.36	0.39	0.39
Site #3	Frequency:	0.66	0.45	0.35	0.45	0.38	0.72	0.59	0.86	0.69	0.62	0.97	0.48	0.38	0.45	0.17	0.31	0.28	0.21
Site #4	Frequency:	0.38	0.25	0.34	0.25	0.25	0.53	0.53	0.47	0.88	0.72	1	0.50	0.25	0.22	0.28	0.19	0.03	0.16
Site #5	Frequency:	0.55	0.40	0.45	0.38	0.40	0.55	0.33	0.53	0.83	0.75	1	0.85	0.53	0.25	0.15	0.15	0.15	0.23
Site #6	Frequency:	0.32	0.32	0.40	0.32	0.53	0.58	0.68	0.58	0.71	0.61	1	0.92	0.21	0.30	0.16	0.16	0.24	0.21
Site #7	Frequency:	0.60	0.29	0.57	0.45	0.37	0.60	0.60	0.69	0.91	0.46	1	0.57	0.20	0.09	0.26	0.06	0.26	0.34
Site #8	Frequency:	0.21	0.10	0.13	0.15	0.39	0.41	0.72	0.49	0.41	0.31	1	0.23	0.23	0.10	0.13	0.10	0.05	0.10
Site #9	Frequency:	0.18	0.15	0.18	0.11	0.21	0.38	0.59	0.41	0.79	0.38	0.97	0.74	0.27	0.06	0.12	0.09	0.09	0.06
Site #10	Frequency:	0.18	0.18	0.12	0.12	0.32	0.26	0.38	0.41	0.59	0.32	0.97	0.65	0.12	0.03	0.06	0.12	0.03	0.06
Site #11	Frequency:	0.52	0.28	0.52	0.80	0.68	0.64	0.64	0.72	0.36	0.48	0.84	0.56	0.56	0.56	0.64	0.32	0.32	0.24
Site #12	Frequency:	0.40	0.24	0.24	0.68	0.56	0.40	0.48	0.32	0.16	0.32	0.72	0.28	0.32	0.36	0.40	0.36	0.28	0.28

Site	Locus:	127	128	129	130	131	132	133	134	135	136	137
Site #1	Frequency:	0.32	0.49	0.68	0.32	0.49	0.76	0.95	0.95	0.98	0.78	0.78
Site #2	Frequency:	0.31	0.54	0.67	0.39	0.64	0.80	0.95	0.87	1	0.90	0.77
Site #3	Frequency:	0.24	0.59	0.59	0.48	0.28	0.72	0.55	0.72	0.86	0.72	0.55
Site #4	Frequency:	0.22	0.53	0.78	0.44	0.38	0.56	0.91	0.81	0.69	0.91	0.84
Site #5	Frequency:	0.38	0.58	0.70	0.38	0.30	0.85	0.95	1	0.98	0.88	0.85
Site #6	Frequency:	0.24	0.55	0.71	0.32	0.26	0.74	0.79	0.76	0.92	0.68	0.66
Site #7	Frequency:	0.34	0.80	0.66	0.66	0.69	0.91	0.94	0.97	0.94	0.94	0.91
Site #8	Frequency:	0.15	0.21	0.23	0.10	0.18	0.46	0.59	0.54	0.44	0.49	0.28
Site #9	Frequency:	0.12	0.27	0.38	0.17	0.09	0.38	0.82	0.77	0.79	0.24	0.47
Site #10	Frequency:	0.06	0.26	0.38	0.06	0.03	0.47	0.68	0.62	0.68	0.35	0.50
Site #11	Frequency:	0.32	0.4	0.44	0.68	0.44	0.36	0.60	0.52	0.60	0.48	0.80
Site #12	Frequency:	0.20	0.32	0.32	0.16	0.24	0.12	0.52	0.56	0.48	0.36	0.32

Figure 12 Dendrogram of UPGMA analysis by Dice's similarity coefficient