Molecular and Field Analyses of *Bathyplectes* spp. (Hymenoptera: Ichneumonidae) in Alfalfa Systems in Virginia

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Abstract

Bathyplectes anurus (Thomson) and *B. curculionis* (Thomson) (Hymenoptera: Ichneumonidae) are specialist parasitoids introduced to the United States for classical biological control of alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). Adults of both species are morphologically similar, generally described as wasps ≈3–4 mm long with black, robust bodies. The *Bathyplectes* spp. occupy sympatric ranges throughout much of the United States and often coexist within alfalfa fields. In Virginia, *B. anurus* is the primary biological control agent, accounting for more than 90% of alfalfa weevil parasitization. A previous study, however, found that the levels of control by *B. anurus* varied among the three alfalfa-growing regions of Virginia, and noted that little is known about the mechanisms or factors responsible for the differential levels of parasitization. Of the factors that may affect host parasitization, three were addressed in this study: population density and spatial distribution of host and parasitoid, the effectiveness of parasitoids in response to host density as measured by the population functional response, and genetic variation among and between populations of parasitoids. GPS-referenced quadrat sampling was conducted in alfalfa fields in the Piedmont and southwestern region to assess alfalfa weevil population levels and spatial distribution, and the level and distribution of parasitization by *B. anurus* and *B. curculionis*. Results showed that there was no significant difference in alfalfa weevil densities among alfalfa fields in the Piedmont and southwestern

region, and also no significant difference between regions in the level of parasitization. Mean alfalfa weevil density per 0.2-m^2 quadrat per sampling date was 11.0 in the Piedmont and 8.0 in the southwestern region; mean percent parasitization per 0.2-m^2 quadrat per sampling date was 9.1% in the Piedmont and 9.9% in the southwestern region. The spatial distributions of alfalfa weevil larvae and parasitization were found to be aggregated in all alfalfa fields sampled. The population functional response of *B. anurus* attack on alfalfa weevil was determined qualitatively to be Type II in all alfalfa fields sampled, except the field located at Blacksburg in the southwestern region. The Type II functional response suggests that, in general, the parasitoid has an inverse density dependent attack response to the density of alfalfa weevil larvae in the two regions of Virginia.

Molecular analyses were conducted to 1) derive genetic sequences for *B. anurus* and *B. curculionis*, which could be used to identify and differentiate the two species, and 2) test for genetic variation within and between regional populations of *Bathyplectes*. Sequences for each species were obtained for the D2–D3 region of the 28S ribosomal DNA (28S rDNA) gene; sequence identity between *B. anurus* and *B. curculionis* was 96.6%. The 28S rDNA sequences were also obtained for another *Bathyplectes* species, possibly *B. stenostigma*, and for four taxa of Hymenoptera that hyperparasitize *B. anurus* and *B. curculionis* cocoons. Sequences were obtained for *B. anurus* and *B. curculionis* for a portion of the mitochondrial gene cytochrome oxidase subunit 1 (COI); sequence identity between *B. anurus* and *B. curculionis* was 89.7%. Genetic variation in the COI gene was observed for both species. One variation of the COI sequence expressed by 25% of *B. anurus* specimens (Haplotype 2) might indicate regional genetic variation correlated to temperature, in particular the average number of days per year that

a region experiences temperatures above 32°C. Two major COI sequence haplotypes found in *B. curculionis* specimens might be indicative of incipient speciation.

DEDICATION

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CHAPTER 1

Introduction and Literature Review

1.1 Introduction

In Virginia, alfalfa, *Medicago sativa* L., comprises over 50 thousand hectares of cropland, primarily in three geograpahic regions: the Piedmont, Shenandoah Valley, and southwestern region. A key insect pest of alfalfa, the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae), was first reported in Virginia in 1952 (Evans, 1959). Numerous parasitic insects including *Bathyplectes anurus* (Thomson) (Hymenoptera: Ichneumonidae), a solitary, koinobiont endoparasitoid wasp, were imported from Europe by the U.S. Department of Agriculture (USDA) to control this insect pest (Brunson and Coles, 1968; Radcliffe and Flanders, 1998). In Virginia, *B. anurus* is thought to be responsible for approximately 92% of all parasitization of alfalfa weevil larvae (Kuhar et al., 2000). However, as has been observed in other states such as North Carolina (Campbell et al., 1975) and Tennessee (Copley and Grant, 1998), the levels of parasitization by *B. anurus* on alfalfa weevil can vary geographically. In Virginia, Kuhar et al. (1999) found parasitization levels to range from 16–32% in the Piedmont region, to 45–73% in the Shenandoah Valley and 46–71% in the southwestern region.

Very little is known about the causes of differential parasitization by *B. anurus* among the regions or the mechanisms that operate to maintain the variations. However, there has been much speculation about possible causes without the support of hypothesis-driven analyses. In general, parasitization levels on pests of crop plants can be influenced by factors relating to crop management practices, the population and spatial ecology of host-parasitoid interactions, and the genetics of the host and/or parasitoid. With respect to management practices, the number and

timing of insecticide applications has been cited as an important cause of the disparate impact of *B. anurus* on alfalfa weevil populations (e.g., Hower and Davis, 1984; Luna, 1986). Los (1982), however, found no significant differences in levels of alfalfa weevil parasitization between insecticide-treated and untreated alfalfa fields. More recently, Dellinger (2003) found that although insecticide treatments significantly lowered levels of parasitization in treated fields, this effect was often only temporary. As such, there is little evidence that insecticide application is responsible for the geographic variability in parasitization observed in alfalfa systems in Virginia.

Parasitization levels can also be influenced by climatic factors (e.g., temperature), host and parasitoid densities, parasitoid behavior and heterogeneity in parasitism, and by the spatial structure of the landscape. Temperature has been shown to affect the way a host responds to parasitoid attacks, as in the case of *Drosophila* spp. in which the process of encapsulation (a cellular immune response to parasitoid invasion) is often more effective at higher temperatures (Kraaijeveld and Godfray, 1999). Kuhar et al. (2001) found that winter temperatures in the Piedmont in Virginia are about 2°C warmer, and as a result, populations of alfalfa weevil larvae are higher and peak two to four weeks earlier than in the Shenandoah Valley and southwestern region. However, Kuhar et al. (2001) also found that *B. anurus* activity was synchronized with the period of peak alfalfa weevil larval populations in each of the regions. Therefore, although climate can influence the dynamics of populations and is a strong determinant of the distribution of alfalfa weevil strains (Radcliffe and Flanders, 1998), climate alone cannot explain the differences in parasitization levels among the regions of Virginia. This is probably because climate usually interacts with other factors to influence ecological processes and to shape the dynamics of populations (Leirs et al., 1997).

Parasitization levels also can be affected by both host and parasitoid density (Van Driesche, 1983). Kuhar et al. (1999), therefore, suggested that the differences in the densities of the alfalfa weevil populations among the regions of Virginia were responsible for the varying levels of parasitism. However, this assertion was made without knowledge of the effects of alfalfa weevil density on *B. anurus* attacks (the functional response) or of the distribution of these attacks in response to host densities.

Although host density is an important determinant of parasitization rates, equally important are parasitoid behavior and heterogeneity in host-parasitoid interactions (Reeve et al., 1994; Maron and Harrison, 1997; Hassell, 2000; Umbanhowar et al., 2003), and the spatial structure of the agricultural landscape (e.g., Menalled et al., 2003). Parasitoids generally respond to the spatial pattern of their hosts in three main ways. They may aggregate positively in response to the host (Hassell and May, 1973; Murdoch and Oaten, 1989; Murdoch, 1994); aggregate independent of host density (Murdoch et al., 1984; Reeve et al., 1994); or exhibit inverse aggregation whereby dense patches of host contain fewer parasitoids (Hassell, 1984). Studies of the patch dynamics of parasitoids and their hosts have found that direct and inverse relationships in the aggregated behavior of parasitoids are common. Fewer studies have observed parasitoid aggregation independently of host density (e.g., Pacala and Hassell, 1991; Reeve et al., 1994). Laboratory studies of alfalfa weevil-*B. anurus* interactions found that the parasitoid tended to aggregate independently of alfalfa weevil larval densities and, as such, acted as a density-independent mortality factor on alfalfa weevil populations (Latheef et al., 1977; Dowell, 1979). If this is true of alfalfa weevil-*B. anurus* interactions in Virginia, we should not expect regional variation in alfalfa weevil density to result in the marked differences in parasitization levels observed between the regions.

The spatial structure and pattern of species resources can influence host-parasitoid dynamics (Landis and Marino, 1998) by affecting such processes as dispersal (Fahrig and Merriam, 1994; Jonsen and Fahrig, 1997) and parasitoid foraging behavior (Reeve et al., 1994). For example, landscape structure has been found to affect parasitism of silverleaf whitefly (*Bemisia argentifollii*) differently in agricultural systems in Florida, where the cropping landscape is more heterogeneous, than in California where the landscape is more uniform (Brewster et al., 1997; Brewster et al., 1999). In a study of generalist and specialist insects in landscapes containing alfalfa fields, Jonsen and Fahrig (1997) found that the structure of the landscape influenced movement, behavior, and demography of alfalfa weevil populations. However, these authors did not study the extent of parasitism within the landscapes. Recently, Menalled et al. (2003) showed that structure of the agricultural landscape influenced temporal dynamics of parasitization of armyworm (*Pseudaletia unipuncta*) populations. How the structure of the landscapes in the alfalfa-growing regions of Virginia affects alfalfa weevil parasitization by *B. anurus* has not been determined.

Genetic variation in host and parasitoid populations has accounted for differences in parasitoid efficiency in several systems (Henter and Via, 1995; Hufbauer and Via, 1999; Kraaijeveld and Godfray, 1999; Hufbauer et al., 2004). Molecular evidence supports the hypothesis that alfalfa weevil populations in the U.S. belong to a single species with three strains (western, Egyptian, and eastern) with overlapping distributions (Hsiao, 1993). Although little is known about the genetic variation of alfalfa weevil populations in Virginia, *B. anurus* has been shown to develop equally well in all three strains under laboratory conditions (Maund and Hsiao, 1991), suggesting that host variation should have little impact on parasitoid efficiency in the

Virginia alfalfa system. Little is known about the genetic variation of parasitoids of the alfalfa weevil in Virginia.

It is likely, therefore, that factors related to the population and spatial ecology of alfalfa weevil and parasitoid interactions and genetic variation among parasitoid populations are responsible for the differential parasitization levels observed in the alfalfa-growing regions of Virginia. Obvious questions then are: what is the relationship between alfalfa weevil density and parasitization by the primary parasitoid, *B. anurus*, in alfalfa systems in Virginia; and, to what extent do parasitoid populations in these systems vary molecularly? An extensive review of the literature on alfalfa cropping systems, the alfalfa weevil, and its parasitoid is provided before attempting to answer these two questions.

1.2 Alfalfa: A Global Crop

1.2.1 Economic Importance of Alfalfa

Cultivated alfalfa, *Medicago sativa* L., is considered the world's most important temperate forage legume, prized for its high nutritional value, soil improvement characteristics, low input requirements, and ecological benefits (Barnes et al., 1988). An estimated 32 million hectares worldwide are in alfalfa production, one-third of which are in the United States, the world's single largest producer of alfalfa (Veronesi et al., 2010). Other major producers of alfalfa are Eastern Europe and Argentina which, together with United States, account for 70% of total alfalfa forage production (Michaud et al., 1988).

Alfalfa is most commonly preserved as hay or silage for livestock feed, especially for use in dairy production, but is also compressed as pellets or cubes for small animal feed. The crop is also grown for human consumption (Mueller et al., 2008). Sprouts of alfalfa seed are a popular

fresh food, and approximately one-third of the U.S. honey crop is produced from alfalfa. The dollar value of alfalfa hay and other products is estimated to be $145 billion annually in the United States, approximately $500 billion annually worldwide (Small, 2011).

1.2.2 Alfalfa as an Efficient Producer of Plant Protein

The crude protein content of alfalfa generally averages between 15% and 22%, twice the amount of protein produced by soybeans per unit area (Putnam et al., 2001). Alfalfa is typically grown as a perennial crop with stands generally kept in production for three to six years though stands can persist longer. Alfalfa can produce as much as 49 metric tonnes of dry matter per hectare in an unirrigated system, and up to 120 metric tonnes under irrigation, all of which is usable (Small, 2011). As with other legumes, alfalfa fixes atmospheric nitrogen through an association with the symbiotic bacteria, *Sinorhizobium meliloti*, housed in the nodules on alfalfa roots; therefore, alfalfa can be grown without additional nitrogen fertilizer, making it a low-input, energy-efficient perennial crop for protein production (Vance et al., 1988).

1.2.3 Ecological Benefits of Alfalfa

The nitrogen fixed in the soil by alfalfa, between 50 and 463 kilograms per hectare per year, is readily available to crops sown after alfalfa in a crop rotation, such as corn or wheat, reducing the amount of nitrogen that needs to be applied to the subsequent crop (Vance et al., 1988). The dense growth habit of alfalfa suppresses weeds common to annual crops, reducing the need to apply herbicides to crops that follow alfalfa in a rotation. The extensive root system and long taproot, which can grow as deep as 9 meters, enable alfalfa to access water and nutrients held deep in the soil. The root system also holds soil together, aiding in soil retention.

Additionally, natural chemicals exuded from alfalfa roots and carbon and nitrogen made available by dead and decaying roots and root nodules provide an excellent environment for the growth of beneficial microorganisms, which improves soil health and increases cycling of soil nutrients in the rhizosphere. Organic acids produced in the rhizosphere also make soils more friable, improving soil tilth and water penetration properties (Putnam et al., 2001; Veronesi et al., 2010; Small, 2011).

As a perennial crop, alfalfa provides a temporally stable habitat for numerous insects, birds, and mammals. Alfalfa is a highly-palatable food source for herbivores, which in turn provides a variety of prey for predators higher up the food chain. The dense growth of alfalfa provides nesting cover for birds and animals. Alfalfa fields are viewed as insectaries due to the diversity of beneficial insects that inhabit the crop, including the minute pirate bug, many species of predatory wasps, flower flies, and ladybugs. Strips of alfalfa planted next to and within other crops can provide a reservoir of predators and parasitoids useful in Integrated Pest Management (IPM) programs, and planting alfalfa in rotation with annual crops helps break the pest and disease cycles of those crops (Summers, 1998; Putnam et al., 2001; Stamps et al., 2009).

1.2.4 Taxonomy and Geographic Distribution

Alfalfa, *Medicago sativa* L., is a wild-cultivated complex of interfertile diploid ($2n$=16) and tetraploid ($4n$=32) subspecies of Eurasian origin. Cultivated alfalfa is generally classified as *M. sativa* ssp. *sativa*, and all forms are tetraploid. However, three other subspecies of the *M. sativa* complex are relevant to the domestication of cultivated alfalfa, as natural hybridizations and intentional crossings between subspecies conferred traits that facilitated the adaptation of

alfalfa to diverse conditions, greatly extending its range (Lesins and Lesins, 1979; Small and Bauchan, 1984).

M. sativa ssp. *caerulea* (= *coerulea*), a diploid, is presumed to be the parent species of cultivated alfalfa. Characterized by violet-blue flowers and strongly-coiled, glabrous seed pods, it is morphologically indistinguishable from the tetraploid subspecies *sativa* (Small, 1985). The native range of this *M. sativa* ssp. *caerulea* extends from northeastern Turkey to central Asia, encompassing the area considered to be the center of alfalfa domestication, the Trans-Caucasus and Iran. A deep taproot enables this subspecies to grow well in arid regions where summer rainfall is limited (Muller, 2003; Small, 2011).

M. sativa ssp. *falcata*, which occurs at both ploidy levels, is characterized by yellow flowers and crescent-shaped, glabrous seed pods. This subspecies is cold-hardy and tolerant of acid soils, and has been crossed with cultivated alfalfa in northern Europe and the eastern United States to confer cold-hardiness and tolerance of acidic soils (Muller, 2003). The native range of subspecies *falcata* is extensive, ranging from Western Europe to East Asia, as far north as Siberia and Scandinavia. Though dry steppe conditions are preferred, ecotypes of subspecies *falcata* are tolerant of conditions ranging from cold and wet to hot and arid (Small, 2011). Crosses between cultivated alfalfa and subspecies *falcata*, classified as subspecies x*varia*, exhibit variegated flowers and weakly-coiled, glabrous seed pods (Quiros and Bauchan, 1988).

M. sativa ssp. *glomerata*, which also occurs at both ploidy levels, is native to the mountainous regions of Algeria, Tunisia, and Italy. This subspecies is characterized by yellow flowers and strongly-coiled, glandular seed pods. Glandular hairs and seed pod coiling are characteristics that offer protection against certain insect pests (Muller, 2003; Small, 2011).

1.2.5 Introduction of Alfalfa to North America

Alfalfa was initially introduced to North America in the 16th century when the Portuguese and Spaniards colonized Mexico and Peru. In the 1800's, missionaries from Mexico introduced alfalfa into Texas, Arizona, New Mexico, and California, which by 1836 was presumably being grown in many parts of the region. Spanish alfalfas were also introduced from Mexico into Colorado (Michaud et al., 1988). However, alfalfa gained importance as a forage crop following its introduction from Chile, dubbed "Chilean clover," into California during the gold rush of the mid-1800s. This crop established easily in the abundant sunshine and arid conditions of the southwest, gaining favor with local stockmen. Grown under irrigation, alfalfa was soon the predominant forage crop produced in the western United States (Michaud et al., 1988; Veronesi et al., 2010). Alfalfa was taken to Utah by Mormon settlers, who grew it for seed, and from there the range expanded into neighboring states until, by 1894, it was a major forage crop in Kansas. By the late 1800's, alfalfa was also being grown in parts of Montana, Iowa, Missouri, and Ohio (Michaud et al., 1988).

The Spanish and Chilean alfalfas were not particularly cold-hardy, and attempts to expand the range of alfalfa northward were largely unsuccessful until immigrant Wendelin Grimm introduced a hybrid from his native Germany into Minnesota in 1857. A cross of subspecies *sativa* and subspecies *falcata*, this hybrid provided the stock from which winter-hardy strains were developed. In 1871, alfalfa developed from the Grimm hybrids was first introduced in Ontario, Canada (Michaud et al., 1988; Russelle, 2001). Between 1858 and 1910, other winter-hardy alfalfas were introduced from Europe and Russia into the upper Midwest and eastern Canada (Veronesi et al., 2010).

Alfalfa was separately introduced by European colonists into eastern North America, with various records of the 1700's detailing attempts by colonists to grow alfalfa from New England to Georgia. Alfalfa thrived on the calcareous soils of New York, but otherwise fared poorly in the east. This lack of early success is likely attributable to acidic soils and humid growing conditions not favorable to the European cultivars of the time (Michaud et al., 1988; Russelle, 2001).

Nine historical introductions of alfalfa germplasm into the United States have been recorded. These include introductions of intermediately cold-hardy strains from the Near East and France in 1947, and introductions of non-hardy strains from Peru in 1899, from India in 1913 and 1956, and from Africa in 1924 (Barnes et al., 1988).

1.2.6 Arthropod Pests of Alfalfa

Alfalfa supports an extremely diverse range of organisms. For example, researchers have identified approximately 1,000 arthropod species that inhabit California alfalfa fields, and nearly 600 arthropod species that inhabit alfalfa in upstate New York. Of these, most cause little or no damage to alfalfa and many, such as predators and parasitoids, are beneficial. However, the few arthropods that are considered pests can cause significant economic losses, estimated at $260 million annually in the United States alone (Summers, 1998).

The most important arthropod pest of alfalfa is the alfalfa weevil (Coleoptera: Curculioinidae), which causes damage primarily through feeding of the larvae on plant leaves. Hemipteran pests, which primarily feed on plant juices, include the potato leafhopper, *Empoasca fabae* (Harris); several species of aphid such as the pea aphid, *Acyrthosiphon pisum* (Harris), and spotted alfalfa aphid, *Therioaphis maculate* Buckton; meadow spittlebug, *Philaenus spumarius*

L.; threecornered alfalfa hopper, *Stictocephala festina* (Say); and tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois). Lepidopteran pests, which cause damage through larval feeding on plant tissues, include the alfalfa caterpillar, *Colias eurytheme*; beet armyworm, *Spodoptera exigua* (Hubner); and yellowstriped armyworm, *Spodoptera praefica* (Grote). The clover root curculio (Coleoptera: Curculionidae), *Sitona hispidulus* (F.), causes damage primarily through larval feeding on root nodules and crowns (Wilson et al., 1979; Godfrey and Yeargan, 1987; Summers, 1989; Summers, 1998).

1.3 Alfalfa Weevil

1.3.1 Taxonomy

The taxonomy of the alfalfa weevil historically has been confused, with species classified under the genera *Phytonomus* and *Hypera*. Species names for alfalfa weevil in its Palearctic range have included *Phytonomus variabilis* (Herbst), *P. posticus* (Gyllenhal), and *Hypera variabilis* (Herbst) (Warner, 1962). In North America, Titus (1911) adopted the name *Hypera postica* (Gyllenhal) for alfalfa weevil discovered in 1904 near Salt Lake City, Utah. Alfalfa weevil discovered in the eastern United States near Annapolis, Maryland, in 1951, was also identified as *H. postica*. These came to be commonly known as the western alfalfa weevil and the eastern alfalfa weevil, respectively. Alfalfa weevil discovered in 1939 near Yuma, Arizona, was presumed to be a different species and was identified as *H. brunneipennis* Boheman, the Egyptian alfalfa weevil (Hsiao, 1996; Radcliffe and Flanders, 1998).

A review of alfalfa weevil nomenclature following the conventions of the International Code of Zoological Nomenclature, 1961, determined that, based on type-species, the generic names *Hypera* and *Phytonomus* are absolute synonyms, with *Hypera* taking precedence. All

specific names published for alfalfa weevil prior to *Rhyncaenus posticus* Gyllenhal in 1813 were invalidated. Therefore, the valid scientific name for alfalfa weevil is *Hypera postica* (Gyllenhal) (Warner, 1962).

1.3.2 Geographic Origins and Distribution

Alfalfa weevil is indigenous to and widely distributed in continental Europe, northern Africa, and Asia to Xinjiang, China (Hsiao, 1993; Radcliffe and Flanders, 1998). Though cultivated alfalfa and other *Medicago* species are preferred host plants, alfalfa weevil will also feed on a few closely-related legumes such as white sweet clover (*Melilotus alba*), red clover (*Trifolium pretense*), hairy vetch (*Vicia villosa*), and Chinese milk vetch (*Astragalus sinicus*) (Titus, 1909; Byrne and Blickenstaff, 1968; Kuwata et al., 2005).

The economic importance of alfalfa weevil varies across its native range and might be largely influenced by climate. For instance, Michelbacher and Leighly (1940) stated that in Morocco and coastal Italy, where summers and winters are mild, alfalfa weevil was well distributed but caused only occasional, localized damage; however, in Turkestan, which is characterized by long, severe winters and hot summers, alfalfa weevil was reported to be a serious pest. The authors posited that in regions where seasonal temperatures remain close to the optimal range for alfalfa weevil activity and development, approximately 10°-25°C (Sweetman, 1929; Zahiri et al., 2010), conditions favor parasitoids as well as weevils and provide greater exposure of alfalfa weevils to parasitization. However, in regions characterized by severe winters and/or hot, dry summers, parasitoid activity is curtailed and weevils cause more damage due to mass feeding during the shortened growing season (Michelbacher and Leighly, 1940).

Worldwide distribution of alfalfa weevil now includes Japan and northwest India, where it is a widespread economic pest (Kuwata et al., 2005; Chandra and Pandey, 2011); south Asia (Brunei Darussalam, Indonesia, the Philippines, and Malaysia) and Oceania (Australia, Papua New Guinea, Samoa and America Samoa), where it a localized, quarantined pest (EPPO, 2009); and throughout North America, including all 48 contiguous states of the United States (Hsiao, 1993).

The alfalfa weevil invasion of North America occurred through three separate introductions from different points of origin. The first discovery of alfalfa weevil was in 1904 near Salt Lake City, Utah, and was believed to have originated in Europe near the borders of France, Italy, and Switzerland. Upon its establishment in the Salt Lake Valley, the weevil spread to adjacent areas at a rate of approximately 16 to 30 kilometers per year (Radcliffe and Flanders, 1998) and by 1948 was recorded in 12 western states (Cothran, 1966). The range of this first introduction, now referred to as the western weevil, has since expanded to 17 states and Alberta, Canada, but does not extend east of longitude 100°. The western weevil seemingly prefers regions of cool and cold climate, which includes the entirety of the Pacific Northwest; the mountain states; and most of North Dakota, South Dakota, Nebraska, and Kansas (Radcliffe and Flanders, 1998). Southward spread of the weevil seems to be inhibited by a climatic barrier of high summer temperatures (Michelbacher and Leighly, 1940), which limits the weevil to the Oklahoma and Texas panhandles, northern New Mexico, northern Arizona, and the coastal and mountain regions of California (Radcliffe and Flanders, 1998).

The second introduction of alfalfa weevil was discovered in 1939 in Yuma, Arizona (Wehrle, 1940). Slight morphological differences, such as a broader thorax and less-pronounced color pattern, along with its establishment in the hot climate of Yuma, led to identification of the

weevil as *Hypera brunneipennis* Boheman, the Egyptian alfalfa weevil, which is indigenous to the Mediterranean region and the Nile Valley of Egypt (Michelbacher and Leighly, 1940). Preferring hot, dry climates, the Egyptian weevil spread throughout Arizona, into southern and central California, south to northern Mexico, and east to western New Mexico and the southwest corner of Colorado. The Egyptian weevil spread north into Utah and southern Nevada, where its range overlaps that of the western weevil. Overlap also occurs in northern Arizona, the northwestern region of New Mexico, the southwest corner of Colorado, and along edges of the Central Valley in California (Hsiao, 1993; Radcliffe and Flanders, 1998).

The third introduction of the alfalfa weevil, referred to as the eastern weevil, was discovered in 1951 in Maryland near Baltimore and by 1952 was in Delaware, Virginia, New Jersey and Pennsylvania as well (Poos and Bissell, 1953). The point of origin is not known, but is believed to be a different region of Europe than that of the western weevil. The eastern weevil prefers warm, wet climates, and is more adaptable and aggressive than the western and Egyptian weevils. Spreading at a rate of more than 80 km per year (Manglitz et al., 1981), by 1964 the eastern weevil infested 25 eastern states (Cothran, 1966), and by 1975 had spread to all eastern and central states and also into Ontario and Quebec, Canada. The range of the eastern weevil west of 100° longitude extends through the Plains states, Oklahoma, Texas, southeast Montana, the northeast corner of Wyoming, southern and eastern Colorado, and the eastern two-thirds of New Mexico, overlapping the range of the western weevil in these states. The Rocky Mountains seem to provide a barrier to further westward movement of the eastern weevil in the mountain states (Hsiao, 1993; Radcliffe and Flanders, 1998).

1.3.3 Subspecies of Alfalfa Weevil

The classification of *Hypera postica* in North America as the western alfalfa weevil and the eastern alfalfa weevil distinguishes more than the points of introduction; these designations also indicate subspecies, or strains, that exhibit differences in ecological preferences, biological characteristics, field behaviors, and reproductive compatibilities (Sell et al., 1978; Hsiao and Stutz, 1985).

Morphologically, the western, eastern, and Egyptian alfalfa weevils are nearly indistinguishable. Newly-emerged larvae are approximately 1 mm long, yellow-green in color, with a black head capsule and faint white dorsal stripe. Larvae darken to a medium green as they develop and the dorsal stripe becomes more prominent. Fully-grown, fourth instars reach approximately 10 mm in length. Adult weevils are gray to brown with a longitudinal elytral stripe of dark brown setae, and range in size from approximately 4 mm in the males to 5 or 6 mm in the females (Titus, 1910). In field populations, the Egyptian weevil is generally the largest of the three weevils, the western weevil the smallest, and the eastern weevil intermediate in size (Hsiao, 1993). Michelbacher and Leighly (1940) noted that the elytral stripe of the Egyptian weevil tends to be lighter and more uniform than that of the western weevil.

Behavioral differences have been noted in the field that sometimes aid in identification. For instance, Egyptian and eastern weevils prefer to pupate on aerial parts of plants, while western weevils prefer to pupate in ground litter (Hsiao, 1993; Bundy, 2005). After pupation, Egyptian and eastern weevils aestivate in the summer as adults and resume activity in the fall, with mating and oviposition occurring late fall through spring (Hsiao, 1993). During aestivation and diapause, Egyptian weevils aggregate under bark or litter outside the field; eastern weevils also leave the field to aestivate but do not aggregate (Hsiao, 1993; Bundy, 2005). After

pupation, western weevils oviposit in late spring through summer, and then scatter to diapause as adults through the fall and winter (Hsiao, 1993; Erney et al., 1996; Bundy et al., 2005).

An important biological difference between alfalfa weevil strains is the presence of *Wolbachia pipientis* Hertig, a maternally-inherited, rickettsia endosymbiont found only in the western weevil. *Wolbachia* induces unidirectional reproductive incompatibility between western weevils and eastern or Egyptian weevils. For instance, eggs produced from the pairing of an eastern female with a western male are infertile, as are the eggs produced from a pairing of an Egyptian female with a western male. Pairing of a western female with an eastern or Egyptian male produces viable eggs, but the sex ratio of progeny is predominantly female. This reproductive incompatibility does not occur between eastern and Egyptian weevils (Leu et al., 1989; Hsiao, 1993; 1996).

Another important biological difference concerns the weevil response to *Bathyplectes* species parasitoids. The eggs of *Bathyplectes curculionis*, which parasitizes larvae of the alfalfa weevil, are strongly encapsulated by eastern and Egyptian weevils, but only weakly by western weevils. For this reason, *Bathyplectes curculionis* is more effective as a biological control agent of the western weevil than of the eastern and Egyptian weevil. Eggs of the parasitoid *Bathyplectes anurus*, which also attacks alfalfa weevil larvae, are rarely encapsulated by any of the weevil strains (Hsiao, 1996).

Though the Egyptian weevil has been classified as a separate species, *Hypera brunneipennis* Boheman, behavioral and biological similarities between the Egyptian weevil and eastern weevil have led researchers to question the validity of identifying the Egyptian weevil as a separate species rather than a different strain of *Hypera postica*. Moreover, traits exhibited by the western weevil that are not exhibited by the eastern and Egyptian weevils, such as infection

with *Wolbachia*, suggest that the western weevil is genetically distinct from the eastern and Egyptian weevils (Sell et al., 1978; Bundy et al., 2005).

1.3.4 Genetic Analyses of Alfalfa Weevil Strains

Cytogenetic analysis conducted by Hsiao and Hsiao (1984) of the eastern, western and Egyptian weevils revealed that all three strains have the same 22 chromosomes with identical karyotypes and C-banding patterns, which would preliminarily invalidate classification of the Egyptian weevil as a separate species. Allozyme analyses conducted by Sell et al. (1978) on gene loci from four enzymes for geographically distinct populations of eastern weevil and western weevil found significant variability between the two strains and among geographic populations that would indicate two genetically distinct populations, of separate origins, of *H. postica* in the United States. A larger allozyme study conducted by Hsiao and Stutz (1985) on gene loci from 12 enzymes, for geographically distinct populations of all three strains, found that Egyptian and eastern weevils are genetically more similar to each other than to the western weevil, but that all three strains are genetically similar overall. Of the variation measured for all populations, only 2.4% were attributable to genetic differences between populations; the remaining 97.6% of measured variation were within-population variation.

Hsiao (1996) sequenced and compared 1,302 base pairs from three mitochondrial DNA regions for the eastern, western, and Egyptian weevils and *Hypera postica* from Budapest, Hungary, and Xinjiang, China. Sequences obtained were a partial sequence (513 base pairs) of the 12S rRNA gene, a partial sequence (499 base pairs) of the 16S rRNA gene, and the internal transcribed spacer region (498 base pairs). Comparison of the sequences for all alfalfa weevil strains showed an overall average of 2% sequence divergence between the five weevil strains,

which further supported the conclusion of the allozyme analyses conducted by Hsiao and Stutz (1985) that the weevil strains belong to the same species.

Erney et al. (1996) sequenced and compared three mitochondrial DNA amplicons for the eastern, western, and Egyptian weevils. The first amplicon (478 base pairs) contained portions of the CO-I and CO-II genes, and the entire intervening $tRNA^{Leu(UUR)}$ gene. The second amplicon (300 base pairs) contained parts of the Cytochrome b and ND1 genes, and the entire intervening $tRNA^{ser(UCN)}$ gene. The third amplicon (253 base pairs) contained portions of the ND1 and large ribosomal genes, and entire intervening $tRNA^{Leu(CUN)}$ gene. Sequences for the eastern and Egyptian weevils were nearly identical, differing in only two nucleotides of all three amplicons. These results seem to confirm that the eastern and Egyptian weevils are of the same species. Comparison of sequences between the western weevil and the eastern and Egyptian weevils showed nucleotide substitutions in all three amplicons, with an overall sequence diversion of 5%.

1.3.5 Biological Control of Alfalfa Weevil

Research into means to control alfalfa weevil began soon after discovery of the pest in Utah. In 1910, E.G. Titus of the Utah Agricultural Experiment Station published a bulletin describing the life cycle and ecology of the alfalfa weevil, with recommendations for control of the pest in the field. Use of the insecticides of the day, namely arsenicals such as Paris Green, was deemed impractical for a couple of reasons. First, insecticide applied to a food crop would necessitate a lengthy pre-harvest interval, during which time new larvae would hatch and damage the crop; second, the frequent applications that would be necessary to protect the crop throughout the growing season could be cost prohibitive. Thus, emphasis was placed on the implementation

of cultural methods to control the pest. These included: clearing debris and leaf litter that would provide shelter to hibernating adults; harvesting the first crop soon after peak egg laying to remove as many eggs as possible from the field; and brush-dragging the field after cutting to destroy larvae and pupal casings.

Interest in biological control of the alfalfa weevil was immediate, as this would provide a low-cost, low-labor means of managing alfalfa weevil. Titus (1910) recorded several birds, mammals, and predatory insects seen to feed on alfalfa weevil and other insects in the field, but noted that no parasitoids had been reared from alfalfa weevil larvae nor was the weevil found to be susceptible to entomophagous bacteria and fungi. From 1911 and 1913, researchers from the USDA Bureau of Entomology traveled to Europe to locate natural enemies of alfalfa weevil in its native range. Of the several parasitoid species imported and released in Utah alfalfa fields, two successfully colonized: the ichneumon *Bathyplectes curculionis* (Thomson), and the pteromalid *Dibrachoides dynastes* (Förster). Further work in biological control of alfalfa weevil was suspended for several years due to World War I (Reeves, 1917; Chamberlin, 1924; Smith, 1930; Michelbacher and Leighly, 1940).

By the end of World War I, the alfalfa weevil had spread from Utah to Idaho, Wyoming, and Colorado (Cothran, 1966). Interest in biological control was renewed by the discovery that, as the weevil had spread into new territory, the parasitoid *Bathyplectes curculionis* had followed and was present in nearly all of the area occupied by alfalfa weevil. Researchers returned to Europe in 1921 to study parasites of alfalfa weevil at all life stages, particularly species that might effectively colonize in the United States under a wide range of climatic conditions. In addition to *Bathyplectes curculionis* and *Dibrachoides dynastes*, several species were exported to and released in the western United States: the larval parasites *Bathyplectes corvina* (Thomson),

later reclassified as *Bathyplectes anurus*, and the eulophid *Tetrastichus incertus* (Ratzeburg); egg parasites *Peridesmia phytonomi* Gahan and *Spintherus* species, both pteromalids; and the prepupal and pupal parasite *Necremnus leucarthros* (Nees), an eulophid (Smith, 1930; Flanders, 1935; Strong, 1935).

Attempts at biological control of the Egyptian alfalfa weevil, discovered in 1939 in Yuma, Arizona, began in 1942 with the release of *Bathyplectes curculionis* adults in the Yuma area (McDuffie, 1945). Colonization of the parasitoid in Yuma was initially slow; however, as the Egyptian alfalfa weevil spread into southern California several years later, *Bathyplectes curculionis* accompanied the weevil. Control of the Egyptian alfalfa weevil by *B. curculionis* was not uniform, generally being much higher in the southern California coastal region than in the hot, dry Yuma desert and California Imperial Valley. Moreover, researchers noted that eggs of *B. curculionis* were sometimes encapsulated by the weevil host, rendering them nonviable (van den Bosch, 1953; van den Bosch and Dietrick, 1959). From 1960 to 1962, study of natural enemies of the Egyptian weevil in the Near East countries of its native range led to the importation and release of several parasitic species: the mymarid *Patasson* sp. (= *Anaphoidea*), an egg parasite; the ichneumon *Bathyplectes anurus* (Thomson), a larval parasite; and prepupal and pupal parasites *Dibrachoides druso* (Walker) (= *dynastes* Förster) and *Habrocytus* sp., both pteromalids (Fisher et al., 1961; Clancy, 1969). Two of these species, *B. anurus* and *D. druso*, eventually established. However, despite the establishment of natural enemies, biological control alone remained insufficient for management of the Egyptian alfalfa weevil (Windbiel et al., 2004).

Biological control of the eastern alfalfa weevil, discovered in Maryland in 1951, was first attempted from 1953 to 1955 through releases of *Bathyplectes curculionis*, obtained from Utah,

in Maryland and Delaware. By 1957, *B. curculionis* had failed to establish at these sites. A second attempt in 1959–1960, with releases of *B. curculionis* collected from California, successfully established the parasitoid at sites in New Jersey, Delaware, and Virginia (Puttler et al., 1961).

From 1957 to 1980, the USDA Agricultural Research Service (ARS) conducted a comprehensive program to establish biological control agents of the eastern alfalfa weevil. Inoculative releases were made in the eastern United States of 12 parasitic species, most of which were collected in Europe by personnel of the USDA European Parasite Laboratory in France and shipped to the Beneficial Insects Research Laboratory (formerly located in Moorestown, New Jersey, and later moved to Newark, Delaware) (Bryan et al., 1993). Six of the species released during this program became established: the ichneumons *Bathyplectes curculionis* (Thomson), *B. anurus* (Thomson), and *B. stenostigma* (Thomson); the eulophid *Tetrastichus incertus* (Ratzenburg); and the brachonids *Microctonus aethiopoides* (Loan) and *M. colesi* (Drea). Stock material of these six species was later provided by the USDA for release against the alfalfa weevil in Canada (Dysart and Day, 1976). A second phase of this comprehensive biological control program was conducted by the USDA Animal and Plant Health Inspection Service – Plant Protection Quarantine (APHIS-PPQ) from 1980 to 1988, the purpose of which was to redistribute established alfalfa weevil parasites throughout the United States. During this phase, several species were recovered that had been released in prior biological control efforts but had not established, including *Dibrachoides dynastes* (Förster), *Patasson luna* (Girault), and *Peridesmia discus* (Walker). Large-scale production of parasites for distribution involved mass-rearing of *D. dynastes* and *T. incertus* at the APHIS National

Biological Control Laboratory at Niles, Michigan, and field collection of other species (Bryan et al., 1993).

Of the many parasite species released in North America to manage alfalfa weevil, greatest efficacy has been achieved with *Bathyplectes anurus*, *Bathyplectes curculionis*, *Microctonus aethiopoides*, and *Tetrastichus incertus*. Additionally, *Zoophthora phytonomi* Arthur, a fungal pathogen of undetermined origin, has emerged as an important mortality factor of alfalfa weevil in Ontario, the eastern United States, and California (Radcliffe and Flanders, 1998).

1.4 The *Bathyplectes* in North America

1.4.1 Taxonomy

Bathyplectes anurus, *Bathyplectes curculionis*, and *Bathyplectes stenostigma* were originally classified by Thomson in 1883 as *Canidia anura*, *Canidia curculionis*, and *Canidia stenostigma*, respectively. In 1911, Szepligeti determined the genus *Bathyplectes* Förster to be synonymous with *Canidia* Holmgren, with *Bathyplectes* taking precedence (Viereck, 1920; Chamberlin, 1926).

Synonyms found in the literature for *Bathyplectes stenostigma* are *Bathyplectes* sp. "bagged," *Bathyplectes contracta*, and *Bathyplectes* n.sp. (Puttler, 1967; Brunson and Coles, 1968; Dysart and Coles, 1971; Dysart and Day, 1976). Synonyms in the literature for *Bathyplectes anurus* are *Bathyplectes anura* (Coles and Puttler, 1963) and *Bathyplectes corvina* (Chamberlin, 1926; Essig and Michelbacher, 1933; Gyrisco, 1958; Fisher et al., 1961; Clancy, 1969; Dysart and Day, 1976), though this synonym would be erroneous as *B. corvina* (Thomson) is a separately described species. References to *B. corvina* in early literature as a natural enemy

of the alfalfa weevil describe the jumping cocoon of the species, a principle diagnostic feature for identifying cocoons of *B. anurus* (Dysart and Day, 1976). *B. corvina* is not referenced in contemporary literature discussing biological control of alfalfa weevil in North America, whereas *B. anurus* is prominently discussed. No common synonyms have been noted in the literature for *B. curculionis*.

1.4.2 Geographic Origins and Distribution

Bathyplectes curculionis is a Eurasian species whose native distribution includes most of Europe and several countries in the Middle East. Chamberlin (1926) stated that *B. curculionis* was widely distributed throughout Europe, and was specifically collected in Germany, Switzerland, France, Italy and Sicily, and in the former Soviet Union (Bryan et al., 1993). Surveys in the Middle East have confirmed that *B. curculionis* is a key biological control agent of *Hypera postica* and *H. brunneipennis* in Egypt, Iran, and Iraq (van den Bosch, 1964; Gonzalez et al., 1980.) In North America, multiple introductions of *B. curculionis* in the western and eastern United States have succeeded in establishing the insect across most of the country (Bryan et al., 1993). *B. curculionis* is also established in Ontario, Canada (Harcourt, 1990).

Bathyplectes anurus is indigenous to Europe and the Middle East, with a native range similar to that of *B. curculionis*. Chamberlin (1924) stated that, in Europe, *B. anurus* was found in practically all regions from which *B. curculionis* was collected, and seemed to be the dominant parasitoid in some areas. A survey of *Bathyplectes* in Egypt, Iran, and Iraq found that *B. anurus* was most prevalent in regions having moderately warm summers and cold to very cold winters, such as the mountains of Iran, and was absent in the hot, arid regions of Egypt and Iraq that were more suitable to *B. curculionis* (Gonzalez et al., 1980). In North America, *B. anurus* is

established throughout much of the United States and is the primary parasitoid of alfalfa weevil in the Northeast, several southern and Midwestern states, and Ontario, Canada (Radcliffe and Flanders, 1998). *B. anurus* has recently become established in Japan for biological control of alfalfa weevil in Chinese milk vetch (Shoubu et al., 2005).

Bathyplectes stenostigma is native to central and northern Europe, with a general range between latitudes 47º North and 56º North and longitudes 2º East to 31º East (Dysart and Coles, 1971), roughly central France to northern Belarus. Dysart and Day (1976) reported that most *B. stenostigma* released in North America originated in Sweden. In North America, *B. stenostigma* is distributed throughout the Intermountain states and the northern Great Plains, with the heaviest concentrations in Colorado and Utah, and is a biological control agent of localized importance in areas too cold for colonization by *B. anurus* (Bryan et al., 1993; Radcliffe and Flanders, 1998).

1.4.3 Morphological, Biological, and Behavioral Differences of *Bathyplectes*

Besides the original descriptions by Thomson (1883), very little has been published about the morphology of the adult *Bathyplectes*, which are said to closely resemble one another. *B. anurus* and *B. curculionis* are generally described as wasps approximately 3–4 mm long with black, robust bodies (Thomson, 1883; Dysart and Day, 1976). Coseglia et al. (1977) noted that the antennae of *B. curculionis* have 18–25 segments. *B. stenostigma* is described as similar in morphology to *B. anurus* and *B. curculionis* except that the body is less robust and the color is predominantly dark brown rather than black (Dysart and Day, 1976).

No information was found in the literature regarding characteristics by which adult males of each species can be identified. Adult females are distinguishable in the field by the ovipositor, which is shortest in *B. anurus* and longest in *B. curculionis* (Clancy, 1969). The ovipositor of *B.*

anurus is approximately 0.96 mm in length and is uncurved, while the ovipositor of *B. curculionis* curves upward and is approximately 1.38 mm in length (Dowell, 1977; Dowell and Horn, 1977). The ovipositor of *B. stenostigma* is slightly shorter than that of *B. curculionis*, approximately 1.33 mm long, and is uncurved like that of *B. anurus* (Dowell, 1977). Species are more readily distinguished by their distinctive larval cocoons. Cocoons of nondiapausing *B. curculionis* are thin-walled and flexible, approximately 3–4 mm long and 2 mm in diameter, and light brown in color with a white or cream-colored equatorial stripe (Dysart and Day, 1976; Weaver, 1976). Cocoons of diapausing *B. curculionis* and *B. anurus* are more heavily constructed and inflexible, medium to dark brown in color, and ringed by a white or cream-colored equatorial stripe. For cocoons of *B. anurus*, the equatorial strip is thin, sharply defined, and raised from the surface of the cocoon; that of *B. curculionis* cocoons is flush to the surface, broader, and with softly-defined edges (Brunson and Coles, 1968; Dysart and Day, 1976; Weaver, 1976). Cocoons of *B. anurus* can also be identified by the propensity to jump when disturbed or in response to environmental conditions, a trait not shared by *B. curculionis* (Chamberlin, 1926; Fisher et al., 1961; Brunson and Coles, 1968; Day, 1970; Dysart and Day, 1976). Cocoons of *B. stenostigma* are similar in size to those of *B. anurus* and *B. curculionis*, but are generally dull brown in color (occasionally white), have a rough, crinkled texture resembling a paper bag, and lack an equatorial band. Within this cocoon is a fragile inner cocoon that houses the developing larva (Brunson and Coles, 1968; Dysart and Coles, 1971; Dysart and Day, 1976).

B. anurus and *B. stenostigma* are univoltine species that undergo obligatory diapause. Eggs oviposited in host larvae hatch in approximately four days. If more than one egg is oviposited in a single host, supernumeraries fight for survival until only one larva remains

(Dysart and Coles, 1971; Dowell and Horn, 1977). The *B. anurus* larva develops through five instars over an 18–21 day period (Bartell and Pass, 1980). After the host has spun its pupal casing, the parasitoid emerges from the host, killing it at that time, to construct its cocoon within the host pupal casing. Larvae transform to diapausing adults in the fall and overwinter within their cocoons in the adult stage (Dowell and Horn, 1977). The *B. stenostigma* larva develops through four (possibly five) instars over approximately ten days, then kills and consumes the host before the host can spin its pupal casing. The parasitoid larva constructs its cocoon in leaf litter on the ground, enters diapause, and overwinters as a larva within its cocoon. Transformation to adult occurs within the cocoon the following spring (Dysart and Coles, 1971; Dowell, 1977). *B. curculionis* is bivoltine and undergoes a diapause that is partially dependent upon daylength (Dowell and Horn, 1977). Eggs oviposited in host larvae hatch in approximately four to five days and, if more than one egg has been oviposited in a host, the first larva to eclose generally destroys the remaining eggs, or supernumeraries fight for survival until only one larva remains. The larva develops through five instars, which generally takes 8–13 days for nondiapausing forms and 13–days for diapausing forms. After the host spins its pupal casing, the parasitoid kills and consumes the host and constructs its cocoon in the host pupal casing (Bartell and Pass, 1978). Nondiapausing adults emerge from cocoons approximately two weeks later, and continue to parasitize larvae in the field. All larvae that develop from the eggs oviposited by nondiapausing adults form diapausing cocoons. Diapausing larvae overwinter in cocoons in the larval stage, and pupate into adults just prior to emergence the following season (Chamberlin, 1926; Latheef and Pass, 1975).

All three *Bathyplectes* species are solitary endoparasitoids of alfalfa weevil larvae. Though they can successfully parasitize all stages, *B. curculionis* and *B. stenostigma* prefer first

and second instars, and spend most time probing alfalfa shoot tips for host larvae in a non-random search pattern. *B. anurus* attacks second and third instars, and searches randomly for host larvae mainly among leaves and outer tips. Differences in host stage preference and search pattern create partial temporal and spatial refuges that facilitate utilization of a single host species by multiple members of a parasitoid guild (Dowell, 1977; Dowell and Horn, 1977). Temporal separation between the *Bathyplectes* can also be attributed to the timing of adult flight activity. Where the ranges of the *Bathyplectes* are sympatric, such as in north central Europe, peak flight of *B. anurus* occurs earliest in the season, followed by *B. curculionis*, then *B. stenostigma*, and lastly the partial second generation of *B. curculionis* (Dysart and Coles, 1971). According to Dysart and Day (1976), this sequence of activity also occurs in the sympatric ranges of *Bathyplectes* in North America, though Smilowitz et al. (1972) noted that in some northern states, such as Pennsylvania and Ohio, the peak flight of *B. anurus* adults occurs later and overlaps the peak flight of first generation *B. curculionis*. Second generation activity of *B. curculionis* adults can extend into late summer or fall if sufficient host larvae are present and climatic conditions are mild (Michelbacher and Leighly, 1940; Parrish and Davis, 1978).

Biological studies of the three *Bathyplectes* species have determined several characteristics that favor *B. anurus* over *B. curculionis* and *B. stenostigma* as a biological control agent of alfalfa weevil. *B. anurus* specimens average 32 ovarioles per ovary compared with 27 per ovary for *B. stenostigma* and 23 per ovary for *B. curculionis* (Dowell, 1978; Yeargan, 1979), and exhibit greater fecundity, with an average of 921.5 eggs produced versus 423.6 eggs produced by *B. stenostigma* and 390.7 produced by *B. curculionis* (Yeargan, 1979). A certain percentage of the *Bathyplectes* females emerge with non-functional ovaries, which in one study by Dowell (1976) averaged 14% in *B. anurus* (n = 14), 12% in *B. curculionis* (n = 50), and 9% in

B. stenostigma (n = 9). However, a separate study conducted by Yeargan and Pass (1978) found the incidence of non-functional ovaries to be zero in *B. anurus* specimens, and 28% in *B. curculionis* specimens; Day (1983) found the incidence of non-functional ovaries to be 1.3% in *B. anurus* specimens (n = 80) compared with 9.3% in *B. curculionis* (n = 75). Eggs of *B. curculionis* are subject to encapsulation by larvae of the eastern and Egyptian strains of alfalfa weevil, but not by larvae of the western alfalfa weevil (van den Bosch, 1964; Puttler, 1967; Berberet et al., 1987; 2003). Eggs of *B. anurus* and *B. stenostigma* are not subject to encapsulation by alfalfa weevil larvae (Puttler, 1967; Dowell, 1977; Harcourt, 1990).

Comparisons of host searching and handling behavior have found that *B.* anurus exhibits the fastest host searching and handling, and *B. curculionis* exhibits the slowest. Regarding larval competition, *B. anurus* is more aggressive at eliminating supernumeraries than *B. stenostigma*, and *B. curculionis* is the least aggressive. However, *B. curculionis* exhibits the greatest dispersal rate, averaging more than 48 km per year and as much as 320 km per year, whereas dispersal rates for *B. anurus* and *B. stenostigma* are much lower, averaging less than 8 km per year for *B. anurus* and less than 16 km per year for *B. stenostigma* (Dowell, 1977; Harcourt, 1990). Despite the advantage in colonizing new territory exhibited by *B. curculionis*, Harcourt (1990) found that higher reproductive capacity, more rapid host search and handling, and more aggressive behavior enables *B. anurus* to outcompete, and eventually displace, *B. curculionis* in eastern populations of alfalfa weevil. Kuhar et al. (2000), studying parasitization of alfalfa weevil in Virginia during the period 1997–1999, found that 92.3% of parasitoids emerging from alfalfa weevil larvae hosts were *B. anurus*; less than 8% were *B. curculionis*, though *B. curculionis* had been introduced in Virginia and became established in the state prior to *B. anurus* (Brunson and Coles, 1968; Bryan et al., 1993).

1.4.4 Hyperparasitism of *Bathyplectes*

Bathyplectes curculionis is subject to hyperparasitism by numerous species of Hymenoptera, predominantly of the families Pteromalidae, Ichneumonidae, and Chalcididae, that attack the developing parasitoid in the cocoon. Published studies of *B. curculionis* hyperparasitism reported levels as low as 7% in southeastern Nebraska to as high as 50% in Colorado (Simpson et al., 1979; Rethwisch and Manglitz, 1986). Successful hyperparasitoids exhibit one or more competitive characteristics such as multivoltinism, gregariousness, extended longevity, or lack of diapause, and often display greater biotic potential than the host which can impact the effectiveness of the primary parasitoid as a biological control agent (Hamlin et al., 1949; Puttler, 1966; Pike and Burkhardt, 1974; Simpson et al., 1979).

Twenty-two parasitoid species have been reported as hyperparasitoids of *B. curculionis* in North America. In studying regional reports of *B. curculionis* hyperparasitism, Rethwisch and Manglitz (1986) concluded that hyperparasitoid species seemed to be associated with particular alfalfa weevil strains. Eight species were determined to be associated only with the western strain of alfalfa weevil: the pteromalids *Trichomalopsis albopilosus* (Graham), *T. americanus* (Gahan), *T. leguminis* (Gahan), and *Sceptrothelys grandiclava* Graham; the ichneumons *Mesochorus agilis* Cresson and *M. nigripes* Ratzeburg; *Spilochalcis side* (Walker), a chalcid; and the eulophid *Tetrastichus bruchophagi* Gahan. Six species were determined to be associated only with the eastern alfalfa weevil strain: the ichneumons *Agrothereutes* sp., *Coccygomimus* sp., *Gambrus ultimus* (Cresson), and *Itoplectus conquisitor* (Say); *Spilochalcis torvina* (Cresson), a chalcid; and *Eupelmus* sp., an eupelmid. The remaining eight species were determined to be associated with both the western and the eastern alfalfa weevil strains: the pteromalids *Catolaccus aeneoviridis* (Girault), *Dibrachus cavus* (Walker), *Pteromalus* sp., *Sceptrothelys*

intermedia (Walker), and *Trichomalopsis viridescens* (Walsh); *Gelis* sp., an ichneumon; *Spilochalcis albifrons* (Walsh), a chalcid; and the eupelmid *Eupelmella* (=*Eupelmus*) *vesicularis* (Retzius). *Mesochorus nigripes* (Ratzeburg) is a known hyperparasitoid of *B. curculionis* in association with the Egyptian alfalfa weevil (Fisher et al., 1961). A majority of species are native to North America or are thought to have been established prior to the introduction of *B. curculionis*, including the pteromalids *Trichomalopsis viridescens*, *T. leguminis*, *T. americanus*; the ichneumons *Mesochorus agilis* and *Gelis* sp.; and the chalcid *Spilochalcis albifrons*. Species believed to have been recently introduced include the ichneumon *Mesochorus nigripes* and the pteromalids *Sceptrothelys grandiclava*, *S. intermedia*, *Trichomalopsis albopilosus*, and *Dibrachys cavus* (Day, 1969; Pike and Burkhardt, 1974; Coseglia et al., 1977).

Simpson et al. (1979), studying hyperparasitism of *B. curculionis* in Colorado, found that most hyperparasitoid species attacking the larval stage preferred larvae of one cocoon type, nondiapausing or diapausing, over the other; thus, the level of hyperparasitism by a particular species would in part be dependent upon the abundance of the preferred cocoon type when hyperparasitoids are in the field. In their study, the species found to be most detrimental to larvae in diapausing cocoons were *Sceptrothelys grandiclava* and *Mesochorus nigripes*; the species most detrimental to larvae in nondiapausing cocoons were *M. agilis* and *Trichomalopsis americanus*. *T. americanus* was also found to parasitize larvae in diapausing cocoons, but at lower numbers. Abu and Ellis (1975), studying the biology of *B. anurus* in Ontario, reported that diapausing cocoons were most often hyperparasitized by *Gelis* sp.

Few references to hyperparasitism of *Bathyplectes anurus* cocoons are found in the literature. Day (1970) hypothesized that the jumping activity of *B. anurus* cocoons provided protection from attack by other insects, and laboratory research exposing *B. anurus* and *B.*

curculionis cocoons to attack from *Dibrachys cavus* showed hyperparasitization of *B.* curculionis to be five to eight times greater than hyperparasitization of *B. anurus*. Reported hyperparasitoid species of *B. anurus* in North America are the pteromalids *Catolaccus aenoviridis*, *Dibrachys cavus*, and *Trichomalopsis viridiscens*; the ichneumon *Gelis* sp.; and the chalcid *Spilochalcis albifrons* (Day, 1969).

Bathyplectes stenostigma is reported to be hyperparasitized by the ichneumon *Mesochorus nigripes* and the pteromalid *Dibrachys cavus* (Dysart and Coles, 1971; Coseglia et al., 1977).

1.4.5 Genetic Analyses of *Bathyplectes*

Quicke et al. (2005) published a partial sequence of the 28S ribosomal RNA gene for *Bathyplectes curculionis*, obtained as part of a larger molecular study to determine phylogenetic relationships among subfamilies of Ichneumonidae. The sequence of 643 base pairs, on file with GenBank under Accession No. AY593068, was obtained from *B. curculionis* specimens collected in Germany. No other sequences for *B. curculionis* are on file with GenBank, nor are sequences for *B. anurus* or *B. stenostigma*.

Dellinger (2003) conducted random amplified polymorphic DNA (RAPD) analysis of *Bathyplectes anurus* specimens, collected over a two-year period from the Piedmont and southwestern region of Virginia, to determine population-level genetic variation. A low level of genetic variation detected between populations was likely attributable to genetic variation among individuals of the population. No other references to genetic analyses of *B. anurus* have been found in published literature.

1.5 *Bathyplectes*-Alfalfa Weevil Interactions in Alfalfa Fields

One means of determining the efficacy of an insect natural enemy as a biological control agent is by examination of its functional response to the densities of the target host. Studies have examined the functional response of *B. anurus* in its attack on the alfalfa weevil under laboratory conditions (Latheef et al., 1977; Dowell, 1979), but there have been no studies of this interaction in the field. The laboratory-derived functional response, which Berryman (1999) preferred to call a behavioral response, provides a measure of the efficacy of a natural enemy (NE) by offering insights into its search and attack behaviors. Solomon's (1949) partitioning of the NE response to increasing prey density into the functional and numerical effects inspired Holling (1959a) to ask whether the parameters of these responses were universal to all acts of predation. This led to an analysis of the components of predation and the classic experiment in which a human subject was used as a predator and sandpaper disks as the prey. The result was the disk equation,

$$N_A = \frac{aT_T N_0}{1 + aT_H N_0} \qquad (1)$$

where N_A represents the number of disks removed, N_0 is the density of disks, T_T the time interval available for searching, T_H handling time, and a the rate of discovery (rate of search multiplied by probability of finding a given disk). Equation (1) described what Holling (1959b) referred to as a Type II decelerating functional response in which the number of prey attacked per predator increases at a decreasing rate or the proportion of prey attacked declines asymptotically with increasing prey density (Juliano, 2001). Holling (1961) saw the disk equation as the "basic behavioral functional response equation" because it served as the starting point for the development of other functional responses and a link to responses of insect natural enemies in real systems.

Functional response models other than the Type II response have been described. In a Type I response, the number of prey attacked increases linearly to a plateau with increasing prey density. Type III is an accelerating response, in which the number of prey attacked per NE individual increases in a sigmoidal manner with increasing prey density. In a Type IV response, the number of prey attacked per NE increases initially but later decreases with increasing prey density (Holling, 1959b; Hassell, 1978; Fujii et al., 1986; Berryman, 1999).

That there are at least four possible types of functional response has prompted studies of how best to determine the response for a particular natural enemy and the most efficient method for deriving the parameters of the functional response model (Fujii et al., 1986; Juliano and Williams, 1987; Fan and Pettit, 1994). However, regardless of the method used to fit the functional response model to data, questions remain as to which functional response is more common among predators and parasitoids, and the circumstances under which they might exhibit a particular type of functional response.

The numerical response or change in predator density with a change in prey numbers is the other important NE response mentioned by Solomon (1949). This response generally has received less attention from ecologists than the functional response (Crawley, 1975), although this seems to be changing with recognition of its importance in interactions between prey and natural enemy.

The efficacy of a predator or parasitoid in its attack on a prey will be influenced by its ability to overcome the various biotic and abiotic factors that affect its searching behavior and attack probability among individual prey. Factors that can alter the vulnerability of prey to attack are likely to be those that will have the greatest impact on the functional response. These factors include all the components of the disk equation such as host density, time the natural

enemy and prey are exposed to each other, search and discovery rate, and handling time (Holling, 1961; Hassell, 1978). In addition, exploitation and interference competition among NE individuals, and environmental conditions such as temperature (Holling, 1961), also can potentially affect the functional response and, consequently, the dynamics and regulation of the prey population. The development of mathematical models and theories of NE-prey interactions alone or in combination with controlled laboratory experiments have helped to improve our understanding of the interactions. However, many deficiencies still exist in our knowledge of the responses of predators to their prey in natural and agroecosystems. For example, most functional response models have been prey or resource-dependent and have assumed that prey density is the only factor affecting attack rate (e.g., Holling, 1959b). However, some have suggested that realistic functional responses should be consumer-dependent (Kratina et al., 2009), whereby attack rate depends on densities of both prey and natural. Yet others have discussed the idea of ratio-dependent functional responses (Abrams, 1994; Abrams and Ginzburg, 2000) where predation rate depends on the ratio of prey to natural enemy rather than on absolute numbers. Kratina et al. (2009) noted that despite disagreement about which model provides a more realistic framework for predicting NE-prey dynamics, a full understanding of these dynamics can be achieved only by considering the multiple dependencies of functional responses.

If knowledge of the functional response is all that is needed to assess the bio-efficiency of a natural enemy against a pest, it should be relatively easy to predict the success of the natural enemy as a biological control agent based solely on the type of functional response. A biocontrol agent having a Type III functional response likely will be favored over one with a Type II response, because a Type III response suggests that the agent is capable of a direct density-dependent attack at low prey density and has the ability to learn and alter its search rate

as prey density changes; this implies, at least in theory, stable prey-enemy interaction and regulation of the prey population (Hassell, 1978; Berryman, 1999; Fernandez-Arhex and Corley, 2003). However, after an extensive review of the literature, Fernandez-Arhex and Corley (2003) found that there were many cases of Type II functional response among parasitoids used in biological control and relatively few cases of successful biological control programs involving parasitoids with a Type III functional response. The findings further suggested that there was no clear relationship between the type of functional response and success of an agent in biological control. This led Fernandez-Arhex and Corley (2003) to caution against placing too much emphasis on the functional response determined by laboratory experiments when evaluating an agent for biological control and to suggest that effort should be focused on the behavior and interactions of natural enemies under natural conditions.

Very few attempts have been made to examine the functional response of natural enemies under natural conditions (Abrams and Ginzburg, 2000); in cases where this has been attempted, the laboratory- and field-measured responses tended to differ. For example, O'Neil (1997) found a lack of correspondence between the laboratory-measured and field-measured functional response for the generalist predator *Podisus maculiventris*. One reason for the disparity is that laboratory-generated functional responses are almost always prey-dependent, whereas in the field the functional response of a natural enemy depends on both prey and NE densities (NE-dependent response), the ratio of prey and NE densities (ratio-dependent response), or the densities of the prey, NE, and other species (multispecies-dependent response) (Abrams and Ginzburg, 2000; Kratina et al., 2009). It is clear then that precise measurements of NE density are required for determining searching and attack efficiency (functional response) under natural conditions (Hassell, 1982). However, the difficulty of satisfying this requirement appears in part

to be responsible for the rarity of field-measured or population functional responses (Abrams and Ginzburg, 2000).

Population functional response, which is assessed in the field, measures mean parasitization across host population as a function of mean host population per host patch, and can be used as a surrogate measure of adaptation of the parasitoid to the host and local environment (Ives et al., 1999). A graph of percent parasitization versus the number of host per patch provides a visual assessment of the spatial variation or functional form of attack. By the shape of the plot, the population functional response can be characterized as density dependent, which increases linearly; inversely density dependent, in which increase is linear to an asymptote, after which increase decreases in rate; or density independent (Pacala and Hassell, 1991; Hassell, 2000).

Key to determining the population functional response is an understanding of spatial heterogeneity in the host-parasitoid system (Hassell, 2000). Spatial distribution of hosts in the field may be random, in which no discernible spatial pattern occurs; uniform, in which host seem to be evenly distributed; or aggregated, in which host are clustered or grouped in patches. The spatial distribution of host population and NE attack can be inferred by the relationship between variance (σ^2) and mean (μ) of population samples, such that variance equal to mean ($\sigma^2 = \mu$) represents a random dispersion; variance less than mean ($\sigma^2 < \mu$) represents a uniform dispersion; and variance greater than mean ($\sigma^2 > \mu$) represents an aggregated dispersion (Elliott, 1977).

Parasitoid response to host spatial pattern can be one of three basic types: parasitoids can aggregate positively in response to the host (Hassell and May, 1973), aggregate independently of host density (Reeve et al., 1994), or exhibit inverse aggregation whereby dense patches of hosts contain fewer parasitoids (Hassell, 1984). Studies of the patch dynamics of host-parasitoid

interactions have found that direct and inverse aggregation of parasitoids in response to hosts are common (Pacala and Hassell, 1991). Parasitoid aggregation in response to host distribution may be quantified by the equation:

$$\beta_i = w\alpha_i^{\mu}$$

where μ represents an index of parasitoid aggregation and w represents a normalization constant so that the sum of the β_i values is unity. An index value of $\mu = 0$ represents an even distribution of searching parasitoids across patches; $\mu<0$ represents an inverse aggregation of parasitoids to host per patch; and $\mu>0$ represents positive parasitoid aggregation in response to host density that increases with the value of μ towards infinity (Hassell and May, 1973; Hassell, 2000).

The CV^2 rule (CV^2 = [variance/mean]^2) can also be used to assesses the dynamical effects of heterogeneity in parasitism and stability of host-parasitoid interactions (Pacala and Hassell, 1991; Hassell, 2000). The rule states that overall population densities will be locally stable if the coefficient of variation squared of the density of searching parasitoids in the vicinity of each host exceeds unity (Jones et al., 1992). CV^2 can be partitioned into two components: a host-density-dependent component (HDD) and a host-density-independent component (HDI), both of which can provide further insights into host-parasitoid dynamics. Derivation of CV^2 involves fitting field data to the distribution of parasitism and local host density per patch. Analysis of CV^2 assumes that the parasitoid is a specialist on a single host and, as such, its interaction with the host is synchronized (Pacala and Hassell, 1991; Jones et al., 1992).

1.6 Research Rationale and Objectives

Though *Bathyplectes anurus* is considered to be one of the most effective parasitoids against alfalfa weevil larvae, particularly in the eastern United States, the level of control achieved by *B. anurus* has been found to vary regionally and within states. Biological control of

alfalfa weevil in the northeast has led to a 73% reduction in the use of insecticides to control the pest; however, though also established in southeastern alfalfa-growing states, *B.* anurus has failed to achieve the level of control exhibited by the parasitoid in its northern range (Radcliffe and Flanders, 1998). In addition, the levels of parasitization have been found to vary between the two alfalfa-growing regions of Virginia.

The hypothesis of this research is that the geographic patterns of alfalfa weevil parasitization by *Bathyplectes anurus* are influenced by the extent to which populations of the parasitoid are locally adapted to the environment of the host or by genetic variations within and/or between regional populations of the parasitoid. The main objectives of this research, therefore, are:

1. To study *B. anurus* interactions with the alfalfa weevil directly in alfalfa fields;
2. To determine the molecular composition of parasitoids of the alfalfa weevil in Virginia. This objective has two tasks:
    a. Develop methods of molecular taxonomy by which *B. anurus* can be genetically identified and differentiated from closely-related species of *Bathyplectes* and from hyperparasitoids; and
    b. Determine and measure genetic variation within and between regional populations of *B. anurus*.

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CHAPTER 2

Bathyplectes anurus and Alfalfa Weevil Interactions in Alfalfa Fields in Virginia

2.1 Introduction

Alfalfa, *Medicago sativa* L., is cultivated in Virginia primarily in three geographic regions: the Piedmont, the Shenandoah Valley, and the southwestern region (Kuhar et al., 1999). A key insect pest in these and other alfalfa-growing regions throughout the U.S is the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae). A number of parasitic insects were imported from Europe by the U.S. Department of Agriculture (USDA) to control alfalfa weevil (Brunson and Coles, 1968; Radcliffe and Flanders, 1998), including *Bathyplectes anurus* (Thomson) (Hymenoptera: Ichneumonidae). Although *B. anurus* is responsible for approximately 92% of all parasitization of alfalfa weevil larvae in Virginia (Kuhar et al., 2000), levels of parasitization have been found to vary by region, with lower levels observed in the Piedmont compared with the Shenandoah Valley and southwestern region (Kuhar et al., 1999). Currently, little is known about the factors or mechanisms responsible for the differential levels of parasitization by *B. anurus* on the alfalfa weevil among the regions. However, there has been much speculation about possible causes without the support of hypothesis-driven analyses.

Information in the literature on insect host-parasitoid interactions points to certain factors that may affect parasitization levels. These factors include host and/or parasitoid genetics (Hufbauer et al., 2004); host population levels; the number of parasitoids searching and their individual effectiveness (their functional response to host density); the extent of mutual interference among parasitoids, the degree of heterogeneity (or pseudointerference) that renders some hosts more susceptible to parasitization than others (Hassell and Waage, 1984); and

temperature (Kraaijeveld and Godfray, 1999). The factors may be grouped into three categories: Management Practices, Genetics, and Population and Spatial Ecology of host and parasitoid.

With respect to Management Practices, the number and timing of insecticide applications has been cited as the most important cause of the disparate impact of *B. anurus* on alfalfa weevil populations (e.g., Hower and Davis, 1984; Luna, 1986). However, additional studies suggest that insecticide application may not be an important determinant of the differential levels of parasitization among the regions (Los, 1982; Dellinger, 2003).

Genetic variation in host and parasitoid populations has accounted for differences in parasitoid efficiency in several systems (Henter and Via, 1995; Hufbauer and Via, 1999; Kraaijeveld and Godfray, 1999; Hufbauer et al., 2004). Molecular evidence supports the hypothesis that alfalfa weevil populations in the U.S. belong to a single species with three strains (western, Egyptian, and eastern) that have overlapping distributions (Hsiao, 1993). Although little is known about the genetic variation of alfalfa weevil populations in Virginia, *B. anurus* has been shown to develop equally well in all three strains under laboratory conditions (Maund and Hsiao, 1991), suggesting that host variation should have little impact on parasitoid efficiency in the Virginia alfalfa system.

Dellinger (2003) attempted to study genetic variability between *B. anurus* collected from alfalfa fields in the Piedmont and southwestern region using random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). Preliminary results suggested that there may be some genetic differences between populations of the parasitoid in the two regions. This indicated a need for additional studies on the genetics of *B. anurus* from the three alfalfa-growing regions of Virginia, the results of which are reported elsewhere.

Parasitization levels can be affected by the landscape structure, climatic factors such as temperature, and by host and parasitoid densities and parasitoid behavior. The spatial structure and pattern of species resources can influence host-parasitoid dynamics (Landis and Marino, 1998) by affecting dispersal (Fahrig and Merriam, 1994; Jonsen and Fahrig, 1997), parasitoid foraging behavior (Reeve et al., 1994), and the temporal dynamics of parasitism (Menalled et al., 2003). Temperature has been shown to affect the way a host responds to parasitoid attacks, as in the case of *Drosophila* spp., in which the process of encapsulation (a cellular immune response to parasitoid invasion) is often more effective at higher temperatures (Kraaijeveld and Godfray, 1999). Kuhar et al. (1999) found that winter temperatures in the Virginia Piedmont are about 2°C warmer, and as a result, populations of alfalfa weevil larvae are higher and peak 2–4 weeks earlier than in the Shenandoah Valley and southwestern region. However, Kuhar et al. (2001) showed that the period of *B. anurus* activity was synchronized with the period of peak alfalfa weevil larval populations in each of the regions, suggesting that although climate influences the dynamics of populations and is a strong determinant of the distribution of alfalfa weevil strains (Radcliffe and Flanders, 1998), climate does not explain differences in parasitization levels among the regions of Virginia. This is probably because climate usually interacts with other factors to influence ecological processes and to shape the dynamics of populations (Leirs et al., 1997).

Both host and parasitoid density can affect parasitization levels (Hassell, 2000). This may have prompted Kuhar et al. (1999) to suggest that differences in the densities of the alfalfa weevil populations among the regions of Virginia are responsible for the varying levels of parasitization. However, this assertion was made without knowledge of the effects of alfalfa weevil density on *B. anurus* attacks (the functional response) or of the distribution of these

attacks in response to host densities. The objective of this project is to address the knowledge gap with respect to *B. anurus*-alfalfa weevil interactions by comparing parasitoid attack and host and parasitoid distributions directly in alfalfa fields in the Piedmont and southwestern region of Virginia.

2.2 Materials and Methods

2.2.1 Study Regions

GPS-referenced quadrat sampling was conducted in two of the three alfalfa-growing regions of Virginia: the Piedmont and southwestern region (SWR) (Figure 2.1). Six alfalfa fields were selected to assess the within-field distributions of alfalfa weevil and parasitoids, to determine percent parasitism per field and region, to identify primary parasitoids, and to determine population functional response of *B. anurus.*

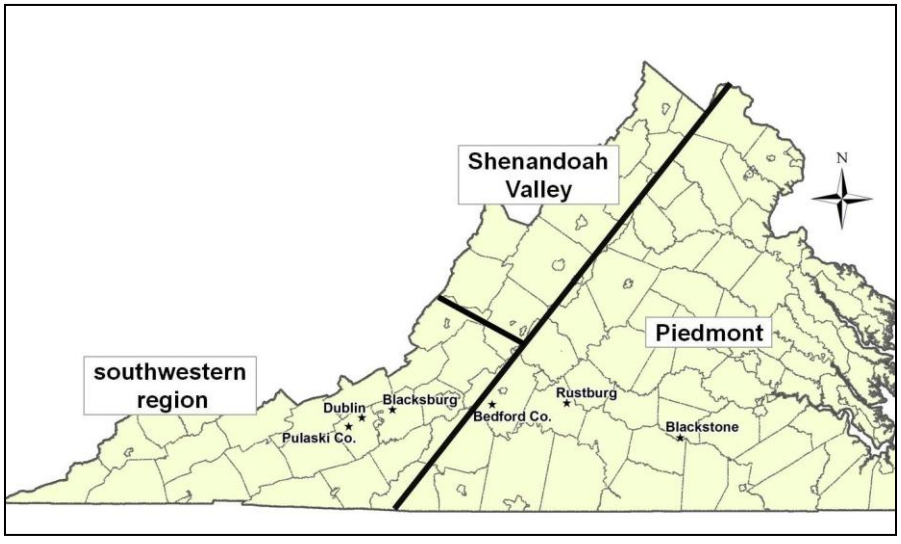


Figure 2.1. Alfalfa growing regions of Virginia and locations of fields sampled.

The fields selected for sampling were 2- to 7.7-ha perennial mixed stands of alfalfa and orchardgrass or alfalfa and tall fescue that had been in production a minimum of three years to ensure stability of field ecology. Fields were geographically distant from each other and were located in separate towns and/or counties. Table 2.1 lists the location, region, and geographic coordinates of approximate center of each field that was sampled.

Table 2.1. Geographic coordinates of fields sampled 2012–2013.

Field Location	Latitude, Longitude of Field Center
Blackstone	37.026700° N, 078.051214° W
Rustburg	37.257521° N, 078.991032° W
Bedford County	37.247217° N, 079.610973° W
Pulaski County	37.102959° N, 080.798801° W
Dublin	37.159693° N, 080.689860° W
Blacksburg	37.212673° N, 080.435946° W

All fields were surveyed in Spring 2013. The fields in Blackstone and Rustburg were also surveyed in Spring 2012 to collect parasitization data, but mainly to obtain specimens for genetic analyses.

2.2.2 Field Descriptions

Blackstone, Nottoway County (Piedmont Region). The field surveyed in Blackstone was a plot of 7.7 ha fronting Virginia Highway 40 just outside the town limits of Blackstone. The stand was a mix of Roundup Ready^{TM} alfalfa and tall fescue that had been in continuous production since 2006. The field was treated each spring with glyphosate herbicide to control weeds, primarily chickweed, and lambda-cyhalothrin insecticide to control alfalfa weevil,

applied per label instructions. In 2012, treatments were applied on March 19 along with supplemental fertilizer and boron. Treatment date for 2013 could not be confirmed with the producer. The field was surveyed in Spring 2012 and Spring 2013. In 2012, sampling for alfalfa weevil was conducted on March 15, April 12, and April 30. Sampling in 2013 was conducted on April 3, April 24, and May 1.

Rustburg, Campbell County (Piedmont Region). The field surveyed in Rustburg was a 2.8-ha plot fronting New Chapel Road just outside the town limits of Rustburg. The stand was a mix of Pioneer^{TM} alfalfa and orchardgrass that had been seeded in 2006 or 2007. The field was treated each spring with lambda-cyhalothrin insecticide to control alfalfa weevil, applied at 0.12 liters per hectare. In 2012, insecticide was applied March 23-24. Treatment date in 2013 was the week of April 15. The field was surveyed in Spring 2012 and Spring 2013. In 2012, alfalfa weevil sampling was conducted on March 15, April 12, and May 2. Sampling dates in 2013 were April 8, April 24, and May 1.

Bedford County (Piedmont Region). The field surveyed in Bedford County was a 2.4-hectare plot within a large agricultural tract just south of Joppa Mill Road (State Route 747) near the town of Moneta. The stand was a mix of alfalfa and tall fescue seeded in 2008. The field was not treated with insecticide or herbicide. The producer indicated that the alfalfa is usually cut just prior to bloom to reduce alfalfa weevil pest pressure. Sampling for alfalfa weevil was conducted in 2013 on April 8, April 24, and May 1.

Pulaski County (Southwestern Region). The field surveyed in Pulaski County was a 6.9-ha plot within a dairy farm off Buena Vista Road, north of Robinson Tract. The stand was a mix of Genoa variety alfalfa and tall fescue, seeded in 2008. In general, the field received no insecticide treatments, though the field was sprayed in 2012 for alfalfa weevil due to unusually

high pest pressure. No insecticide was applied to the field in 2013. The field was sampled in 2013 on April 10, April 26, and May 4.

Dublin, Pulaski County (Southwestern Region). The field surveyed in Dublin was a plot of approximately 4.7 ha fronting Highland Road just off Route 100, north of the New River Valley airport. The stand was a mix of alfalfa and tall fescue seeded in 2008. In general, the field received no insecticide treatments, though the field was sprayed in 2012 for alfalfa weevil due to unusually high pest pressure. No insecticide was applied to the field in 2013. Sampling was conducted in 2013 on April 10, April 26, and May 4.

Blacksburg, Montgomery County (Southwestern Region). The field surveyed in Blacksburg was a 3.2-ha plot near Plantation Road on the Virginia Tech campus, across from the Virginia Tech piggery. The stand was a mix of tall fescue and Southern States variety Evermore alfalfa that had been seeded in the fall of 2008. No herbicide or insecticide treatments were applied to the field. The Blacksburg field was sampled in 2013 on April 10, April 26, and May 4.

2.2.3 Sampling Protocol

Quadrat sampling was conducted in each field on three dates to collect time-series data of alfalfa weevil and parasitoid interactions. The first sampling was conducted early in the season, when alfalfa had attained a height of approximately 2.5 cm and sweep net sampling was positive for alfalfa weevil larvae. The second field visit was made approximately 3 weeks later at the approximate peak of alfalfa weevil activity. The third sampling was conducted just prior to the first alfalfa mowing date, generally the first week of May.

Fifteen samples were collected from each field on each sampling date using a stratified random sampling method. The field was visually divided into three strata, and five points were randomly selected within each stratum. Stratum sampling order was shuffled on each field visit for additional randomization. The GPS coordinates of the locations selected in each stratum were recorded using a Garmin hand-held GPS unit. A 0.2-m^2 quadrat was placed on the coordinate and all foliage within the quadrat was collected and bagged in a sealable 3.78-liter food storage bag; alfalfa weevil and parasitoid specimens on the ground within the quadrate were also collected. Each bag was labeled with the sample ID and sampling date and placed on ice for transport to the Virginia Tech laboratory for specimen rearing.

2.2.4 Specimen Rearing

In the laboratory, each foliage sample was transferred to a 13-cm x 7.9-cm x 27-cm paper sack. The sack was sealed, labeled with the sample ID and sampling date, and stored at ambient room temperature until alfalfa weevil larvae in each sample had completed development to the adult stage, approximately two to three weeks. Bags were occasionally unsealed and contents lightly misted with distilled water to prevent specimen desiccation.

After rearing, the number of alfalfa weevil adults and parasitoid cocoons in each sample were counted and logged. Parasitoid cocoons were identified to species and stored on moist cotton in 59-ml condiment containers with ventilated lids. Containers were labeled with the sample ID, sampling date, and number and species of parasitoid. Alfalfa weevil adults were destroyed.

2.2.5 Data and Statistical Analyses

Data Mapping. GPS coordinates for field perimeters and sampling points were mapped using ArcMap version 10.1 (Esri, 2012). Geographic coordinate system used was the World Geographic System (WGS) 1984. Satellite imagery of each field for map background was obtained from Esri World Imagery.

Between-Region Comparisons of Alfalfa Weevil Density and Parasitization. Data for each field on the total number of alfalfa weevil larvae collected and total number of larvae parasitized were compared by region to determine whether there were any significance differences between regions. Analyses were performed in JMP Pro 10.0 using the nonparametric Wilcoxon/Kruskal-Wallis test (Zar, 2010) with a significance level of $\alpha = 0.05$. Data points analyzed ($n = 18$) in each test represented the pooled totals collected at the fifteen sampling points for each field ($n = 6$) on each date ($n = 3$).

Spatial Dispersion of Alfalfa Weevil and Parasitization. The spatial distribution of host population and natural enemy attack can be inferred by the relationship between variance (σ^2) and mean (μ) of population samples, such that variance equal to mean ($\sigma^2 = \mu$) can be used to infer a random dispersion; variance less than mean ($\sigma^2 < \mu$) represents a uniform dispersion; and variance greater than mean ($\sigma^2 > \mu$) represents an aggregated dispersion (Elliott, 1977; Krebs, 1998).

The ratios of variance to mean were calculated for each field of the total number of alfalfa weevil larvae collected and number of larvae parasitized for all 45 sampling points to infer spatial dispersion of alfalfa weevil larvae and parasitization. Results were tested by chi-square (χ^2) analysis for agreement with a Poisson series at a 95% confidence level ($P < 0.05$) using the formula (Elliott, 1977; Krebs, 1998),

$$\chi^2 = \frac{s^2(n-1)}{\bar{x}} \qquad (1)$$

where s^2, n - 1, and $\bar{x}$ are the variance, degrees of freedom, and mean of the samples, respectively.

***Functional Response*.** Regression analyses of the sampling data were carried out in JMP Pro 10.0 (SAS, 2012) to determine the population functional response (Hassell, 2000; Juliano, 2001) of *B. anurus* attack on alfalfa weevil in each field. Two analyses were run on each data set to determine: 1) the relationship of number of alfalfa weevil larvae attacked to alfalfa weevil density per sampling point, and 2) the relationship of proportion of alfalfa weevil larvae parasitized to alfalfa weevil density per sampling point (Juliano, 2001). Points at which zero larvae were collected were excluded from the analyses. Log-scale trend lines were fitted to the data to visualize the functional response curves. For the Blackstone field, data for the 2012 sampling were only graphed as virtually no parasitoid activity was observed in Blackstone in 2013. For the Rustburg field, the 2012 and 2013 data were graphed to compare functional response over time in the same field.

2.3 Results

2.3.1 Field Sampling

***Blackstone, Nottoway County (Piedmont Region)*.** In Spring 2012, the total number of alfalfa weevil larvae collected in 45 samples (quadrats) was 761, for an average of 16.9 larvae/sample. Most alfalfa weevil larvae were collected in the sampling of March 15 (n = 727), prior to insecticide application. Samplings conducted after field treatment, on April 12 and April 30, yielded larvae totals of 28 and 6 alfalfa weevil larvae, respectively. Thirty-three larvae were

parasitized by *B. anurus*, for a parasitization level of 4.3%. Of these, 29 (88%) were collected in the sampling of March 15, prior to insecticide application.

In 2013, field sampling began on April 3, more than two weeks later than the previous year due to cooler weather and later onset of alfalfa weevil activity. The total number of larvae for 45 samples was 191, for an average of 4.2 larvae per sample. Most larvae were collected in the second sampling, conducted April 24 (n = 101). The sampling conducted April 3 yielded 69 larvae; 21 larvae were collected in the sampling of May 1. One parasitized larva was collected in total, a parasitization level of 0.5%. A field map of the 2012 sampling points is shown in Figure 2.2 (see also Map 1 in Appendix A). The map is labeled with the ratio of parasitized larvae/number of alfalfa weevil larvae collected at each sampling point. The 2013 sampling points are shown in Map 2 of Appendix A.

Rustburg, Campbell County (Piedmont Region). In the 2012 survey, the total number of alfalfa weevil larvae collected in 45 samples (quadrats) was 305, for an average of 6.8 larvae per sample. Most larvae were collected in the sampling of March 15 (n = 187), prior to insecticide application. The sampling conducted on April 12, approximately three weeks after insecticide application, yielded 118 larvae; no larvae were collected in the sampling of May 2. A total of 15 larvae were parasitized, 15 by *B. anurus* and two by *B. curculionis*, for a parasitization level of 5.6%. Twelve parasitized larvae were collected in the sampling of March 15; five were collected in the sampling of April 12.

In 2013, 726 total alfalfa weevil larvae were collected for an average of 16.1 larvae per sample. Of the total, more than 93% were collected in the first sampling on April 8 (n = 678); the second sampling, conducted on April 24 approximately 7 to 9 days after insecticide application, yielded 19 larvae, and the sampling on May 1 yielded 29 larvae.

Figure 2.2. Map of GPS-referenced points sampled with a 0.2-m^2 quadrat in the Blackstone field (Piedmont region) in Spring 2012. Blue, red, and yellow labels indicate quadrats sampled on first, second, and third sampling dates, respectively; labels represent the ratio of larvae parasitized/number of larvae collected at each quadrat. (Imagery source: Esri et al., 2014.)

Parasitized larvae totaled 219, for a parasitization level of 30.2%. *B. anurus* accounted for 211 parasitized larvae; *B. curculionis* parasitized eight larvae. Most parasitized larvae were collected in the sampling of April 8 (n = 202), prior to insecticide treatment of the field, including six of the eight larvae parasitized by *B. curculionis*. Three parasitized larvae were collected on April 24 in samples after insecticide application and 14 were collected on May 1, of which two were parasitized by *B. curculionis*.

Field maps of the Rustburg sampling points for 2012 and 2013 are presented as Map 3 and Map 4, respectively, in Appendix A. The maps are labeled with the ratio of parasitized larvae/number of alfalfa weevil larvae collected at each sampling point. The map for 2013 is also labeled with the number of larvae parasitized by *B. curculionis* obtained at locations within the field.

Bedford County (Piedmont Region). The total number of alfalfa weevil larvae collected in 45 sampling points (quadrats) was 963, for an average of 21.4 larvae per sample. The sampling of April 8 yielded 544 larvae; samplings of April 24 and May 1 yielded 253 larvae and 166 larvae, respectively. In total, 437 alfalfa weevil larvae were parasitized for a parasitization level of 45.4%. Percent parasitism increased with sampling date, though the number of parasitized larvae simultaneously decreased. In the sampling of April 8, 170 of 544 larvae were parasitized (31.2%); in the sampling of April 24, 138 of 253 larvae were parasitized (54.5%); and in the sampling of May 1, 129 of 166 larvae were parasitized (77.7%). Parasitized larvae were recovered from 43 of the 45 points sampled.

B. anurus accounted for 94.5% of parasitization. *B. curculionis* parasitized 24 specimens, of which 21 were collected in the sampling of April 8; the other three were recovered from the sampling of April 24. A field map of the 2013 sampling points, labeled with the ratio

of parasitized larvae/number of alfalfa weevil larvae collected at each sampling point, is shown as Map 5 in Appendix A. Also labeled are number of larvae parasitized by *B. curculionis* obtained at locations within the field.

Pulaski County (Southwestern Region). The total number of alfalfa weevil larvae obtained from 45 sampling points (quadrats) was 202, for an average of 4.5 larvae per sample. Most larvae were collected in the sampling conducted April 26 (n = 121); 28 larvae were collected in the sampling of April 10, and 53 collected in the sampling of May 4. Parasitized larvae totaled 76, all by *B. anurus*, for a parasitization level of 37.6%. Seventy-five percent of parasitized larvae were collected in the sampling of April 26; the other 25% were collected in the sampling of May 4. No parasitized larvae were collected in the sampling of April 10. Map 6, Appendix A, shows the sampling points labeled with the ratio of parasitized larvae/number of alfalfa weevil larvae collected at each point.

Dublin, Pulaski County (Southwestern Region). The total number of alfalfa weevil larvae collected for 45 sampling points (quadrats) was 117, for an average of 2.6 larvae per sample. Sixty-eight percent of larvae were collected in the sampling of May 4 (n = 80); 15 larvae were collected in the sampling of April 10, and 22 larvae were collected in the sampling of April 26. Parasitized larvae totaled 49, all by *B. anurus*, for a parasitization level of 41.9%. Thirty-seven of the parasitized larvae (75.5%) were collected in the sampling of May 4; the rest were collected in the sampling of April 26. No parasitized larvae were collected in the sampling of April 10. The Dublin field sampling points, labeled with the ratio of parasitized larvae/number of alfalfa weevil larvae collected at each point, are shown in Map 7, Appendix A.

Blacksburg, Montgomery County (Southwestern Region). The total number of alfalfa weevil larvae collected from 45 sampling points (quadrats) was 356, for an average of 7.9 larvae

per sample. The majority of larvae, 217 (61%), were collected in the sampling of April 26; the samplings of April 10 and May 4 yielded 17 larvae and 122 larvae, respectively. Total larvae parasitized were 210, for a parasitization level of 59%. *B. anurus* accounted for all parasitization. Fifty-nine percent of parasitized larvae (n = 124) were collected in the sampling of April 26; 41% (n = 86) were collected in the sampling of May 4. No parasitized larvae were collected in the sampling of April 10. Map 8, Appendix A, shows the Blacksburg field sampling points, labeled with the ratio of parasitized larvae/number of alfalfa weevil larvae collected at each point.

2.3.2 Between-Region Comparisons of Alfalfa Weevil and Parasitization

Table 2.2 presents summary data on alfalfa weevil larval levels and percent parasitization by region, field, and date for the Spring 2013 field surveys. As seen in the table, the total number of alfalfa weevil collected in the Piedmont region was approximately 2.8 times the number of larvae collected in the southwestern region; however, a one-way analysis using the Wilcoxon/Kruskal-Wallis test of the number of alfalfa weevil collected per field and sampling date found no statistical difference between regions (χ^2 =1.4211, df = 1, P > 0.05).

Similarly, when the parasitization data obtained in the 2013 field surveys were pooled by region, average percent parasitization observed in the Piedmont region was 34.9% compared with 49.6% in the southwestern region. However, a Wilcoxon/Kruskal-Wallis test of parasitization level per field by sampling date found no statistical difference in percent parasitization between the two regions (χ^2 =0.1275, df = 1, P > 0.05).

Table 2.2. Field and regional comparisons of alfalfa weevil sampling results for Spring 2013.

Region [a]	Field	Sampling Date	No. Alfalfa Weevil	No. Parasitized	Percent Parasitization
Piedmont	Blackstone	4/3/2013	69	1	1.4
		4/24/2013	101	0	0
		5/2/2013	21	0	0
	Rustburg	4/8/2013	678	202	29.8
		4/24/2013	19	3	15.8
		5/1/2013	29	14	48.3
	Bedford County	4/8/2013	544	170	31.3
		4/24/2013	253	138	54.5
		5/1/2013	166	129	77.7
	Total		**1880**	**657**	**34.9**
	Mean ± SE		**208.9 ± 80.9**	**73.0 ± 84.9**	**28.8 ± 9.1**
SWR	Pulaski County	4/10/2013	28	0	0
		4/26/2013	121	57	47.1
		5/4/2013	53	19	35.8
	Dublin	4/10/2013	15	0	0
		4/26/2013	22	12	54.5
		5/4/2013	80	37	46.3
	Blacksburg	4/10/2013	17	0	0
		4/26/2013	217	124	57.1
		5/4/2013	122	86	70.5
	Total		**675**	**335**	**49.6**
	Mean ± SE		**75.0 ± 22.6**	**37.2 ± 43.9**	**34.6 ± 9.2**

[a] SWR = southwestern region

2.3.3 Spatial Dispersion and Population Function Response

For each field, a ratio of variance greater than mean ($\sigma^2 > \mu$) was obtained for the number of alfalfa weevil collected per quadrat and for the number of larvae parasitized per quadrat,

indicating aggregated spatial dispersions of alfalfa weevil larvae and parasitization within the alfalfa fields. The χ^2 value in all cases where the variance:mean ratio indicted an aggregated dispersion showed that the dispersion was significantly different from the null hypothesis of a random dispersion (Table 2.3).

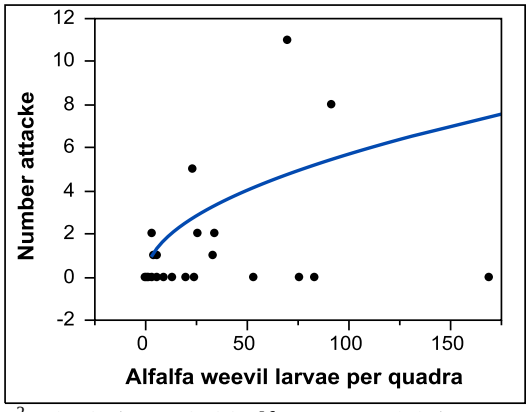
Table 2.3. Results of analysis of spatial dispersion of number of alfalfa weevil and number parasitized per quadrat in alfalfa fields in the Piedmont and southwestern region, Virginia.

	No. AW Larvae	**No. Parasitized**	**No. AW Larvae**	**No. Parasitized**
	Blackstone 2012		**Blackstone 2013**	
Mean	16.91	0.73	4.24	0.02
Variance	1121.26	4.56	21.96	0.02
χ^2 (df=44)	2917.40	273.80	227.67	44.00
Dispersion	Aggregated	Aggregated	Aggregated	Random
	Rustburg 2012		**Rustburg 2013**	
Mean	6.78	0.38	16.13	4.87
Variance	100.95	0.60	642.98	71.75
χ^2 (df=44)	655.30	70.30	1753.60	648.70
Dispersion	Aggregated	Aggregated	Aggregated	Aggregated
	Bedford 2013		**Pulaski 2013**	
Mean	21.40	9.71	4.49	1.69
Variance	312.47	59.39	25.30	6.76
χ^2 (df=44)	642.50	269.10	248.00	176.20
Dispersion	Aggregated	Aggregated	Aggregated	Aggregated
	Dublin 2013		**Blacksburg VT 2013**	
Mean	2.60	1.09	7.91	4.67
Variance	10.75	2.36	113.36	47.73
χ^2 (df=44)	181.80	95.20	630.50	450.00
Dispersion	Aggregated	Aggregated	Aggregated	Aggregated

Graphs of the relationship of the number of alfalfa weevil larvae attacked per quadrat and proportion of weevil attacked showed that overall, there was an inverse density-dependent Type II functional response for *B. anurus* attack on alfalfa weevil larvae in alfalfa fields in the Piedmont and SWR (Figures 2.3 through 2.9).

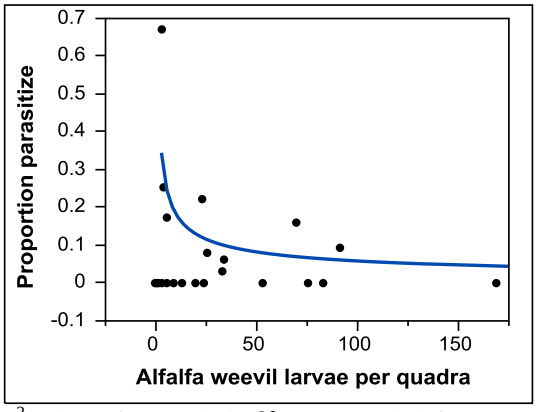
Blackstone (Piedmont Region), Spring 2012

a)



R^2 = 0.46; (F = 5.8786; df = 1,7; P < 0.05)
ln(No. Attacked) = -0.569 + 0.502*ln(AW Density)

b)

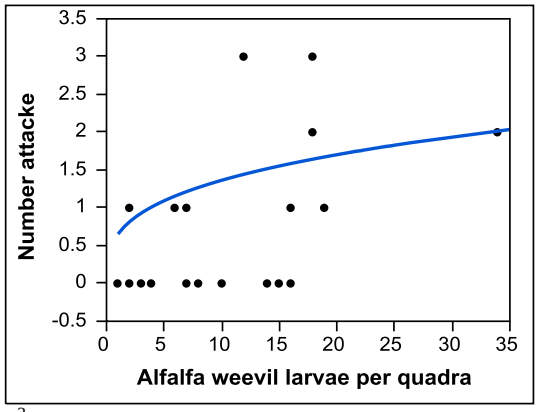


R^2 = 0.44; (F = 5.5973; df = 1,7; P < 0.05)
ln(Prop. Parasitized) = -0.569 - 0.493* ln(AW Density)

Figure 2.3. Relationships of **a)** number of larvae attacked by alfalfa weevil density and **b)** proportion of larvae parasitized by alfalfa weevil density in Spring 2012 at Blackstone, Virginia. The shapes of the trend lines indicate an inverse-density dependent Type II functional response for *B. anurus* attack on the alfalfa weevil.

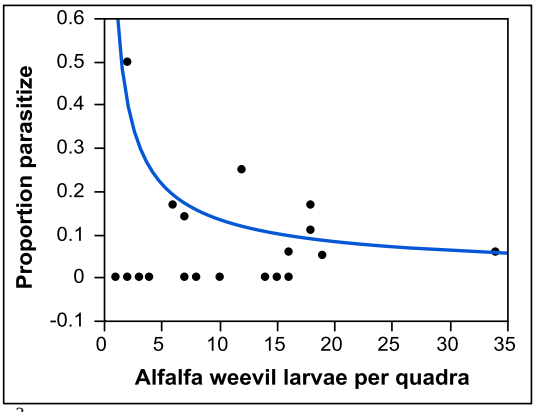
Rustburg (Piedmont Region), Spring 2012

a)



R^2 = 0.28; (F = 3.5087; df = 1,9; P > 0.05)
ln(No. Attacked) = -0.425 + 0.320* ln(AW Density)

b)

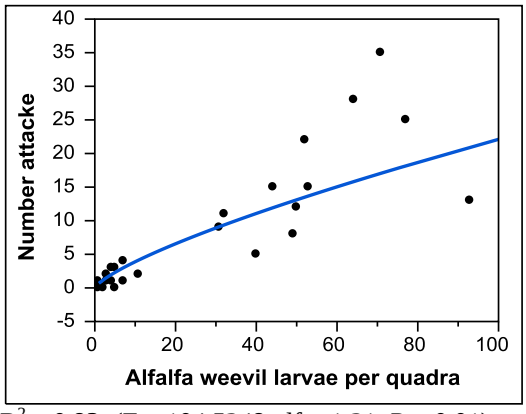


R^2 = 0.62; (F = 14.7174; df = 1,9; P < 0.01)
ln(Prop. Parasitized) = -0.432 - 0.681* ln(AW Density)

Figure 2.4. Relationships of **a)** number of larvae attacked by alfalfa weevil density and **b)** proportion of larvae parasitized by alfalfa weevil density in Spring 2012 at Rustburg, Virginia. The shapes of the trend lines indicate an inverse-density dependent Type II functional response for *B. anurus* attack on the alfalfa weevil.

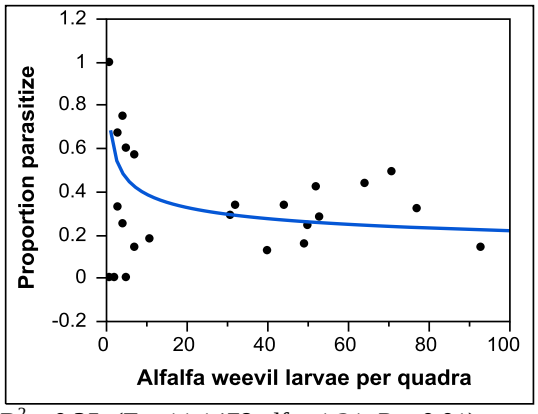
Rustburg (Piedmont Region), Spring 2013

a)



R^2 = 0.83; (F = 104.5248; df = 1,21; P < 0.01)
ln(No. Attacked) = -0.377 + 0.754* ln(AW Density)

b)

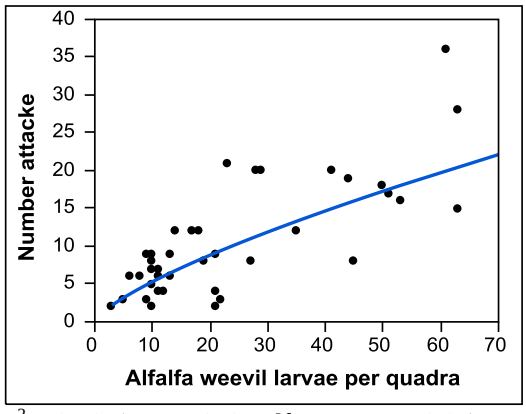


R^2 = 0.35; (F = 11.1478; df = 1,21; P < 0.01)
ln(Prop. Parasitized) = -0.378 - 0.246* ln(AW Density)

Figure 2.5. Relationships of **a)** number of larvae attacked by alfalfa weevil density and **b)** proportion of larvae parasitized by alfalfa weevil density in Spring 2013 at Rustburg, Virginia. The shapes of the trend lines indicate an inverse-density dependent Type II functional response for *B. anurus* attack on the alfalfa weevil.

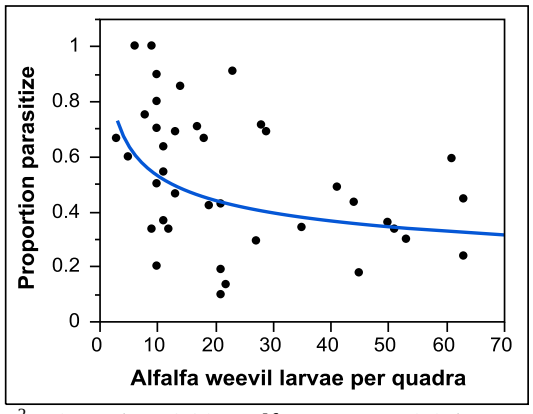
Bedford County (Piedmont Region), Spring 2013

a)



R^2 = 0.56; (F = 52.3505; df = 1,41; P < 0.01)
ln(No. Attacked) = -0.019 + 0.734* ln(AW Density)

b)

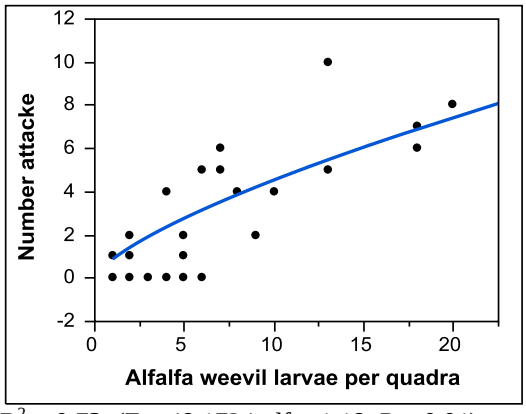


R^2 = 0.14; (F = 6.9075; df = 1,41; P < 0.05)
ln(Prop. Parasitized) = -0.019 - 0.266* ln(AW Density)

Figure 2.6. Relationships of **a)** number of larvae attacked by alfalfa weevil density and **b)** proportion of larvae parasitized by alfalfa weevil density in Spring 2013 at Bedford County, Virginia. The shapes of the trend lines indicate an inverse-density dependent Type II functional response for *B. anurus* attack on the alfalfa weevil.

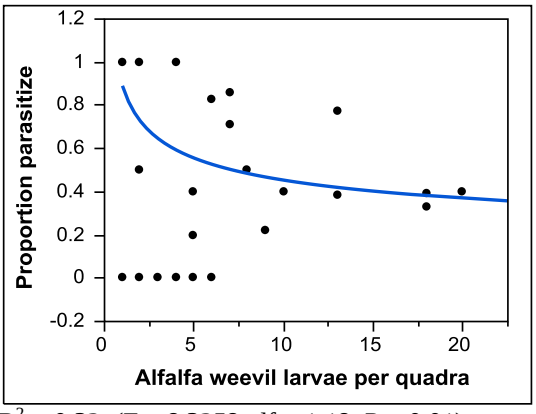
Pulaski County (Southwestern Region), Spring 2013

a)



R^2 = 0.73; (F = 49.1724; df = 1,18; P < 0.01)
ln(No. Attacked) = -0.116 + 0.709* ln(AW Density)

b)

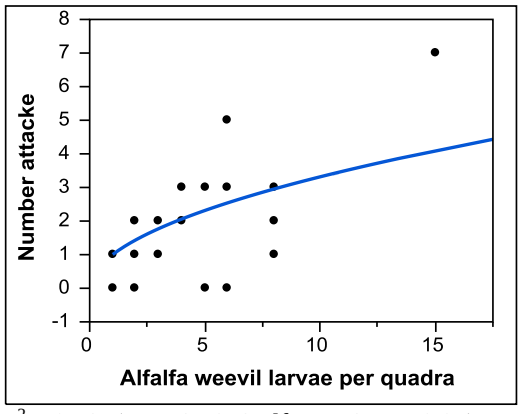


R^2 = 0.32; (F = 8.3258; df = 1,18; P < 0.01)
ln(Prop. Parasitized) = -0.115 - 0.292* ln(AW Density)

Figure 2.7. Relationships of **a)** number of larvae attacked by alfalfa weevil density and **b)** proportion of larvae parasitized by alfalfa weevil density in Spring 2013 at Pulaski County, Virginia. The shapes of the trend lines indicate an inverse-density dependent Type II functional response for *B. anurus* attack on the alfalfa weevil.

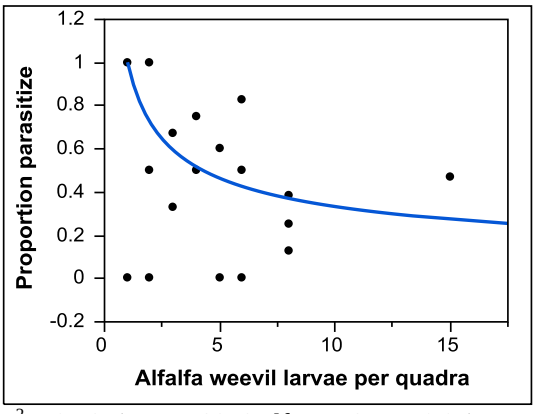
Dublin (Southwestern Region), Spring 2013

a)



R^2 = 0.49; (F = 18.4819; df = 1,19; P < 0.01)
ln (No. Attacked) = 0.005 + 0.519* ln(AW Density)

b)

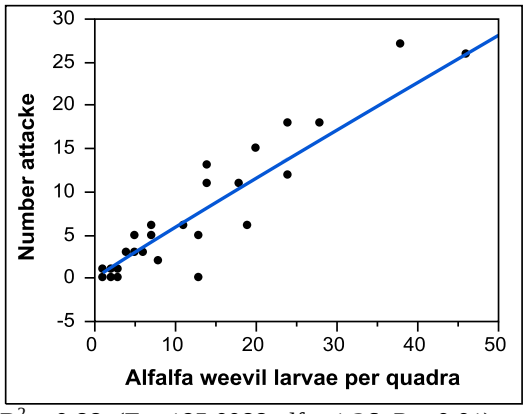


R^2 = 0.46; (F = 15.9816; df = 1,19; P < 0.01)
ln (Prop. Parasitized) = 0.002 - 0.477* ln(AW Density)

Figure 2.8. Relationships of **a)** number of larvae attacked by alfalfa weevil density and **b)** proportion of larvae parasitized by alfalfa weevil density in Spring 2013 at Pulaski County, Virginia. The shapes of the trend lines indicate an inverse-density dependent Type II functional response for *B. anurus* attack on the alfalfa weevil.

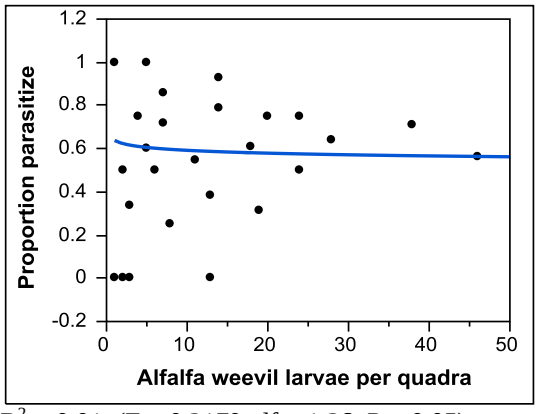
Blacksburg (Southwestern Region), Spring 2013

a)



R^2 = 0.88; (F = 195.0088; df = 1,26; P < 0.01)
ln(No. Attacked) = -0.449 + 0.968* ln(AW Density)

b)



R^2 = 0.01; (F = 0.2170; df = 1,26; P > 0.05)
ln(Prop. Parasitized) = -0.449 - 0.032* ln(AW Density)

Figure 2.9. Relationships of **a)** number of larvae attacked by alfalfa weevil density and **b)** proportion of larvae parasitized by alfalfa weevil density in Spring 2013 in Blacksburg, Virginia. The shape of the trend lines in **a)** indicate a linear Type I functional response for *B. anurus* attack on the alfalfa weevil.

Combined with the analysis of spatial dispersion in each field, results seem to indicate that there is no significant difference between populations in the Piedmont and southwestern regions in the behavioral adaptation and response of *B. anurus* to local populations of alfalfa weevil.

2.4. Discussion

2.4.1 Field Sampling Data

In 2013, the first samplings were conducted in the Blackstone and Rustburg fields on April 3 and April 8, respectively, approximately three weeks later than first sampling in 2012, which was conducted on March 15. This was due to cold weather at the start of 2013 that slowed alfalfa growth and delayed the onset of alfalfa weevil activity. Climatological data obtained from the National Oceanic and Atmospheric Administration (NOAA) National Climate Data Center (2013) showed that the average temperature at Blackstone, generally the warmest of the

areas surveyed, was 3.8°C for January through March 2013 with a mean maximum temperature of only 10.1°C, versus average and mean maximum temperatures of 7.8°C and 14.9°C, respectively, for the same period in 2012. The threshold temperature for alfalfa weevil embryogenesis is 9°C (Roberts et al., 1970), which was rarely attained in the survey areas in Virginia during the first three months of 2013. Cold weather is also the most likely reason for substantially lower number of alfalfa weevil larvae collected in Blackstone in 2013 (n = 191) than in the same field in 2012 (n = 761), and the absence of parasitoid activity at the beginning of the 2013 season. Based on sampling data obtained from the 2012 survey of the Blackstone field, which saw alfalfa weevil and parasitization levels decrease sharply after insecticide application, the absence of parasitized larvae in the samplings of April 24 and April 30 was not considered unusual.

The total number of alfalfa weevil larvae collected at Rustburg in 2013 (n = 726) was greater than the total number collected in 2012 (305), probably due in part to the delay in sampling on account of weather. As in Blackstone, average temperature observed for January through March 2013 was much cooler than during the same period in 2012, and sampling was delayed an additional five days to April 8 because of a snowstorm on April 4.

It is interesting to note that in 2012, the number of alfalfa weevil larvae collected on April 12 (n = 118), approximately three weeks after insecticide application, was similar to the number of larvae (n = 187) collected in the sampling of March 15 prior to treatment of the field. This was not observed in 2013, which saw the number of alfalfa weevil larvae decrease sharply in the two samplings conducted after insecticide application. Parasitization level was significantly higher in 2013 (30.2%) compared with 2012 (5.6%). Also of interest is that, though

the number of parasitized larvae collected after insecticide treatment was lower than prior to treatment, the percent parasitization increased after spraying (Table 2.2).

At Bedford County, the number of alfalfa weevil larvae collected in this field (n = 963) was greater than collected in any of the other fields. Of interest is that, though alfalfa weevil population was high, insecticide treatments were not applied to the field; the producer stated that alfalfa weevil pest pressure was reduced by cutting the alfalfa before bloom. Also of interest is that, though the number of larvae parasitized decreased during the season, the proportion parasitized increased (Table 2.2).

The Bedford County field was one of two fields surveyed from which larvae parasitized by *B. curculionis* were collected; the other field was at Rustburg. Parasitization by *B. curculionis* in the Bedford field was 5.5%, similar to the level observed by Kuhar et al. (2000).

Low numbers of alfalfa weevil larvae and the absence of parasitized larvae collected in the first sampling of the three fields in southwestern regions are most likely attributable to the cool weather experienced for the first part of 2013. NOAA (2013) monthly climatological data, showed that the average temperature recorded at Blacksburg for the period January through March 2013 was 0.9°C, with a mean maximum temperature of only 6.3°C. Similar temperatures were experienced in Pulaski and Dublin.

2.4.2 Regional Comparison of Alfalfa Weevil Parasitization

As shown in Table 2.2, the parasitization levels of 34.9% for the Piedmont and 49.6%, southwestern region were similar to the parasitization levels observed by Kuhar et al. (1999) in alfalfa fields in the Piedmont (16–32%) and SWR (47–71%). However, whereas the parasitization levels observed by Kuhar et al. (1999) were found to be significantly different,

analysis of the levels obtained from the 2013 data in this study found no significant difference in parasitization between the regions.

The lack of statistical significance at the field level is supported by similarities in data for particular dates. For example, parasitization levels of larvae collected in Bedford County (Piedmont) on April 24 and May 1 were 54.5% and 77.5%, respectively, similar to parasitization levels of larvae collected in Blacksburg (SWR) on April 26 (51.7%) and May 4 (70.5%), though host density in the Bedford County field was almost three times greater than in the Blacksburg field. This would seem to indicate that higher host density in and of itself is not necessarily a factor in differential parasitization of alfalfa weevil by *B. anurus*. Parasitization levels of larvae collected on May 1 in Rustburg (Piedmont) and on May 4 in Dublin (southwestern region) were also similar, at 48.3% and 46.3% respectively, though the Rustburg field had been treated with insecticide two weeks earlier and the Dublin field had not been treated. This supports the observations by Los (1982) and Dellinger (2003) that insecticide treatment may not be an important determinant in variable parasitization by *B. anurus* in alfalfa fields in Virginia.

2.4.3 Spatial Dispersion and Population Functional Response

Key to determining the population functional response is an understanding of spatial heterogeneity in the host-parasitoid system (Hassell, 2000). Spatial distribution of hosts in the field may be random, in which no discernible spatial pattern occurs; uniform, in which host appears to be evenly distributed; or aggregated, in which host are clustered or grouped in patches. Parasitoid response to host spatial pattern can be one of three basic types: parasitoids may aggregate positively in response to the host (Hassell and May, 1973), aggregate independently of host density (Reeve et al., 1994), or exhibit inverse aggregation whereby dense

patches of hosts contain fewer parasitoids (Hassell, 1984). Studies of the patch dynamics of host-parasitoid interactions have found that direct and inverse aggregation of parasitoids in response to hosts are common (Pacala and Hassell, 1991). The aggregated dispersion patterns of alfalfa weevil larvae and parasitization by *B. anurus* suggest that the parasitoid tends to aggregate positively in response to alfalfa weevil density in alfalfa fields in Virginia. No difference was observed between fields of the Piedmont and southwestern region.

The population functional response for a parasitoid, which is assessed from field data, measures mean parasitism across host population as a function of mean host population per host patch, and can be used as a surrogate measure of adaptation of the parasitoid to the host and local environment (Ives et al., 1999). The inversely density-dependent functional response, commonly referred to as Type II (Juliano, 2001), has been observed most frequently with parasitoids used as biological control agents (Fernandez-Arhex and Corley, 2003). The population functional response of *B. anurus* attack on the alfalfa weevil was found to be Type II for all fields with the exception of the Blacksburg field. The aggregation of *B. anurus* attack in patches of high weevil larval densities may be one of the causes for the inverse density dependent Type II functional response of the parasitoid. In situations where parasitoids are seeking larval hosts in the same patch, parasitoid searching efficiency may be reduced as an increasing proportion of encounters between individual parasitoids and hosts will be wasted on hosts that are already parasitized. This pseudointerference with respect to the searching parasitoids (Hassell, 2000) causes the risk of parasitism to vary among host individuals, resulting in a lower proportion of hosts parasitized as their density increases (i.e., inverse density dependent attack).

An overall finding of Type II population functional response does not necessarily mean there is no difference in *B. anurus* attack on alfalfa weevil between the Piedmont and

southwestern regions. Holling's (1959a,b) formula for determining functional response includes two variables related to parasitoid attack: *a*, which measures parasitoid searching efficiency, and T_h, the variable for host handling time. Differences in searching efficiency and handling time may occur in the field between parasitoids or populations of parasitoids having the same functional response, resulting in variable levels of host parasitization (Hassell, 2000). Studies of the searching efficiency and host handling time of *B. anurus* from regional populations can help to determine differences in *B. anurus*-alfalfa weevil interactions in the three regions of Virginia.

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CHAPTER 3

A Diagnostic Procedure for *Bathyplectes* spp. and for Detection of Hyperparasitization Using Restriction Enzyme Analysis of the 28S rDNA Gene

Abstract

The specialist parasitoids *Bathyplectes anurus* and *Bathyplectes curculionis* are morphologically similar, which makes their identification difficult. Also, *B. anurus* and *B. curculionis* are both subject to hyperparasitization in the field by several species of Hymenoptera, and hyperparasitized cocoons cannot be distinguished visually from non-parasitized cocoons. To address the species diagnostic problem, we developed a PCR-RFLP (PCR-restriction fragment length polymorphism) procedure to discriminate *B. anurus* and *B. curculionis* and to test for the presence of hyperparasitoid DNA in their cocoons. Genomic DNA was extracted from parasitoid cocoons which are abundant in the field and readily identifiable to species. A portion of the highly-conserved 28S rDNA gene was PCR amplified and digested using the restriction enzyme, BstBI. Digestion of 28S rDNA with BstBI produces a unique banding pattern for each taxon when visualized by agarose gel electrophoresis, enabling preliminary identification of the parasitoid or hyperparasitoid DNA. The protocol was used to successfully screen DNA extracted from 163 *Bathyplectes* cocoons, of which 15 produced fragment patterns indicating possible hyperparasitization or contamination by other non-target DNA. A successful diagnostic procedure for detecting the presence of non-target DNA increases the confidence with which genomic DNA extracted from *B. anurus* and *B. curculionis* cocoons can be used in molecular analyses of these two species.

3.1 Introduction

The species *Bathyplectes anurus* (Thomson) and *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) are specialist parasitoids introduced to North America for classical biological control of alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae). *Bathyplectes curculionis* and *B. anurus* are Eurasian species whose native distribution includes most of Europe and several countries in the Middle East. Apart from the original descriptions of the two species by Thomson (1883), very little has been published about the morphology of the adult *Bathyplectes*, which are said to bear close resemblance. Both *B. anurus* and *B. curculionis* are generally described as wasps ≈3–4 mm long with black, robust bodies (Thomson, 1883; Dysart and Day, 1976). Although adult females may be distinguished in the field by the length of the ovipositor, which is short (≈ 0.96 mm) in *B. anurus* and longer (≈1.38 mm) in *B. curculionis* (Clancy, 1969; (Dowell, 1977; Dowell and Horn, 1977), the two *Bathyplectes* species are otherwise nearly identical morphologically. As such, molecular analyses were conducted using DNA extracted from larval cocoons, which are easily collected in the field and readily identifiable to species, to develop a diagnostic procedure that could be used to distinguish between adults of the two species.

B. anurus and *B. curculionis* cocoons are subject to hyperparasitism in the field by several species of Hymenoptera, primarily of the families Pteromalidae, Chalcididae, and Ichneumonidae (Simpson et al., 1979). Hyperparasitized cocoons cannot be distinguished visually from non-hyperparasitized cocoons, raising uncertainty as to the identity and genetic purity of DNA extracted from *Bathyplectes* cocoons selected for molecular analyses. To address this problem, a diagnostic PCR-RFLP (PCR-restriction fragment length polymorphism) procedure using restriction enzyme fragment analysis was developed and tested to determine the

presence of hyperparasitoid DNA in 28S rDNA isolates of genomic DNA extracted from *Bathyplectes* larval cocoons. A restriction enzyme cleaves DNA at a specific sequence, or recognition site, producing a pattern of DNA fragments determined by the number and location of recognition sites in the DNA sample of interest. After gel electrophoresis of the enzyme reaction product to separate the DNA fragments by size, the fragment pattern can be visually assessed and the DNA of interest identified by comparison to a positive control (Rodriguez et al., 1997). A region of the 28S rDNA gene was chosen for this diagnostic because the same pair of primers can be used to amplify the gene region of interest in all taxa studied in this project.

3.2 Materials and Methods

3.2.1 Collection of *Bathyplectes* and Hyperparasitoid Adult Specimens

B. anurus, *B. curculionis*, and hyperparasitoid adults were collected and preserved in 90% ethanol as they emerged from laboratory-reared *Bathyplectes* cocoons obtained in Virginia alfalfa fields during the 2012 and 2013 field seasons. Specimens used for the study consisted of nine *B. anurus* and six *B. curculionis* adults; three pteromalids collected from *B. anurus* cocoons; one chalcid collected from a *B. anurus* cocoon; two ichneumons, preliminarily identified as *Gelis sp.*, collected from *B. anurus* and diapausing *B. curculionis* cocoons; and two ichneumons of unknown genus collected from non-diapausing *B. curculionis* cocoons, referred to in this study as Ichneumonidae Taxon 2. Additional specimens of each taxon were preserved and stored for further identification.

3.2.2 Extraction of Genomic DNA and Isolation of 28S rDNA

Genomic DNA was extracted from whole hyperparasitoid and *Bathyplectes* adult specimens and whole *Bathyplectes* cocoons using the Qiagen DNeasy Blood and Tissue Kit and following the spin-column protocol for purification of total DNA from animal tissue (Qiagen, 2006). DNA was eluted in sterile, double-deionized water, and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Prior to extraction of DNA from cocoons, the cocoons were externally cleansed by a light scrubbing with 100% ethanol and rinse with double-deionized water to remove surface particulates, followed by a rinse with 100% ethanol and allowing them to air dry.

Primers (forward: 5' GCGAACAAGTACCGTGAGGG 3'; reverse: 5' TAGTTCACCATCTTTCGGGTC 3') were used to select a region of approximately 700 base pairs of the highly-conserved 28S rDNA gene (Quicke et al., 2005). DNA was amplified in 25-µl reactions using Apex^{TM} 2.0X Taq RED DNA Polymerase Master Mix with 10 pmol each of forward and reverse primer, 1 µl of genomic DNA, and 9.5 µl of sterile, double-deionized water. A negative control reaction of Taq RED DNA Polymerase Master Mix, primers, and water was included as a check for contamination. Polymerase chain reaction (PCR) was performed using a Vapo.Protect Mastercycler Pro thermal cycler (Eppendorf AG, Hamburg, Germany) under the following conditions: initial denaturation for 10 minutes at 95°C; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds; and final extension at 72°C for 7 minutes. PCR product was visualized by electrophoresis on a 1% agarose gel, and amplicon sizes were determined using a 1 kb plus DNA size ladder. The approximately 700-bp amplicons were gel extracted and purified using the QIAquick Gel Extraction Kit per the Quick-Start Protocol (Qiagen, 2010). Purified 28S rDNA isolate was

eluted in sterile, double-deionized water, and quantified by spectrophotometer. Samples for each specimen were submitted for Sanger sequencing.

Sequences obtained for each specimen were aligned using SeqMan Pro Version 10.0 (DNASTAR, Inc.) to obtain consensus sequences for each taxon. Consensus sequences were entered into a restriction enzyme recognition site mapper (Stothard, 2012) to identify recognition sites for a single restriction enzyme that could be used analyze all taxa in this study.

3.3 Results and Discussion

3.3.1 Selection of Restriction Enzyme and Development of Taxon Positive Controls

A recognition site was identified for the restriction enzyme BstBI, an isoschizomer of AsuII (5' to 3': TT│CGAA). Table 3.1 presents the sequence strand lengths and locations of the BstBI recognition site in consensus sequences of each taxon.

Table 3.1. Sequence positions of BstBI restriction enzyme recognition site by taxon.

Taxon	Sequence Strand Length (bp)	Position(s) of BstBI Recognition Site	Sizes of Resulting Sequence Fragments (bp)
B. anurus	715	615	615, 100
B. curculionis	713	344, 613	344, 269, 100
Pteromalidae hyperparasitoid	723	238	485, 238
Chalcididae hyperparasitoid	723	236	487, 236
Ichneumonidae, genus *Gelis*	721	348	373, 348
Ichneumonidae Taxon 2	712	342, 611	342, 269, 101

Restriction enzyme reactions were performed with BstBI per the manufacturer's protocol (New England Biolabs, 2012), using 900 to 1000 ng of 28S rDNA isolate per 50 µl reaction. A

negative control of restriction enzyme, buffer, and sterile, double-deionized water was included as a check for contamination. Results were visualized by gel electrophoresis on 1.5% agarose gels, as presented in Figure 3.1.

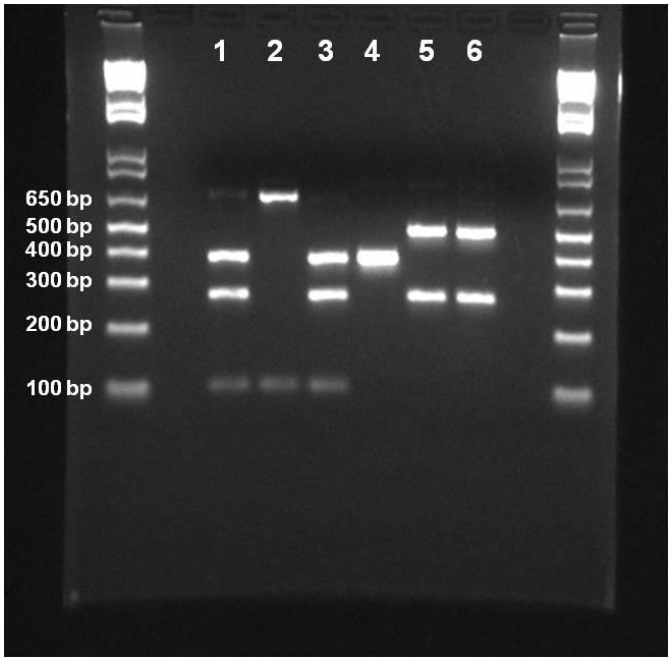

Figure 3.1. Digestion of 28S rDNA from adult *Bathyplectes* and hyperparasitoids by restriction enzyme BstBI, visualized by gel electrophoresis on a 1.5% agarose gel. Far left and far right lanes: 1 kb+ DNA size ladder. **Specimen 1:** *B. curculionis*; **Specimen 2:** *B. anurus*; **Specimen 3:** Hyperparasitoid, Ichneumonidae, Taxon 2; **Specimen 4:** Hyperparasitoid, Ichneumonidae, genus *Gelis*; **Specimen 5:** Hyperparasitoid, Pteromalidae; **Specimen 6:** Hyperparasitoid, Chalcididae.

Reactions were repeated using 28S rDNA isolates of different specimens from each taxon to determine consistency of results. Because Chalcididae and Pteromalidae fragment patterns appeared to be identical, these taxa were grouped as a single hyperparasitoid positive control, superfamily Chalcidoidea. However, restriction enzymes were identified by which these two taxa can easily be differentiated in a separate restriction enzyme fraction analysis, including BshTI (5’ to 3’: A|CCGGT), MboI (5’ to 3’: |GATC) and MluI (5’ to 3’: A|CGCGT). It was also

noted that the fragment patterns of *B. curculionis* and hyperparasitoid Ichneumonidae Taxon 2 appeared identical. Because this hyperparasitoid has only been collected from non-diapausing *B. curculionis* cocoons, which are infrequently collected, this hyperparasitoid positive control was not included in screening of DNA extracted from *B. anurus* and diapausing *B. curculionis* cocoons. However, restriction enzymes that can be used to screen non-diapausing *B. curculionis* cocoons for hyperparasitization by Ichneumonidae Taxon 2 include BfaI (5' to 3': C|TAG), MseI (5' to 3': T|TAA), NlaIII (5' to 3': CATG|) and TaqI (5' to 3': T|CGA).

3.3.2 Testing of Assay on DNA from *Bathyplectes* Cocoons

The assay was tested on 28S rDNA isolates from 105 *B. anurus* cocoons collected in Virginia, 27 *B. curculionis* cocoons collected in Virginia and Colorado, and 31 *Bathyplectes* cocoons of undetermined species collected in Virginia. Assays were conducted by species and geographic origin in groups of 8 to 15 specimens. Positive controls for *B. anurus* and *B. curculionis* were included in all assays to confirm species identification of cocoon specimens. Hyperparasitoid positive controls used in all assays were for Chalcidoidea and Ichneumonidae genus *Gelis*. Negative controls were included in each assay to check for reaction contamination.

3.3.3 PCR-RFLP Analyses of *Bathyplectes* Specimens

Restriction enzyme fragment patterns of all 105 *B. anurus* cocoon specimens matched the fragment pattern of the *B. anurus* adult positive control. To confirm results, 28S rDNA sequences were obtained for 45 *B. anurus* specimens. Sequences for all specimens had 100% identity to the *B. anurus* adult consensus sequence, and the recognition site for BstBI was located successfully in each sequence at or near nucleotide position 615.

Restriction enzyme fragment patterns for 21 of 27 *B. curculionis* cocoon specimens matched the fragment pattern of the *B. curculionis* adult positive control. To confirm results, 28S rDNA sequences were obtained for these 21 *B. curculionis* cocoon specimens. Sequences obtained had 100% identity to the *B. curculionis* consensus sequence, and BstBI recognition sites were successfully located in each sequence near nucleotide positions 344 and 613.

Figure 3.2 presents examples of fragment patterns obtained from nonparasitized *B. anurus* and *B. curculionis* cocoons. It was noted that faint bands appeared in each lane above the 650 bp marker. Because ribosomal DNA is highly repetitive, these bands are believed to be nondigested fragments of 28S rDNA.

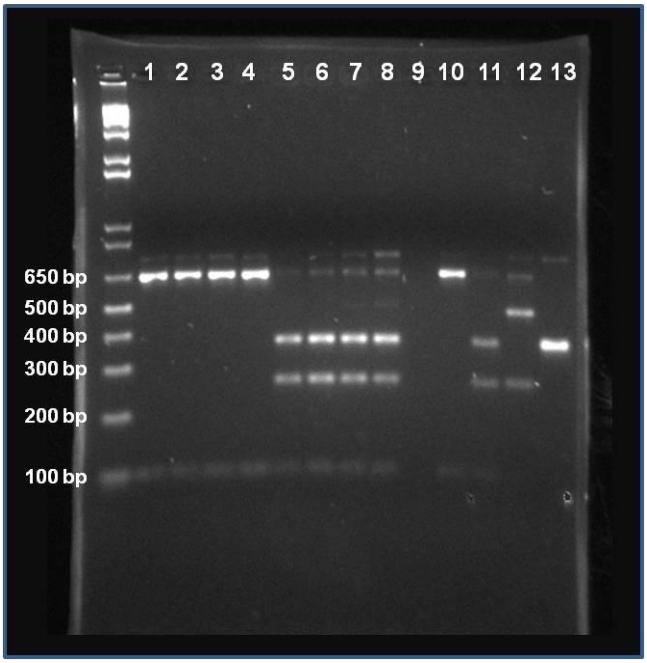

Figure 3.2. Comparison of banding patterns obtained from nonparasitized *Bathyplectes* cocoons after digestion of 28S rDNA by restriction enzyme BstBI, visualized by gel electrophoresis on a 1.5% agarose gel. **Far left lane:** 1 kb+ DNA size ladder; **1 through 4:** *B. anurus* specimens; **5 through 8:** *B. curculionis* specimens; **9:** Negative control; **10 through 13:** Positive controls for *B. anurus*, *B. curculionis*, hyperparasitoid Chalcidoidea, and hyperparasitoid Ichneumonidae genus *Gelis*, respectively. Faint bands above the 650-bp mark are believed to be nondigested fragments of 28S rDNA.

Figure 3.3 compares banding patterns obtained from the DNA of hyperparasitized cocoons (lanes 3 through 8) to banding patterns from nononparasitized cocoons (lanes 1 and 2). Of the six specimens for which fragment patterns did not match the *B. curculionis* positive control, patterns for two specimens matched that of the Chalcidoidea hyperparasitoid positive control (lanes 3 and 4), and patterns of three specimens appeared to be a combination of the Chalcidoidea and *B. curculionis* positive control patterns (lanes 5 through 7). The fragment pattern for one specimen did not match any of the positive controls, and appeared not to have been cleaved by BstBI (lane 8).

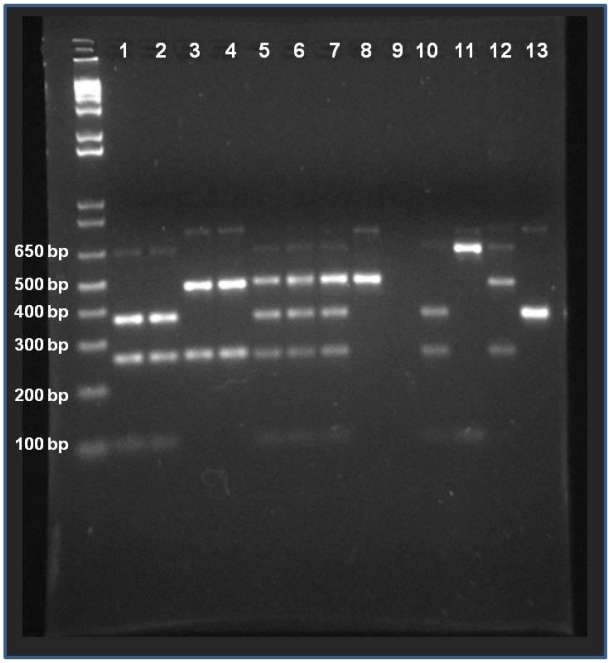

Figure 3.3. Comparison of banding patterns obtained from parasitized and nonparasitized *B. curculionis* after digestion of 28S rDNA by restriction enzyme BstBI, visualized by gel electrophoresis on a 1.5% agarose gel**. Far left lane:** 1 kb+ DNA size ladder. **1 and 2:** Nonparasitized *B. curculionis* specimens; **3 through 8:** Banding patterns of parasitized/corrupted *B. curculionis* specimens; **9:** Negative control; **10 through 13:** Positive controls for *B. curculionis*, *B. anurus*, hyperparasitoid Chalcidoidea, and hyperparasitoid Ichneumonidae genus *Gelis*, respectively. Patterns for specimens 3 and 4 match pattern of Chalcidoidea. Patterns for specimens 5, 6, and 7 appear to be a combination of *B. curculionis* and hyperparasitoid patterns. Pattern for specimen 8 does not match any controls, including that of species of interest.

To confirm results, 28S rDNA sequences were obtained for the specimens for which fragment patterns did not match the *B. curculionis* positive control. Sequences of the two specimens for which patterns matched the Chalcidoidea hyperparasitoid positive control had 99% to 100% identity with the consensus sequence of the Pteromalidae adult, and BstBI recognition sites in each sequence were located near nucleotide position 238, as occurs in sequences of the Chalcidoidea positive controls. Sequences of the three specimens displaying combination fragment patterns could not be aligned due to noise caused by superimposed DNA sequences. However, Basic Local Alignment Search Tool (BLAST) queries of the National Center for Biotechnology Information (NCBI) database were conducted for each sequence strand of these specimens to determine identities of the DNA. Partial alignments were returned for all three specimens to 28S rDNA sequences of several Ichneumonidae in the subfamily Campopleginae, group Dusona, the subfamily and group to which the *Bathyplectes* belong. Partial alignments were also returned for one of the three specimens to 26S rDNA sequences of Basidiomycota, a fungal group. No BLAST hits to Chalcididae or Pteromalidae sequences were returned for any of these specimens. The BstBI recognition site was found near nucleotide position 612 on the reverse strands of the sequences for these three specimens, but was not found on the forward strands.

The sequence of the *B. curculionis* cocoon specimen not matching any positive controls was shorter than sequences obtained for all other specimens, at ≈450 base pairs, and did not align to any of the consensus sequences of taxa included in this study. A BLAST query of the NCBI database returned partial alignments with the 26S rDNA sequences of several plants, primarily in the family Cucurbitacea, but no alignments to sequences of insects or other arthropods. The BstBI recognition site was not found in the sequence for this specimen.

3.3.4 PCR-RFLP Analyses of Unidentified *Bathyplectes* Cocoons

Of the 31 unidentified *Bathyplectes* specimens analyzed, restriction enzyme fragment patterns of 22 specimens definitively matched the pattern of the *B. anurus* adult positive control, and were therefore identified as *B. anurus*. Fragment patterns of the other nine specimens did not match the patterns of any of the positive controls, and of these, sequences were obtained for seven specimens. The sequence of one of these seven specimens aligned to the *B. anurus* consensus sequence, but the sequence trace file showed evidence of contamination by DNA of another organism. Sequences of the other six specimens did not align to any of the consensus sequences of taxa included in this study. BLAST queries of the NCBI database for each specimen returned a significant alignment to a portion of the 18S rDNA gene of *Acanthamoeba castellanii*, a common soil protozoan, but no alignments were returned to sequences for insects or other arthropods. Recognition sites for BstBI were not found in the sequences of any of these seven specimens.

Of 163 *Bathyplectes* cocoons tested, 148 presented fragment patterns that matched the patterns of the *Bathyplectes* adult positive controls. These visual assessments were verified by genetic sequencing of 28S rDNA from 45 *B. anurus* and 21 *B. curculionis* test specimens. In all cases, sequences of the test specimens had 100% identity to the consensus sequence of the adult positive control.

Results also seem to indicate that hyperparasitization or contamination by other non-target DNA can be preliminarily determined by restriction enzyme fragment patterns that do not match the patterns of the *Bathyplectes* adult positive controls. By this assay, genomic DNA of 15 test specimens was preliminarily determined to be corrupted. The fragment patterns for two of these specimens matched the pattern of the Chalcidoidea hyperparasitoid control, which was

verified by genetic sequencing of 28S rDNA from these specimens. The other 13 specimens presented fragment patterns that did not match patterns of any of the positive controls. Genetic sequencing of 28S rDNA for 11 of these specimens verified contamination by non-target plant, fungal, and microorganism DNA.

Overall, results seem to indicate that this PCR-RFLP analysis protocol can be used for positive identification of species of interest for genomic DNA extracted from non-hyperparasitized *B. anurus* and *B. curculionis* cocoons.

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CHAPTER 4

Molecular Differentiation of *Bathyplectes curculionis* and *Bathyplectes anurus* by Partial Sequencing of the 28S rDNA and COI Genes

Abstract

Bathyplectes curculionis and *Bathyplectes anurus* are morphologically similar species of specialist parasitoid established throughout the United States for classical biological control of alfalfa weevil. In Virginia, *B. anurus* is the primary biological control agent of alfalfa weevil; however, effectiveness of *B. anurus* against alfalfa weevil varies between the alfalfa-producing regions of the state which might be attributable to biotic factors including genetic variation. Molecular taxonomy can aid in identifying genetically distinct populations within *B. anurus* and differentiating *B. anurus* and *B. curculionis*. We performed molecular analyses of 28S rDNA and COI sequences for *B. anurus* specimens to detect DNA polymorphism indicative of genetic variation within and between populations. Inter-species identity of 28S rDNA sequences obtained for *B. curculionis* and *B. anurus* specimens was 96.6%; inter-species identity of COI sequences was 89.7%, indicating that these two gene regions can be used for molecular identification and differentiation of *B. anurus* and *B. curculionis*. One COI sequence haplotype expressed by 25% of *B. anurus* specimens (Haplotype 2) might indicate regional genetic variation correlated to temperature. Two major COI sequence haplotypes found in Virginia *B. curculionis* specimens might be indicative of incipient speciation.

4.1 Introduction

The species *Bathyplectes curculionis* and *Bathyplectes anurus* are specialist parasitoids imported from Europe and the Middle East for classical biological control of alfalfa weevil (Fisher et al., 1961). *B. curculionis* and *B. anurus* occupy sympatric ranges throughout most of the United States, and often coexist within fields (Bryan et al., 1993). Adults of the two species are nearly identical morphologically, and are usually identified by sight recognition of the parasitoid cocoons, which, though highly similar, evince characteristics unique to each species (Dysart and Day, 1976). However, variations in cocoon morphology can alter distinguishing characteristics, resulting in misidentification and research error.

In Virginia, *B. anurus* is the most important biological control agent of alfalfa weevil. *B. curculionis* is also established in Virginia, though at low numbers, accounting for less than 10% of the *Bathyplectes* parasitoid complex (Kuhar et al., 2000). A three-year study of alfalfa weevil biological control in Virginia found levels of control to be similar in the Shenandoah Valley and southwestern regions, ranging from 45% to 73% and 46% to 71% respectively; however, the level of control attained in the Piedmont region was much lower, ranging from 16% to 32% (Kuhar et al., 1999).

Biotic factors, including genetic variations, can influence the success of an introduced biological control agent by affecting the extent to which the organism is locally adapted to the new environment or to populations of hosts (Caltagirone, 1985). Additionally, genetic variation of certain gene loci, such as within mitochondrial DNA genes, between regional populations of a species can be indicative of adaptation to local conditions including temperature (Wang et al., 2013). The objectives of this study were 1) to derive molecular sequences for each species of a portion of the 28S ribosomal DNA gene and the mitochondrial gene cytochrome oxidase subunit

1 (COI) by which *B. anurus* and *B. curculionis* can be molecularly identified and differentiated; and 2) to compare COI sequences for *B. anurus* specimens from populations in the Piedmont, Shenandoah Valley, and southwestern regions to determine if differential parasitization of alfalfa weevil by *B. anurus* might be attributable to population-level genetic variation.

4.2 Materials and Methods

4.2.1 Collection of *Bathyplectes* Specimens

Bathyplectes cocoons were reared in the laboratory from field-collected alfalfa weevil larvae obtained in Spring 2012 and Spring 2013 from nine alfalfa fields in the Piedmont, Shenandoah Valley, and southwestern region of Virginia. Each field sampled was located in a separate town or county to represent geographically-distant points within the region. Cocoons were identified to species by morphology, and identity of *B. anurus* cocoons was verified by jumping of the cocoons in response to environmental stimuli, a characteristic unique to *B. anurus*. Most adult *Bathyplectes* used in this study were collected and identified to species upon emergence from cocoons; a few adults were obtained by sweep-netting alfalfa fields. *Bathyplectes* specimens were also obtained from Colorado for outgroup comparisons, and included pinned adult archival specimens identified as *B. curculionis*, and *Bathyplectes* cocoons collected in 2013 from alfalfa field stubble. Table 4.1 shows the collection data of all specimens included in this study.

Table 4.1. Collection data of *Bathyplectes* specimens sequenced in this study.

Specimen IDs	Species	Stage	Collection Date	Location
Bed BC1-10	*B. curculionis*	Larval cocoon	April 2013	Bedford Co., VA (Piedmont)
Rus BC1-8	*B. curculionis*	Larval cocoon	April-May 2013	Rustburg, VA (Piedmont)
Rus BC9-10	*B. curculionis*	Adult	Jan-Feb 2013	Rustburg, VA (Piedmont)
Roc BC1-2	*B. curculionis*	Adult	January 2013	Rockingham Co., VA (Shenandoah Valley)
CO BC1-2	*B. curculionis*	Larval cocoon	June 2013	Pueblo Co., CO
CO BC3-4	*B. curculionis*	Adult archival specimen	June 1993	Boulder Co., CO
App BA	*B. anurus*	Adult	March 2013	Appomattox, VA (Piedmont)
Bla BA1-10	*B. anurus*	Larval cocoon	March 2012	Blackstone, VA (Piedmont)
Bed BA1-10	*B. anurus*	Larval cocoon	April 2013	Rustburg, VA (Piedmont)
Rus BA1-10	*B. anurus*	Larval cocoon	April 2012	Rustburg, VA (Piedmont)
Rus BA11	*B. anurus*	Adult	March 2012	Rustburg, VA (Piedmont)
Aug BA1-15	*B. anurus*	Larval cocoon	April 2012	Augusta Co., VA (Shenandoah Valley)
Aug BA16	*B. anurus*	Adult	March 2012	Augusta Co., VA (Shenandoah Valley)
Aug BA17-18	*B. anurus*	Adult	February 2013	Augusta Co., VA (Shenandoah Valley)
Roc BA1-15	*B. anurus*	Larval cocoon	April 2012	Rockingham Co., VA (Shenandoah Valley)
Roc BA16-17	*B. anurus*	Adult	February 2013	Rockingham Co., VA (Shenandoah Valley)
Dub BA1-5	*B. anurus*	Larval cocoon	March 2012	Dublin, VA (southwestern region)
Dub BA6-10	*B. anurus*	Larval cocoon	May 2013	Dublin, VA (southwestern region)
Pul BA1-4	*B. anurus*	Larval cocoon	April 2012	Pulaski Co., VA (southwestern region)
Pul BA5-10	*B. anurus*	Larval cocoon	April 2013	Pulaski Co., VA (southwestern region)
VT BA1-6	*B. anurus*	Larval cocoon	May 2012	Blacksburg, VA (southwestern region)
VT BA7-10	*B. anurus*	Larval cocoon	April 2013	Blacksburg, VA (southwestern region)
VT BA11-14	*B. anurus*	Adult	February 2013	Blacksburg, VA (southwestern region)
CO BA1	*B. anurus*	Adult archival specimen	June 1993	Weld Co., CO
CO BA2-4	*B. anurus*	Larval cocoon	June 2013	Pueblo Co., CO
CO BS	*B. stenostigma?*	Adult archival specimen	June 1993	Boulder Co., CO

Geographic representation of the 101 Virginia *B. anurus* specimens used in this study was nearly uniform among regions, and included 32 individuals from the Piedmont, 35 individuals from the Shenandoah Valley, and 34 individuals from the southwestern region. Specimens of *B. curculionis*, which comprised less than 8% of the *Bathyplectes* parasitoid complex in Virginia (Kuhar et al., 2000), were mostly obtained from two locations in the Piedmont region and included 10 individuals from Bedford County and 10 individuals from Rustburg. Two *B. curculionis* specimens were also obtained from Rockingham County in the Shenandoah region.

4.2.2 Extraction of Genomic DNA

Genomic DNA was extracted from whole *Bathyplectes* adult specimens and cocoons using the Qiagen DNeasy Blood and Tissue Kit and following the spin-column protocol for purification of total DNA from animal tissue (Qiagen, 2006). DNA was eluted in sterile, double-deionized water, and quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Prior to extraction of DNA from cocoons, the cocoons were externally cleansed by a light scrubbing with 100% ethanol and rinsed with double-deionized water to remove surface particulates, followed by a rinse with 100% ethanol and allowing them to air dry.

4.2.3 Isolation of 28S rDNA and Cytochrome Oxidase I Gene Regions

Polymerase chain reaction (PCR) was performed to amplify regions of approximately 700 base pairs each of the D2-D3 expansion region of the 28S rDNA gene, and the mitochondrial gene cytochrome oxidase subunit 1 (COI). Primers used to select the 28S rDNA gene region of interest were: Forward 5’ GCGAACAAGTACCGTGAGGG 3’; Reverse 5’

TAGTTCACCATCTTTCGGGTC 3' (Quicke et al., 2005). Primers used to select the COI gene region of interest were: Forward 5' ATTCAACCAATCATAAAGATAT 3'; Reverse 5' TAAACTTCTGGATGTCCAAAA 3' (Hebert et al., 2004). DNA was amplified in 25-µl reactions using Apex^{TM} 2.0X Taq RED DNA Polymerase Master Mix with 10 pmol each of forward and reverse primer, 1 µl of genomic DNA, and 9.5 µl of sterile, double-deionized water. A negative control reaction of Taq RED DNA Polymerase Master Mix, primers, and water was included as a check for contamination. PCR conditions for amplification of 28S rDNA were initial denaturation for 10 minutes at 95°C; 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds; and final extension at 72°C for 7 minutes. Conditions for amplification of COI were initial denaturation for 10 minutes at 95°C; 35 cycles of denaturation at 95°C for 45 seconds, annealing at 51°C for 45 seconds, and extension at 72°C for 60 seconds; and final extension at 72°C for 7 minutes.

PCR product was visualized by electrophoresis on a 1% agarose gel, and amplicon sizes were determined using a 1 kb plus DNA size ladder. Amplicons of interest were gel extracted and purified using the QIAquick Gel Extraction Kit per the Quick-Start Protocol (Qiagen, 2010). Purified DNA isolate was eluted in sterile, double-deionized water, and quantified by spectrophotometer. Samples for each specimen were submitted for Sanger sequencing.

4.2.4 Alignment and Analyses of DNA Sequences

Individual sequences of each gene region for each specimen were edited and assembled into scaffolds using SeqMan Pro version 11.0 (DNASTAR, 2013). Scaffolds were aligned in MEGA version 6 (Tamura, Stecher, Peterson, Filipski, and Kumar, 2013), and the alignments exported to Nexus (PAUP 4.0) format. Alignments were analyzed for DNA sequence

polymorphisms using DnaSP version 5 (Librado and Rozas, 2009).

4.2.5 Climate Analysis of *B. anurus* Haplotype Regions

Analysis of the geographic distribution of nucleotide sequence haplotypes among Virginia specimens seems to indicate regional bias expression of Haplotypes 1 and 2. One hypothesis for regional haplotype expression is that genetic variation can indicate an adaptive response to environmental conditions, including temperature. In their study of local adaptation of the small yellow croaker fish, *Larimichtyhs polyactis*, Wang et al. (2013) found genetic variation between regional populations of a particular mitochondrial DNA marker that was strongly correlated with local temperature patterns, and concluded that temperature was highly influential in adaptive differentiation of this gene locus.

To examine regional expression of *B. anurus* Haplotype 2 relative to climate, monthly climatological data reports were obtained from the National Oceanic and Atmospheric Administration National Climate Data Center for specimen collection locations included in this study for the period 1957, the year *B. anurus* was first introduced in Virginia, through 2012. Data categories included in each report were: mean temperature, mean maximum temperature, mean minimum temperature; observed maximum temperature, observed minimum temperature; number of days observed maximum temperature $\geq$ 32ºC, $\leq$0ºC; and number of days observed minimum temperature $\geq$0ºC, <0ºC, $\leq$-18ºC. Rainfall and snowfall totals were also included, but varied in completeness by report and so were not included in this analysis. Climatological data for Dublin are represented by the Blacksburg data set because separate data for Dublin were not available. Also, data for Bedford were not available for the entire reporting period.

A principal components analysis (PCA) was performed to test for multicollinearity among the climatic variables and to identify a subset of representative variables for regression analysis (Dunteman, 1989; Hair et al., 2010). The PCA was carried out on the correlation matrix and the principal components with an eigenvalue ≥ 0.70 were selected for further analysis (Dunteman, 1989; Jolliffe, 2002). The average values for the variables with the largest loadings on each of the selected principal components were used in a stepwise regression analysis to determine which one or group had the greatest influence on Haplotype 2 expression. The analysis was carried out using JMP Pro 10.0 (SAS Institute, 2012).

4.3 Results and Discussion

4.3.1 *B. curculionis* 28S rDNA

28S rDNA sequences were obtained for 24 of 25 *B. curculionis* specimens; a sequence was not obtained for specimen CO BC2. Sequence strands averaged 713 base pairs; editing and alignment of the strands produced a consensus region of 616 base pairs for all sequences. Analysis of the alignment for DNA sequence polymorphisms identified four variable sites, resulting in four sequence haplotypes with identity between haplotypes approaching 100%. Haplotype 1, the majority haplotype, is expressed homozygously in 11 specimens (Bed BC5, Bed BC6, Bed BC9, Bed BC10, Roc BC2, Rus BC3, Rus BC6, Rus BC7, Rus BC9, CO BC1, and CO BC2) and heterozygously, on the forward strand only, in seven specimens (Bed BC2, Bed BC4, Bed BC7, Roc BC1, Rus BC10, CO BC3, and CO BC4). The Haplotype 1 sequence is presented in Figure 4.1.

```
    acgagacgcc  ccgggagtgc  gggaccgcat  cgtgacgcgg  cccatcctcc   50
 51 ctcggttggc  gcaaggccaa  ccttcacttt  cattgcgcct  ttaggcttaa  100
101 cagatcccaa  tgactcgcgc  acatgttaga  ctccttggtc  cgtgtttcaa  150
151 gacgggtcct  gaaagtaccc  aaagcagtag  cgtcgccgac  cggtattaat  200
201 aagagccagt  cccaggacac  cgccagccaa  cagctggcca  ggcccggtga  250
251 cggcgctaag  tccgtacatc  cgggaaacac  tggcctcgct  tgcggcgggc  300
301 cggacgcagt  tcgaattgcg  gctcaatacc  gtgcgagtac  cgccgggcag  350
351 ctggtcagac  ggccgggggt  ctgccacgtg  acgccgtgaa  gcgtgatacg  400
401 tgacaggcgc  ccacccgggt  cgtagaccga  cacccaacgg  gtcgcgacgt  450
451 cctactaggg  gagaagtgca  caacgacgac  acccggcaaa  taaatcatac  500
501 ggacgcgtgc  agcgggaccg  aagtccccaa  catcgcgaac  cgcacaattg  550
551 ccaggatcgc  tgttgatgaa  tctctccatt  cgaacttttg  ggtttctcag  600
601 gtttacccct  gaacgg
```

Figure 4.1. Sequence of the 28S rDNA gene region D2-D3 for *Bathyplectes curculionis* Haplotype 1.

Haplotype 2, expressed only by specimen CO BC3, is characterized by a single nucleotide polymorphism (SNP) at position 69 of the reverse strand, a transversion of t→a, and a transversion of c→a at position 576 of the reverse strand. Haplotype 3 is characterized by a transition of t→c at position 248. This SNP occurs heterozygously, on the reverse strand only, in five specimens (Bed BC2, Bed BC4, Bed BC7, Rus BC10, and CO BC4) and homozygously in three specimens (Bed BC1, Bed BC3, and Rus BC1). Haplotype 4 is characterized by a transition of a→g at position 63. This SNP occurs homozygously in four specimens (Rus BC2, Rus BC4, Rus BC5, and Rus BC8) and on the reverse strand only of one specimen, Roc BC1.

The 28S rDNA sequence for Haplotype 1, the majority haplotype, was compared to the 28S rDNA sequence for *B. curculionis* on file with GenBank, Accession No. AY593068, obtained from a specimen collected in Germany. The Haplotype 1 sequence aligned Plus/Minus to positions 1 through 601 of the GenBank reference sequence. Identity to the GenBank sequence was 95% with 30 SNPs and one gap occurring along the alignment, which would seem

to indicate that the Virginia and Colorado *B. curculionis* specimens sequenced in this study are not the same species as the German *B. curculionis* represented by the GenBank sequence.

Though sample size was limited by the scarcity of *B. curculionis*, the near unanimity of the sequences obtained for Virginia and Colorado specimens included in this study preliminarily indicates that *B. curculionis* in Virginia and Colorado are of the same species and that a 28S rDNA sequence has been derived for molecular identification of *B. curculionis* in the United States. These findings can be verified through a larger sampling of *B. curculionis* specimens from regions in which the species is more abundant, such as the western and mountain states.

4.3.2 *B. anurus* 28S rDNA

28S rDNA sequences were obtained for 52 *B. anurus* specimens from Virginia and two specimens from Colorado. Virginia specimens sequenced were: App BA, Bla BA1 through 7, Rus BA1 through 8, and Rus BA11, from the Piedmont region; Aug BA 1 through 7, Aug BA 16, Roc BA 1 through 8, Roc BA16 and Roc BA17, from the Shenandoah region; and Dub BA1 through 5, Pul BA1 through 4, VT BA1 through 6, VT BA11 and VT BA12, from the Southwest region. The Colorado specimens sequenced were CO BA1 and CO BA2. It should be noted that CO BA1, an adult archival specimen collected in 1993, had been identified originally as *B. curculionis*, but was verified to be *B. anurus* by the genetic sequence obtained in this study.

Sequence strands averaged 715 base pairs; editing and alignment of the strands produced a consensus region of 594 base pairs for all sequences, presented in Figure 4.2. Analysis of the alignment for DNA sequence polymorphisms identified a single variable site, a homozygous transition of a→g at position 271, expressed only in specimen Rus BA8. Sequence identity was 100% among the remaining 53 specimens.

```
     ccccgggagt  gcgggaccgc  atcgtgacgc  ggcccatcct  ccctcggttg   50
  51 gcgcaaggcc  taccttcact  ttcattgcgc  ctttaggctt  aatagatccc  100
 101 aatgactcgc  gcacatgtta  gactccttgg  tccgtgtttc  aagacgggtc  150
 151 ctgaaagtac  ccaaagcagt  agcgtcgccg  accggtattt  atttaagagc  200
 201 cagtcccagg  acaccgccag  ccaacagctg  gccaggcccg  gtgacggcgc  250
 251 taagtccgta  catccgggaa  acactggcct  cgcttgcggc  gggccggacg  300
 301 cagttcaaat  tgcggctcaa  taccgtgcga  gtaccgccgg  gcagctggtc  350
 351 ggacaaccgg  gggtctgcca  catgacgccg  tgaagcgtaa  cacgtgacgg  400
 401 gcacccaccc  gggtcgtaga  ccgacaccca  acgggtcgcg  acgtcctact  450
 451 aggggagaag  tgcacgacga  cgacgcccgg  caaataaatc  atacggacgc  500
 501 gtgccgcgag  accggagtcc  ccgacatcgc  gaaccgcaca  attgccagga  550
 551 tcgctgacga  tgaatctctc  cattcgaact  tttgggtttc  tcag        600
```

Figure 4.2. Sequence of the 28S rDNA gene region D2-D3 for *Bathyplectes anurus*.

Alignment of the *B. anurus* consensus sequence to the Virginia and Colorado *B. curculionis* sequence for Haplotype 1 resulted in 96.6% identity, with 20 SNPs occurring over the 595-base-pair alignment. The *B. anurus* sequence was also compared to the GenBank 28S rDNA sequence for *B. curculionis*, Accession No. AY593068. The *B. anurus* sequence aligned Plus/Minus to positions 2 through 593 of the GenBank *B. curculionis* sequence. Identity to the GenBank reference sequence was 95.9%, with 24 SNPs and 2 gaps occurring along the alignment. These results appear to indicate that *B. anurus* can be molecularly identified and differentiated from *B. curculionis* by this region of the 28S rDNA gene.

4.3.3 28S rDNA Sequence for Third *Bathyplectes* Species

A 28S rDNA sequence was obtained for specimen CO BS, an adult archival specimen collected in 1993 from Boulder County, Colorado. Though the specimen had been identified originally as *B. curculionis*, the 28S rDNA sequence obtained did not match any sequence obtained in this study for *B. curculionis*, nor did the sequence match sequences obtained for *B. anurus*. This specimen is believed to be the morphologically similar sister species, *Bathyplectes*

stenostigma, another specialist parasitoid imported from Europe for biological control of alfalfa weevil. *B. stenostigma* is a biological control agent of local importance throughout the northern Great Plains and Intermountain regions of the United States, including in Colorado where all three species of *Bathyplectes* are established (Bryan et al., 1993; Radcliffe and Flanders, 1998).

The forward and reverse strands of the CO BS 28S rDNA sequence averaged 713 nucleotides which, after editing and alignment, resulted in a sequence of 624 base pairs as presented in Figure 4.3.

Comparison of the sequence for specimen CO BS to the 28S rDNA sequence for Virginia and Colorado *B. curculionis* Haplotype 1 resulted in an alignment of 614 base pairs with 32 SNPs. Identity between these species was 94.8%. Comparison of the sequence for specimen CO BS to the *B. anurus* sequence resulted in an alignment of 591 base pairs with 19 SNPs, and inter-species identity of 96.8%.

```
    atgagaacga  gacgccccgg  gagtgcggga  ccgcatcgtg  acgcggccca   50
 51 tcctccctcg  gttggcgcaa  ggcctacctt  cactttcatt  gcgcctttag  100
101 gcttaacaga  tcccaatgac  tcgcgcacat  gttagactcc  ttggtccgtg  150
151 tttcaagacg  ggtcctgaaa  gtacccaaag  cagtagcgtc  gccgaccggt  200
201 atttgtgtag  agccagtcct  aggacaccgc  cagccaacag  ctggccaggc  250
251 ccggtgacgg  cgctaagtcc  gtacatccgg  gaaacactgg  ccgcgcttgc  300
301 ggcgggccgg  acgcagttca  aatttgcgac  tcaataccgt  gcgagtaccg  350
351 ccgggcagct  ggtcggacaa  ccgggggtct  gccacatgac  gccgtgaagc  400
401 gtaacacatg  acgggcaccc  acccgagtcg  tagaccgaca  cccaacgggt  450
451 cgcgacgtcc  tactagggga  gaagtgcacg  acgacgacac  ccgacaaaaa  500
501 atgacacgga  cgcgtgccgc  gggaccgagg  tcccgggcat  cgcgaaccgc  550
551 acatcgccgg  gatcgctgac  gatgaatctc  tccattcgaa  cttttgggtt  600
601 tctcaggttt  acccctgaac  ggtt
```

Figure 4.3. Sequence of the 28S rDNA gene region D2-D3 for *Bathyplectes* specimen CO BS.

The CO BS sequence aligned Plus/Minus to positions 1 through 607 of the GenBank 28S rDNA sequence for *B. curculionis*, with 25 SNPs identified. Identity between the CO BS

sequence and the GenBank reference sequence was 95.9%. Preliminarily, comparison of the sequences seems to indicate that specimen CO BS is neither *B. curculionis*, as originally identified, nor *B. anurus*. Identification of specimen CO BS as *B. stenostigma* can be molecularly verified by obtaining 28S rDNA sequences for comparison from an adequate test group of properly identified *B. stenostigma* specimens.

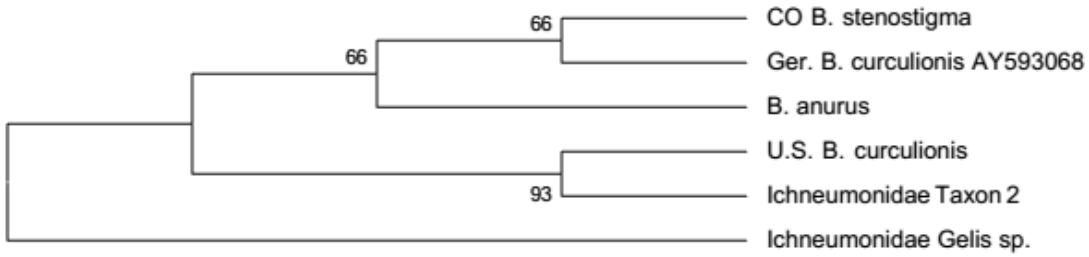
4.3.4 Phylogenetic Analysis of the *Bathyplectes* 28S rDNA Sequences

28S rDNA molecular data were analyzed in MEGA 6 to construct phylogeny of the three *Bathyplectes* species sequenced in this study and the German *B. curculionis* represented by GenBank Accession No. AY593068. Also included in the phylogenetic analysis were 28S rDNA sequences obtained from two taxa of hyperparasitoid collected in the laboratory from *Bathyplectes* cocoons. The first, the ichneumon *Gelis* sp., was collected from *B. anurus* and *B. curculionis* cocoons. The second hyperparasitoid, an ichneumon collected only from non-diapausing *B. curculionis* cocoons and referred to in our study as Ichneumonidae Taxon 2, has not been identified beyond family. However, results of a Basic Local Alignment Search Tool (BLAST) query of the NCBI database using the 28S rDNA sequence show closest identity (95% to 97%) with members of the Ichneumonidae subfamily Campopleginae, the subfamily to which the *Bathyplectes* belong. Identity between sequences of Ichneumonidae Taxon 2 and the *B. curculionis* host is 98.8%.

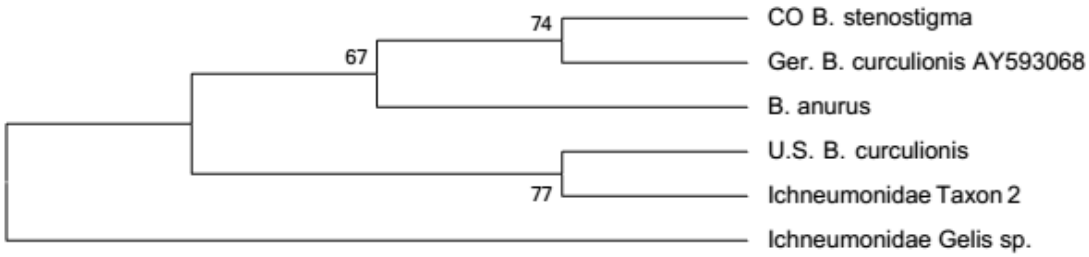
Phylogenies were constructed using the Maximum Parsimony, Maximum Likelihood, and Neighbor-Joining methods. For the Maximum Parsimony analysis, the default setting of Min-mini heuristic approach with search factor of 2 was accepted for construction of the maximum parsimony tree. For the maximum likelihood analysis, the BioNJ method, using the

Maximum Composite Likelihood approach, was selected for construction of the heuristic search tree. The Maximum Likelihood and Neighbor-Joining methods employed the Tamura 3-parameter model with Gamma distribution (Tamura, 1992), selected by a model analysis run in MEGA of the alignment file to be used to construct the phylogeny. The model setting used for gap/missing data treatment was partial deletion with minimum site coverage of 95%. The branch swap filter default setting of "very strong" was accepted. Tree results were tested by a 1000-replicate bootstrap analysis. Figure 4.4 presents the bootstrap consensus trees produced by each analysis method.

a)



b)



c)

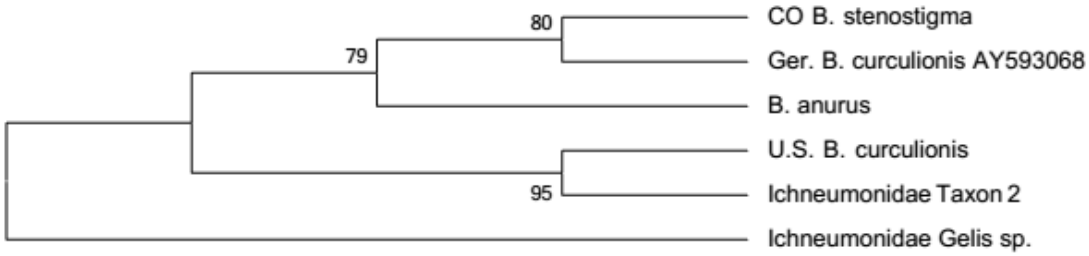


Figure 4.4. Phylogeny inferred by MEGA 6 using a) Maximum Parsimony, b) Maximum Likelihood, and c) Neighbor-Joining methods. Numbers at branch nodes indicate percentage of trees produced with same taxa clusters in 1000-replicate bootstrap analysis.

All three methods produced the same tree, differing only in the percentage of bootstrap replicate trees produced by each method. The hyperparasitoid *Gelis* sp. is the outgroup taxon, which is appropriate as *Gelis* belongs to the Ichneumonidae subfamily Cryptinae, and the other taxa belong to the subfamily Campopleginae.

The grouping of U.S. *B. curculionis* with its hyperparasitoid, Ichneumonidae Taxon 2, does not appear to be unreasonable given the high sequence identity between these two species and recovery to date of the hyperparasitoid solely from *B. curculionis* hosts. Grouping of the purported *B. stenostigma* specimen (CO BS) with the GenBank German *B. curculionis* reference specimen rather than with *B. anurus* was unexpected, given the higher identity between specimen CO BS and *B. anurus* (96.8%) versus identity between CO BS and the GenBank specimen (95.9%). However, a DnaSP analysis of conserved regions between these species indicated that the largest conserved region between the CO BS and GenBank reference sequences is 174 base pairs, but the largest conserved region between the CO BS and *B. anurus* sequences is only 96 base pairs.

4.3.5 *B. curculionis* COI Gene

COI sequences were obtained for 19 of the 25 *B. curculionis* specimens: Bed BC1-2, Bed BC4-8, Bed BC10, Roc BC1-2, Rus BC1, Rus BC3, Rus BC5-7, Rus BC9-10, and CO BC1-2. Sequence strands averaged 678 nucleotides; editing and alignment of the strands produced a consensus region of 595 base pairs for all sequences. Analysis of the alignment for DNA sequence polymorphisms identified 23 variable sites, resulting in five haplotypes. Haplotype 1, the majority haplotype, is expressed by nine specimens: Bed BC1, Bed BC4, Bed BC7, Bed BC8, Rus BC5, Rus BC7, Roc BC2, and the Colorado specimens CO BC1 and CO BC2.

Haplotype 2, which is characterized by 21 SNPs, is also a significant haplotype and is expressed by six specimens: Bed BC2, Bed BC 5, Bed BC10, Rus BC1, Rus BC6, and Rus BC10. Haplotype 3 is a single-position variation of Haplotype 1, characterized by a homozygous transition of c→t at position 499. This occurs only in specimen Rus BC3. Haplotype 4 is a single-position variation of Haplotype 2, characterized by a homozygous transition of g→a at position 218. This is expressed by two specimens, Bed BC6 and Roc BC1. Haplotype 5 is also a single-position variation of Haplotype 2, characterized by a homozygous transversion of a→t at position 392. This occurs only in specimen Rus BC9. Figure 4.5 compares the nucleotide sequences of Haplotypes 1 and 2, the majority haplotypes.

```
Haplotype 1      cacccccctga agggtcaaaa aatgaagtat ttaaatttcg atctgttaaa
Haplotype 2      .......a.. ...a...... .......... .......... ..........  50

Haplotype 1      agtatagtaa ttgctccagc taaaactggt actgctaaaa gaagtaaaat
Haplotype 2  51  .......... ....a..... .......... .......... .......... 100

Haplotype 1      tgttgtaatt ttaattgatc aagtaaaaag agttaattgt tcaaattttt
Haplotype 2  101 ..c....... .......... .........g .......... .......... 150

Haplotype 1      tattaatatt ttttatatta aaaattgtag taataaaatt aattgcacct
Haplotype 2  151 .......... .......... ........t. ....g..... ...a..t... 200

Haplotype 1      ataatagaag atattccggc taaatgtaat gaaaaaatag ctaaatctac
Haplotype 2  201 .......... .......... .......... .......... .......... 250

Haplotype 1      tgatattcct tcatgattta catttaaaga taaaggggga taaacagttc
Haplotype 2  251 .......... .......... .......... .........g .....t.... 300

Haplotype 1      aaccagttcc tactccttga ttagtaatag aacttaaaat taagagtaaa
Haplotype 2  301 .......c.. .......... .......... .......... ...a...... 350

Haplotype 1      attgaaggtg gaagtaatca aaatcttata ttatttattc gggggaaagc
Haplotype 2  351 .........g .......... .......... .......... .a..a..g.. 400

Haplotype 1      tatatcaggt cttcctaata ttaaaggaat taatcaattt ccaaatcctc
Haplotype 2  401 .........a .......... .......... .......... ........a. 450

Haplotype 1      caattataat tggtataact ataaaaaaaa ttataataaa agcatgtgct
Haplotype 2  451 .......... .......... .......... .......... .......... 500

Haplotype 1      gtaacaaaag aattataaat ttgatcattg ttaattaaat atccaggatt
Haplotype 2  501 .......... .......... .........a .......... .a........ 550

Haplotype 1      tcttaattct attcgaataa ttaaacttat tgaagatcca attat
Haplotype 2  551 .......... .......... .......... .......... .....     600
```

Figure 4.5. Sequences of a portion of the COI gene for *Bathyplectes curculionis* Haplotypes 1 and 2. Dots indicate nucleotides in common.

Despite the small sample size, no regional bias was detected between haplotypes among Virginia specimens. More than one haplotype was expressed by specimens collected from the same fields in Bedford County, Rustburg, and Rockingham County. The only regional group to express a single haplotype was Colorado. A larger sampling of *B. curculionis* specimens from regions outside Virginia may verify whether the expression of multiple haplotypes among regional populations is typical.

The COI gene is a protein-coding gene; therefore, translation of the nucleotide sequences to protein sequences was necessary to determine if the SNPs present in each haplotype sequence result in protein coding changes. To translate the COI nucleotide sequences to proteins, a BLAST query was conducted of the NCBI database with the *B. curculionis* COI sequences to find a closely-related reference sequence for alignment and translation. The highest-scoring alignment was to a COI sequence of 658 base pairs for an unclassified Campopleginae, GenBank Accession No. JN300365. Alignment of the *B. curculionis* sequences to the Campopleginae sp. reference sequence occurred Plus/Minus to positions 35 through 629. The reverse complement nucleotide sequences for the *B. curculionis* haplotypes were aligned with the Campopleginae sp. nucleotide sequence in MEGA 6 using ClustalW and then translated to protein using translation table 5, the invertebrate mitochondrial genetic code. Results were verified by comparing the resulting Campopleginae protein sequence to the sequence listed in the GenBank file.

Translation begins at position 1 of the reverse complementary nucleotide sequence, and results in a single open reading frame of 198 amino acids. Though 23 SNPs in the nucleotide sequences differentiate the five haplotypes, only two protein changes occur: in the Haplotype 3 sequence, protein 33 changes from alanine (A) to threonine (T); and in the sequences of

Haplotypes 2, 4 and 5, protein 165 changes from threonine to alanine. The protein sequence for Haplotype 1 is presented in Figure 4.6.

```
          10          20          30          40          50          60
           *           *           *           *           *           *
MIGSSMSLII RMELSNPGYL INNDQIYNSF VTAHAFIMIF FMVMPIMIGG FGNWLIPLML
          70          80          90         100         110         120
           *           *           *           *           *           *
GSPDMAFPRM NNMSFWLLPP SILLLILSSI TNQGVGTGWT VYPPLSLNVN HEGMSVDLAI
         130         140         150         160         170         180
           *           *           *           *           *           *
FSLHLAGMSS IMGAINFITT IFNMKNINKK FEQLTLFTWS IKITTILLLL AVPVLAGAIT
         190
           *
MLLTDRNLNT SFFDPSGG
```

Figure 4.6. COI gene protein sequence for *Bathyplectes curculionis* Haplotype 1.

4.3.6 *B. anurus* COI Gene

COI sequences were obtained for 99 *B. anurus* specimens; sequences were not obtained for specimens App BA, Aug BA1, Aug BA16, Roc BA 17, VT BA12, and CO BA1. Sequence strands averaged 679 nucleotides which, after editing and alignment, produced a consensus region of 597 base pairs for all sequences.

Analysis of the alignment for DNA sequence polymorphisms identified six variable sites, resulting in five homozygous haplotypes and two heterozygous haplotypes. Haplotype 1, the majority haplotype, was expressed by 66.7% of all specimens including 65 Virginia specimens (18 from the Piedmont, 18 from the Shenandoah Valley, 29 from the southwestern region) and one Colorado specimen. Haplotype 2, characterized by homozygous transversion of t→a at position 22, was expressed by 25.2% of specimens including 23 Virginia specimens (9 from the Piedmont, 12 from the Shenandoah Valley, 2 from the southwestern region) and two Colorado specimens. Haplotype 3, expressed only by Shenandoah Valley specimen Aug BA6, is

characterized by homozygous transversion of t→a at position 22 and homozygous transition of c→t at position 60. Haplotype 4, characterized by homozygous transition of c→t at position 93, was expressed only by two southwestern region specimens, Dub BA2 and Dub BA6. Haplotype 5, expressed by Piedmont specimen Bed BA1 and Shenandoah Valley specimen Rus BA10, is characterized by homozygous transition of a→g at position 538. Haplotype 6, expressed by Shenandoah Valley specimen Roc BA13, is characterized by homozygous transversion of t→a at position 22 and a transition of a→g at position 126 on the reverse strand only. Haplotype 7, expressed by Piedmont specimen Rus BA7 and Shenandoah Valley specimen Aug BA9, is characterized by transition of t→g at position 540 on the forward strand only. Figure 4.7 presents the COI nucleotide sequence for Haplotype 1.

```
    agggtcaaaa  aaagaagtat  ttagattacg  atcagttagg  agtatagtga   50
 51 tagctccagc  taatacagga  acagctaata  atagtaaaat  agctgtaatt  100
101 ttaattgatc  aagtaaataa  ggttaattgt  tcaaattttt  tattaatatt  150
151 ttttatatta  aaaattgttg  taataaaatt  aattgctcct  ataattgaag  200
201 atataccagc  aagatgtaaa  gaaaaaatag  ctaaatctac  tgatattcct  250
251 tcatggttga  tatttagaga  aagaggggag  taaacagttc  atcctgttcc  300
301 aactccttga  ttaataattg  atctaaaaat  taataataaa  attgaaggag  350
351 ggagtaatca  aaatcttata  ttatttattc  gaggaaatgc  tatatcagga  400
401 ctacctaata  ttaaaggaat  aagtcaattt  ccaaaaccac  caattataat  450
451 gggtataact  ataaaaaaaa  ttataataaa  ggcatgagct  gtaacaaaag  500
501 aattataaat  ttgatcatta  ttgattaaat  atcctggatt  tcttaattct  550
    attcgaataa  ttaatcttat  agatgatcca  attatgccag  ctcatat
551                                                          600
```

Figure 4.7. Sequence of a portion of the COI gene for *Bathyplectes anurus* Haplotype 1.

To determine if SNPs that differentiate the haplotypes result in protein coding changes, the *B. anurus* COI nucleotide sequences were translated to protein sequences by aligning the sequences to the GenBank Campopleginae sp. COI sequence, Accession No. JN300365, which occurs Plus/Minus from positions 23 through 599 of the reference sequence. The *B. anurus* reverse complement nucleotide sequences were aligned in MEGA 6.0 to the Campopleginae sp.

sequence using ClustalW, then translated to protein by the invertebrate mitochondrial genetic code (translation table 5). Translation was verified by comparison of the MEGA output to the protein sequence listed in the GenBank record for Campopleginae sp.

Translation begins at position 1 of the reverse complementary nucleotide sequence, and results in a single open reading frame of 199 amino acids. Three protein changes occur between the haplotype sequences: for Haplotype 3, a change in protein 180 from alanine (A) to threonine (T); for Haplotype 4, a change in protein 169 from alanine to threonine; and for Haplotype 7, a change in protein 20 from asparagine (N) to histidine (H) on the forward strand only. The protein sequence for Haplotype 1 is presented in Figure 4.8.

```
          10          20          30          40          50          60
           *           *           *           *           *           *
MWAGMIGSSM SLIIRMELSN PGYLINNDQI YNSFVTAHAF IMIFFMVMPI MIGGFGNWLI
          70          80          90         100         110         120
           *           *           *           *           *           *
PLMLGSPDMA FPRMNNMSFW LLPPSILLLI FSSIINQGVG TGWTVYPPLS LNINHEGMSV
         130         140         150         160         170         180
           *           *           *           *           *           *
DLAIFSLHLA GMSSIMGAIN FITTIFNMKN INKKFEQLTL FTWSIKITAI LLLLAVPVLA
         190
           *
GAITMLLTDR NLNTSFFDP
```

Figure 4.8. COI gene protein sequence for *Bathyplectes anurus* Haplotype 1.

4.3.7 Climate Analysis of *B. anurus* Haplotype Regions

For specimens from the southwestern region, 29 of 33 (88%) expressed the majority haplotype, accounting for 44.6% of Virginia Haplotype 1 expression; the Shenandoah Valley (18 of 32 specimens) and the Piedmont (18 of 31 specimens) each accounted for 27.7% of Virginia Haplotype 1 expression. Of the 23 Virginia specimens expressing Haplotype 2, 12 were collected in the Shenandoah Valley, nine were collected in the Piedmont, and two were collected

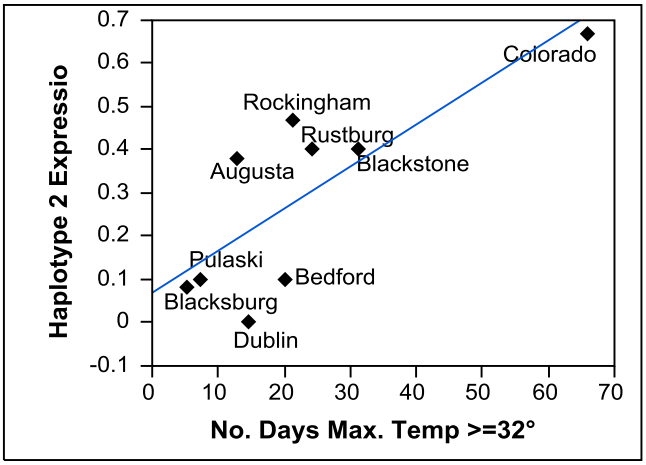
in the southwestern region, accounting for 52.2%, 39.1%, and 8.7% respectively of Haplotype 2 expression. Two of the three Colorado specimens for which sequences were obtained also expressed Haplotype 2. It is interesting to note that of the five remaining haplotypes, four were expressed by specimens from the Piedmont and Shenandoah Valley but not by specimens from the southwestern region, while one, Haplotype 4, was expressed only by specimens from the southwestern region, specifically Dublin. However, as each of these haplotypes was represented by only one or two specimens, population-level analyses of these haplotypes were not practical.

Averages for the climatic variables at each location for the reporting period 1957–2012 are shown in Table 4.2.

Table 4.2. Average annual temperature data by location for period 1957–2012.

Location	Mean Temp °C	Obs. Max Temp °C	Obs. Min Temp °C	No. Days Max≥32°C	No. Days Max≤0°C	No. Days Min≤0°C	No. Days Min>0°C	No. Days Min≤-18°C
Blacksburg	10.91	32.95	-18.59	5.3	18.4	128.0	237.0	2.1
Pulaski	11.61	33.53	-17.88	7.2	12.7	117.2	247.8	1.1
Dublin	11.90	34.57	-14.44	14.6	12.9	112.4	252.6	0.3
Rockingham	11.82	35.55	-18.37	21.3	14.8	117.4	247.6	1.7
Augusta	11.65	34.58	-17.72	12.9	14.5	115.3	249.7	1.6
Blackstone	13.83	36.15	-15.30	31.1	6.5	99.0	266.0	0.5
Rustburg	13.39	35.56	-15.52	24.4	8.8	91.8	273.2	0.5
Bedford	13.68	35.03	-14.37	20.2	6.4	78.4	286.6	0.3
Pueblo CO	11.39	39.53	-25.11	65.9	16.4	159.9	205.1	8.2

The results of the PCA showed that No. Days Min ≤ 0°C, No. Days Max ≥ 32°C and Obs. Max Temp, and No. Days Min ≤-18°C had the largest loading on PC1, PC2, and PC3, respectively. Because Days Max ≥ 32°C was highly correlated with Obs. Max Temp (r = 0.89), the decision was made to select only one of the two variables for the stepwise regression. The analysis showed that only Days Max ≥ 32°C was a significant predictor of Haplotype 2 expression (Figure 4.9).



Summary of Fit

RSquare	0.613729
RSquare Adj	0.558548
Root Mean Square Error	0.15032
Mean of Response	0.288889
Observations (or Sum Wgts)	9

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	0.25131537	0.251315	11.1220
Error	7	0.15817352	0.022596	**Prob > F**
C. Total	8	0.40948889		0.0125*

Figure 4.9. Regression analysis of Haplotype 2 Expression by No. Days Maximum Temperature ≥32°C.

The correlation between maximum temperature and regional expression of Haplotype 2 could indicate that genetic variation is occurring as an adaptive response to locations characterized by hot summers, a hypothesis that could be tested by a much larger sampling of *B. anurus* populations from different regions of the United States. A survey of the *Bathyplectes* in Egypt, Iran, and Iraq, the range of origin, found that *B. anurus* was most prevalent in regions having moderately warm summers and cold to very cold winters, such as the mountains of Iran, and was absent in the hot, arid regions of Egypt and Iraq that were more suitable to *B. curculionis* (Gonzalez et al., 1980).

4.3.8 Comparison of *B. anurus* and *B. curculionis* COI Sequences

The COI sequence for *B. anurus* Haplotype 1 was compared to the COI sequences for *B. curculionis* Haplotypes 1 and 2 to determine identity between the species. The *B. anurus* and *B. curculionis* nucleotide sequences were aligned in MEGA 6 and exported to Nexus (PAUP 4.0) format for analysis in DnaSP.

Identity between the nucleotide sequences of *B. anurus* and *B. curculionis* Haplotype 1 was 89.7%, with 60 DNA sequence polymorphisms identified. Identity between nucleotide sequences of *B. anurus* and *B. curculionis* Haplotype 2 was 89.1%, with 64 DNA sequence polymorphisms identified. This variability is sufficient that COI nucleotide sequences can be used to molecularly differentiate *B. anurus* and *B. curculionis*.

Though the nucleotide sequences are highly differentiated between the species, the protein sequences of *B. anurus* and *B. curculionis* are nearly identical in this gene region. Only three proteins separate *B. anurus* and *B. curculionis* Haplotype 1; four proteins separate *B. anurus* and *B. curculionis* Haplotype 2.

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CHAPTER 5

Summary

Bathyplectes anurus (Thomson) and *B. curculionis* (Thomson) (Hymenoptera: Ichneumonidae) are specialist parasitoids introduced throughout the United States for classical biological control of alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae). Alfalfa weevil, a key pest of alfalfa in many areas of the world, was first reported in Virginia in 1952 (Evans, 1959) in the three main alfalfa-growing regions of the state: the Piedmont, Shenandoah Valley, and southwestern region. In Virginia, *B. anurus* is the primary biological control agent, accounting for more than 90% of alfalfa weevil parasitization. A previous study by Kuhar et al. (1999), however, found that the levels of control achieved by *B. anurus* varied across the state, with significantly less parasitization occurring in the Piedmont. Little is known about the mechanisms or factors responsible for the differential levels of parasitization.

In general, factors that may affect parasitism levels in the field include, management and agronomic practices, such as insecticide use (Jiu and Waage, 1990); environmental factors e.g., temperature (Kraaijeveld and Godfray, 1999); host and/or parasitoid factors such as population levels, the searching efficiency and effectiveness of the parasitoid (functional response to host density); the extent of mutual interference among parasitoids, and the degree of heterogeneity (or pseudointerference) that renders some hosts more susceptible to parasitism than others (Hassell and Waage, 1984); and host and/or parasitoid genetics (Hufbauer et al., 2004). Insecticides may be applied to alfalfa fields in early spring to control alfalfa weevil. However, there is evidence which suggests that in some cases insecticides may have little or no effects on parasitism (e.g., Gerling and Naranjo, 1998), as appears to be the case with the parasitoid-alfalfa weevil interactions in Virginia (Los, 1982; Dellinger, 2003).

Climatic factors such as temperature can affect host distribution and the way a host responds to parasitoid attacks. The process of encapsulation by *Drosophila* spp. in response to parasitoid attack, for example, has been found to be more effective at higher temperatures (Kraaijeveld and Godfray, 1999). Climate is also considered to be a strong determinant of the distribution of the three strains (western, Egyptian, and eastern) of alfalfa weevil (Radcliffe and Flanders, 1998). With respect to Virginia, Kuhar et al. (2001) noted that winter temperatures in the Piedmont are about 2°C warmer than in the other two alfalfa-growing regions, and as a result, populations of alfalfa weevil larvae are higher and peak two to four weeks earlier than in the Shenandoah Valley and southwestern region. However, Kuhar et al. (2001) presented no evidence of the direct effects of temperature on alfalfa weevil-*B. anurus* interactions in Virginia. Climate is known to interact with other factors to influence ecological processes and to shape the dynamics of populations (Leirs et al., 1997). As such, although temperature may play a role in the differential levels of parasitism among the alfalfa-growing regions of Virginia, it is unlikely that this factor is acting alone, but is doing so in combination with other factors.

Among the factors that may affect parasitism, three were addressed in this study of alfalfa weevil-parasitoid interactions: population density and spatial distribution of host and parasitoid, the effectiveness of parasitoids in response to host density as measured by the population functional response, and genetic variation among and between populations of parasitoids.

Chapter 2

GPS-referenced quadrat sampling was conducted in alfalfa fields in the Piedmont and southwestern region to assess alfalfa weevil population levels and spatial distribution, and the level and distribution of parasitization by *B. anurus* and *B. curculionis*. Results showed that

there was no significant difference in alfalfa weevil densities between alfalfa fields in the Piedmont and southwestern region, and also no significant difference between regions in the level of parasitization. In all fields, the spatial distributions of alfalfa weevil larvae and parasitization were found to be aggregated. The population functional response of *B. anurus* attack on alfalfa weevil was determined qualitatively to be Type II in all fields except the Blacksburg field in the southwestern region. The Type II functional response suggests that, in general, the parasitoid has an inverse density dependent attack response to the density of alfalfa weevil larvae in the two regions of Virginia. The Type II functional response is reportedly the most commonly observed attack response for parasitoids that have been used successfully as biological control agents (Fernandez-Arhex and Corley, 2003). *B. anurus* was the dominant parasitoid in all regions, accounting for 97% of all parasitization. Larvae parasitized by *B. curculionis* were collected from Piedmont fields but not in the southwestern region fields.

Chapter 3

Molecular analyses were conducted of the 28S rDNA gene and the mitochondrial COI gene to 1) derive sequences for *B. anurus* and *B. curculionis* that could be used to molecularly identify and differentiate the two species, and 2) test for genetic variation among and between regional populations of *Bathyplectes*. The 28S rDNA gene for *B. anurus, B. curculionis,* and four taxa of hyperparasitoid were used to develop a diagnostic tool for molecular identification of *B. anurus* and *B. curculionis*, and to test DNA extracted from parasitoid cocoons for hyperparasitization. Most DNA utilized in molecular analyses was extracted from parasitoid cocoons, which are readily collected in alfalfa fields and easy to identify to species. *B. anurus* and *B. curculionis* are subject to hyperparasitism in the field, but parasitized cocoons cannot be

distinguished from nonparasitized cocoons. A diagnostic procedure was developed and tested to screen the DNA extracted from cocoons for the presence of hyperparasitoid DNA through restriction enzyme fragment pattern analysis of isolates of 28S rDNA.

Chapter 4

Sequences of the 28S rDNA gene region D2-D3 and for a portion of the mitochondrial gene cytochrome oxidase subunit 1 (COI) were derived for *B. anurus* and *B. curculionis* specimens collected from regional populations in Virginia and for specimens obtained from Colorado for outgroup comparisons. Identity between sequences obtained for Virginia and Colorado specimens of the same species was near 100%. The 28S rDNA sequences obtained for *B. curculionis* specimens from Virginia and Colorado did not match a *B. curculionis* sequence on file with GenBank for a German *B. curculionis* specimen. Through analysis of the 28S rDNA sequences, two adult specimens from a Colorado archive were found to have been incorrectly identified as *B. curculionis*; one specimen was determined to be *B. anurus* and the other specimen was determined to be a third *Bathyplectes* species, possibly *B. stenostigma*.

COI sequences for each species were analyzed for genetic variation among and between populations. Six variations of the COI sequence were detected in 33% of all *B. anurus* specimens in this study. These findings agree with the preliminary results obtained by Dellinger (2003) of genetic variability between populations of *B. anurus* in the Piedmont and southwestern region. Most variations were expressed by only one or two individuals; however, one sequence variation (Haplotype 2) was expressed by 25% of *B. anurus* individuals, primarily specimens collected from the Piedmont, Shenandoah Valley, southwestern region, and Colorado. Regression analysis of Haplotype 2 expression relative to climatological data for all regions

indicated a significant relationship (R^2 = 0.61; P = 0.013) between genetic variation in the COI sequences and the occurrence and duration of temperatures above 32°C. These findings are of particular interest because a survey by Gonzales et al. (1980) of *B. anurus* and *B. curculionis* distribution in Egypt, Iran, and Iraq, the range of origin of the two species, found that *B. anurus* was most prevalent in regions having moderately warm summers and cold to very cold winters, such as in the mountains of Iran, and was absent in the hot, arid regions of Egypt and Iraq that were more suitable to *B. curculionis*.

Analysis of the COI sequences for *B. curculionis* specimens identified two major sequence variations expressed by individuals collected from the same regions and fields in Virginia that might indicate incipient speciation.

Future Research

Behavioral functional response experiments should be conducted in the laboratory using *B. anurus* specimens collected from the different regions to measure attack rate and host handling time. The laboratory response can be compared with the population functional response that was qualitatively determined in this study.

Molecular analyses of *B. curculionis* specimens collected from regions of the United States and Europe in which *B. curculionis* is prevalent should be conducted to determine a) if *B. curculionis* in the United States are a single species that has diverged from the European *B. curculionis*, and b) if the two COI sequences obtained for Virginia specimens occur in *B. curculionis* populations of other regions in the United States; if incipient variation is occurring, is it a local phenomenon or widespread?

Molecular analyses of *B. anurus* specimens collected from a larger sampling of fields in Virginia and of *B. anurus* specimens collected from regions throughout the United States should be conducted to obtain a better estimate of the strength of the relationship between genetic variation observed in the COI sequences and regional climatic conditions, in particular, the occurrence and duration of high summer temperatures.

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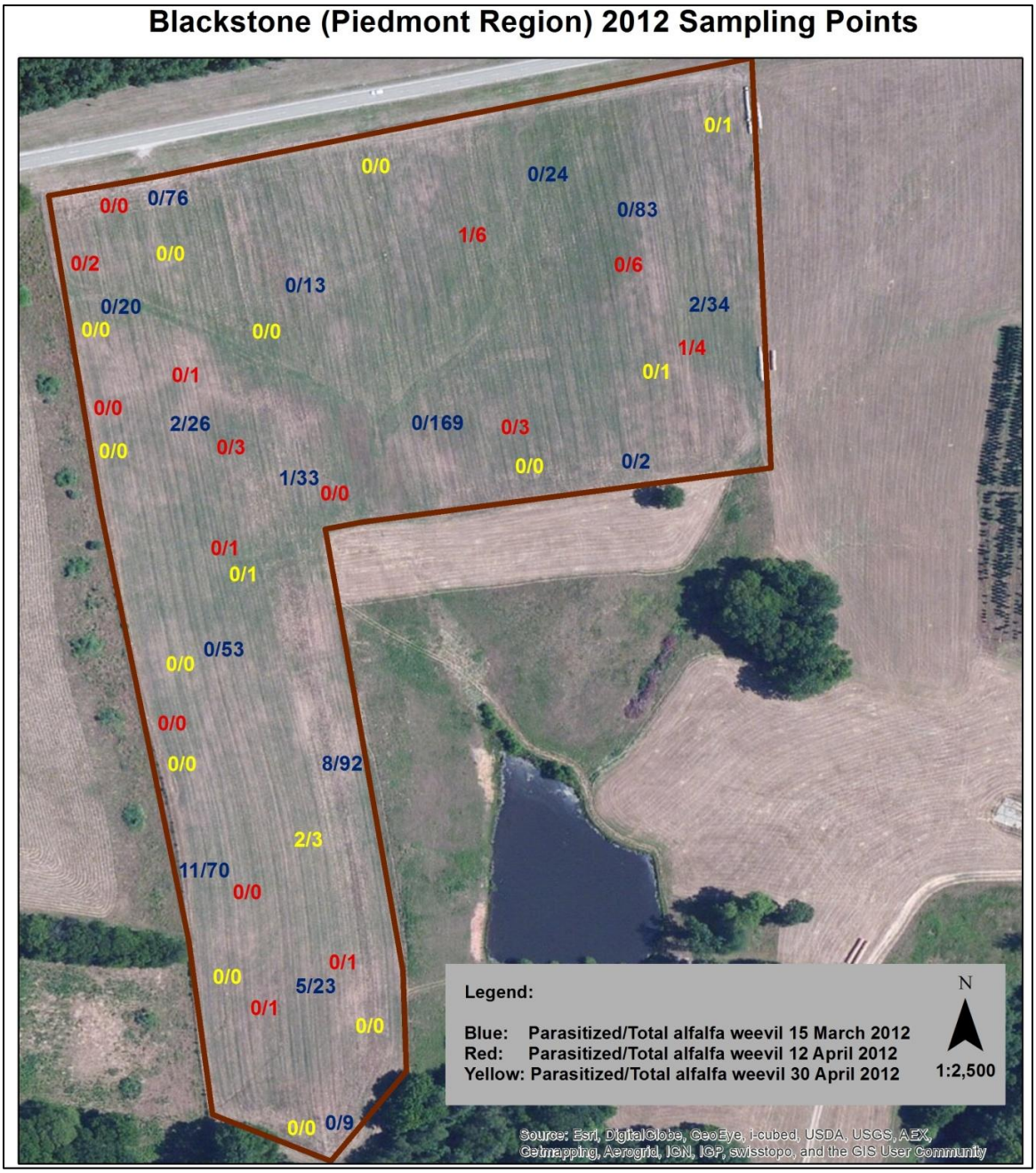
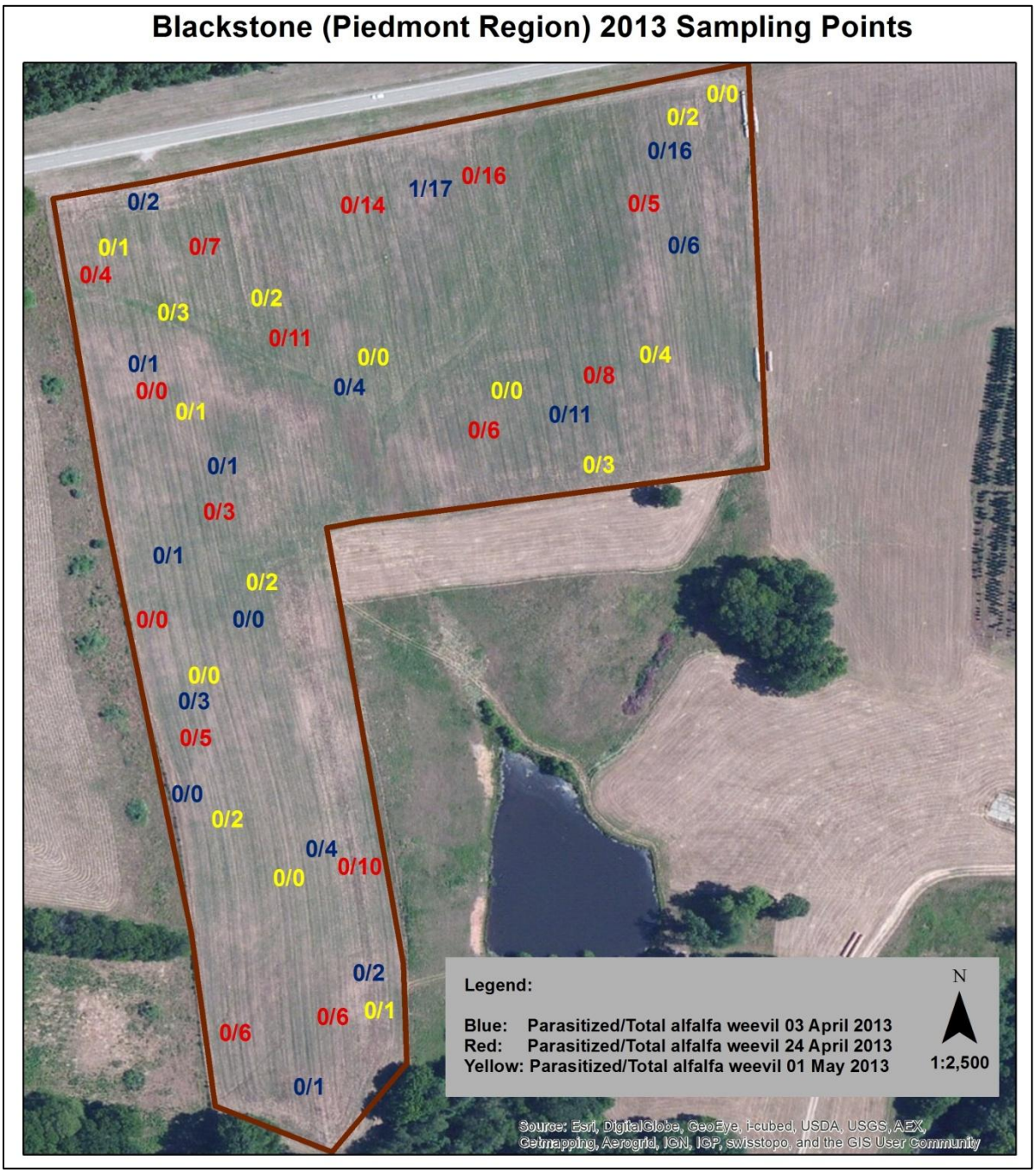
APPENDIX A

Field Maps of 2012 and 2013 Quadrat Sampling Points



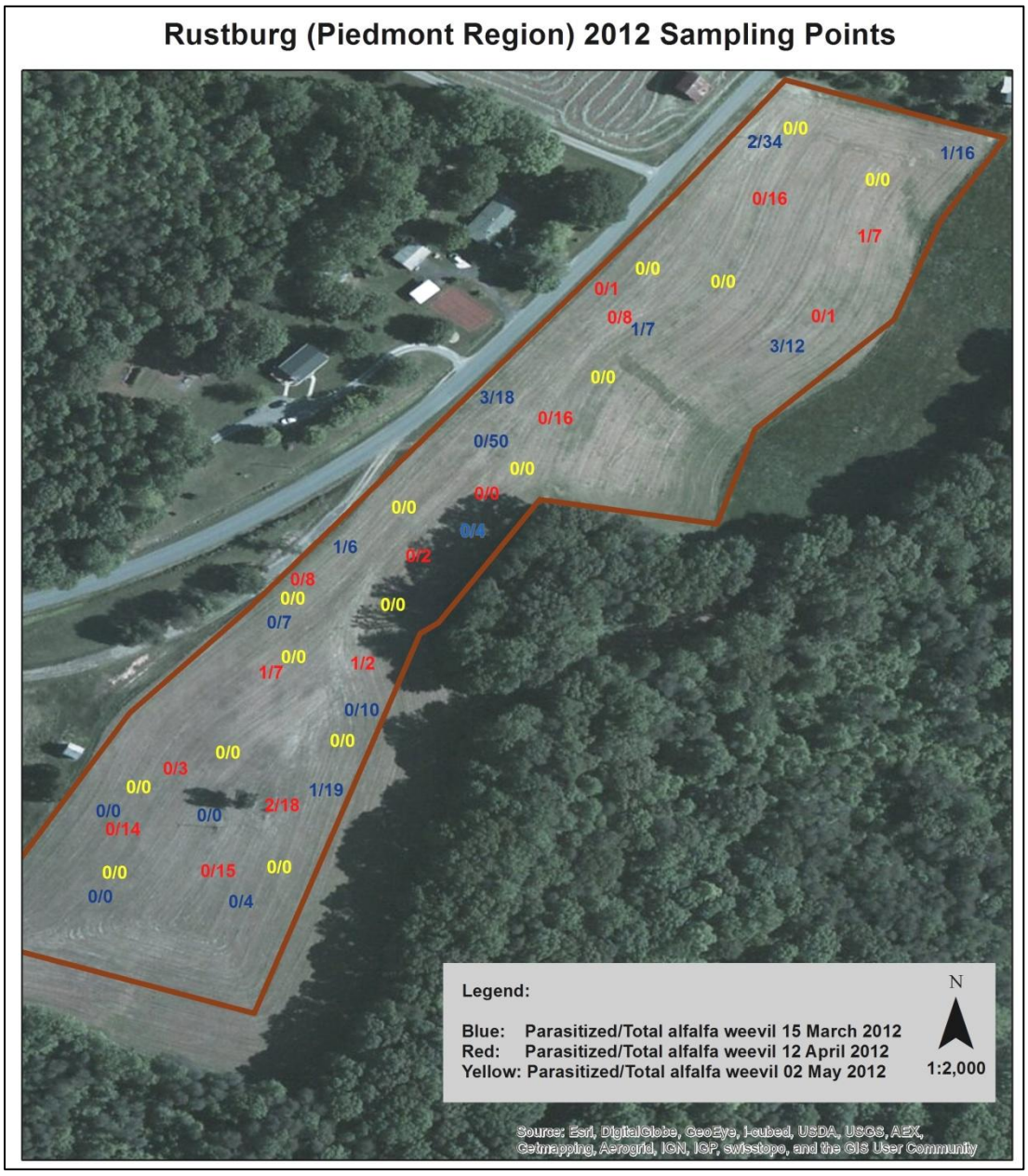
Map 1. Map of GPS-referenced points sampled with a 0.2-m^2 quadrat in the Blackstone field (Piedmont region) in Spring 2012. Labels indicate the ratio of larvae parasitized/number of larvae collected at each quadrat.

Imagery source: Esri et al., 2014. *http://goto.arcgisonline.com/maps/World_Imagery*.



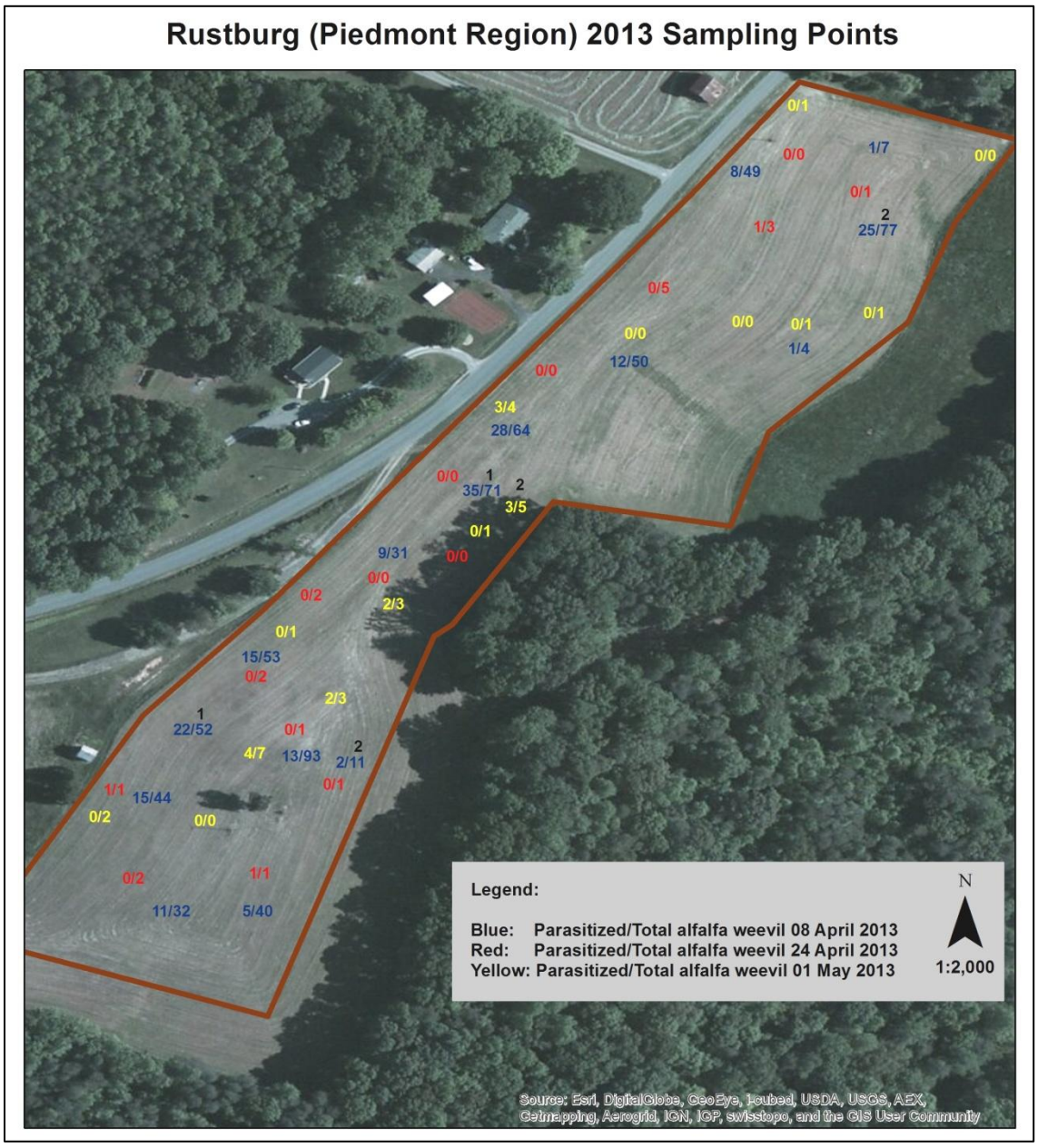
Map 2. Map of GPS-referenced points sampled with a 0.2-m^2 quadrat in the Blackstone field (Piedmont region) in Spring 2013. Labels indicate the ratio of larvae parasitized/number of larvae collected at each quadrat.

Imagery source: Esri et al., 2014. *http://goto.arcgisonline.com/maps/World_Imagery*.



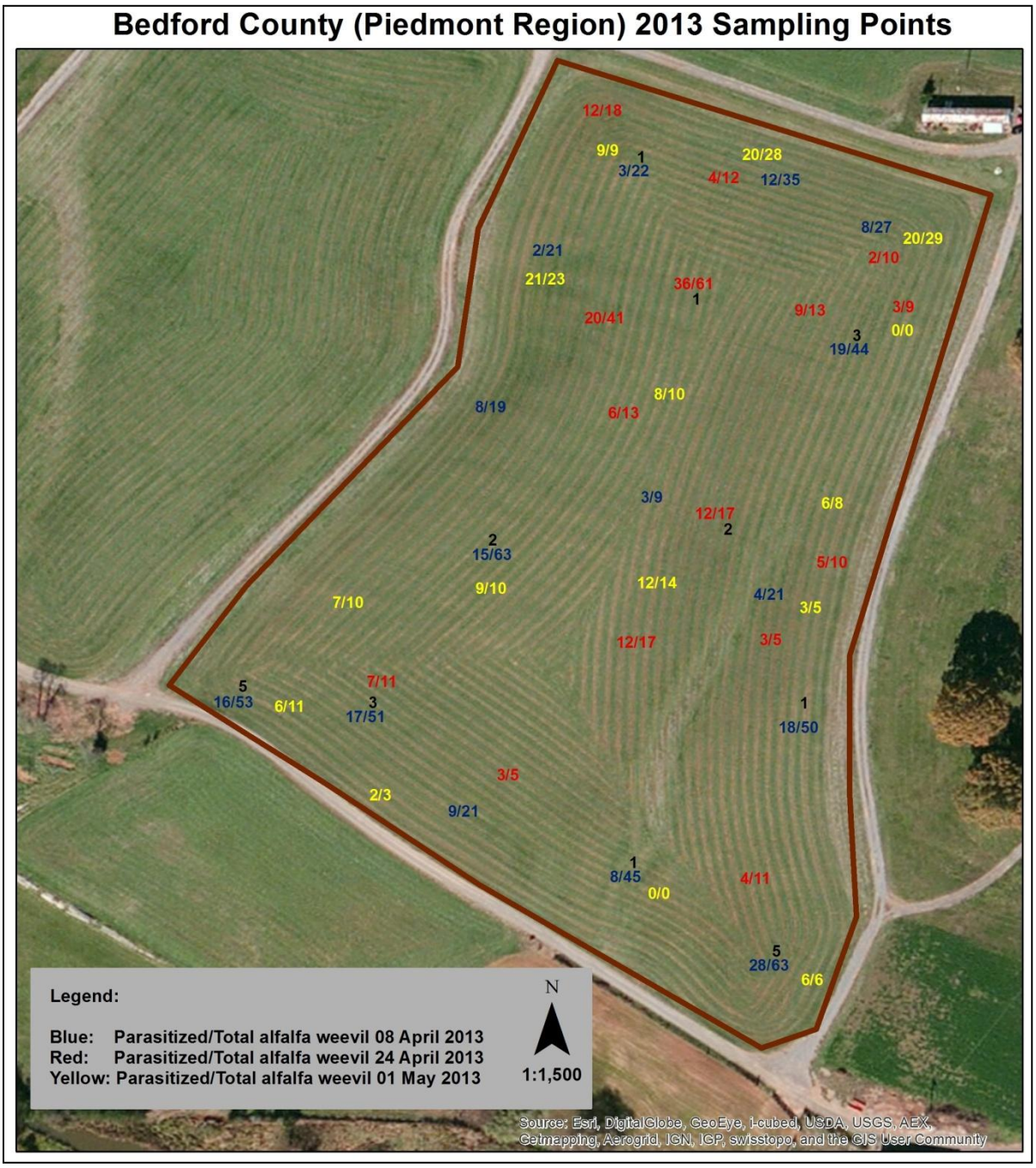
Map 3. Map of GPS-referenced points sampled with a 0.2-m^2 quadrat in the Rustburg field (Piedmont region) in Spring 2012. Labels indicate the ratio of larvae parasitized/number of larvae collected at each quadrat.

Imagery source: Esri et al., 2014. *http://goto.arcgisonline.com/maps/World_Imagery*.



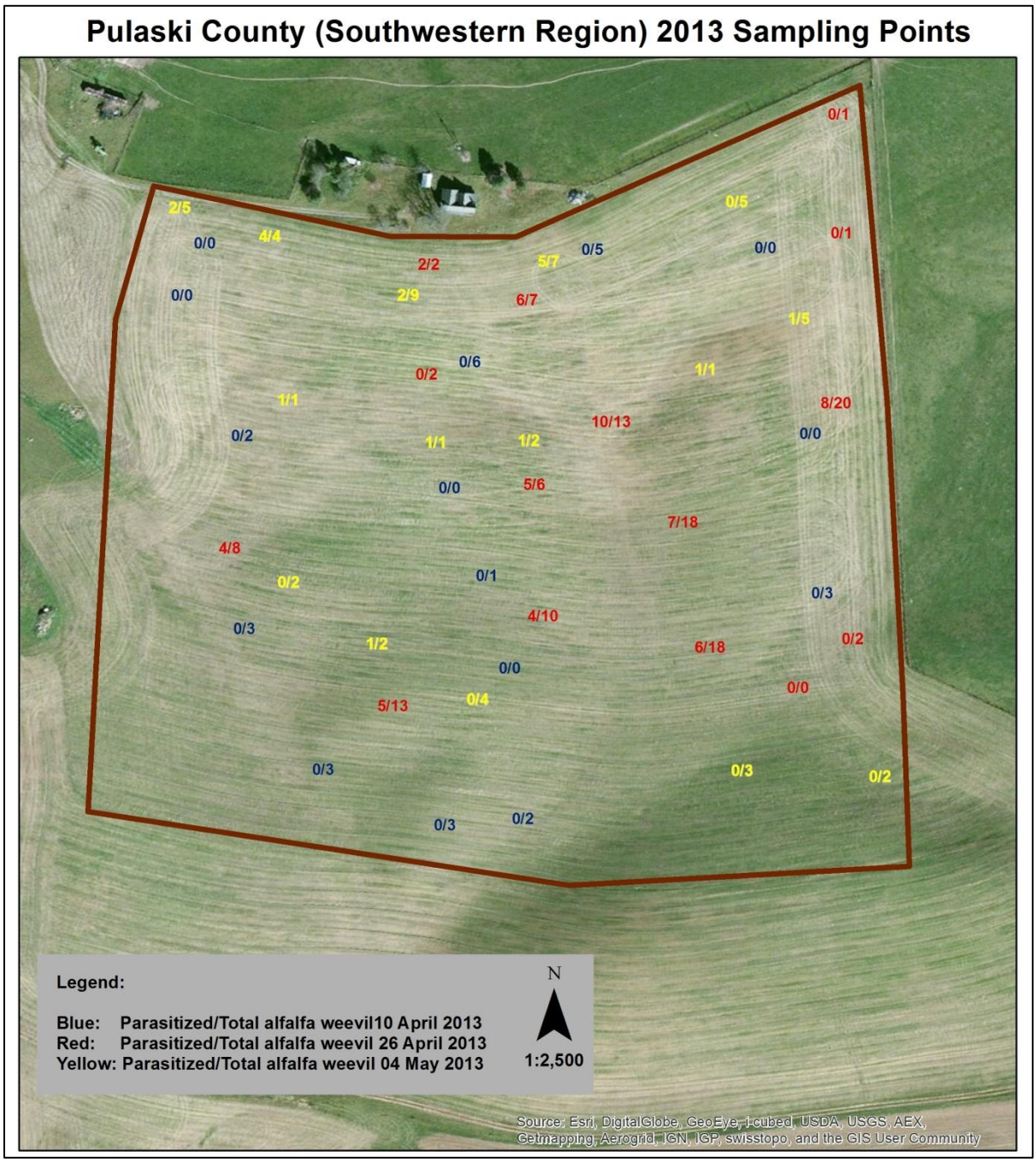
Map 4. Map of GPS-referenced points sampled with a 0.2-m^2 quadrat in the Rustburg field (Piedmont region) in Spring 2013. Labels indicate the ratio of larvae parasitized/number of larvae collected at each quadrat. Black labels indicate the number of larvae parasitized by *B. curculionis* obtained at locations within the field.

Imagery source: Esri et al., 2014. *http://goto.arcgisonline.com/maps/World_Imagery*.



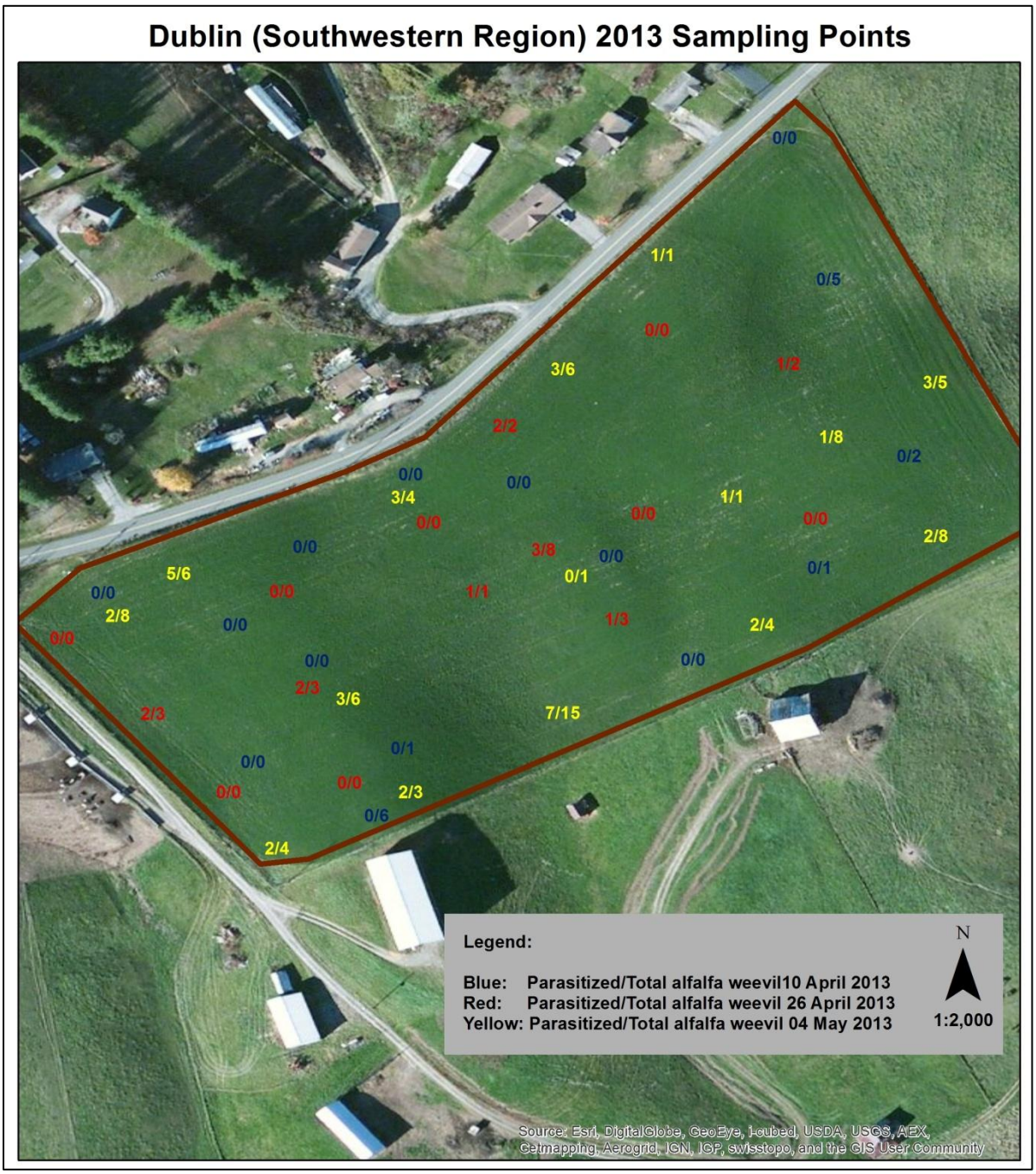
Map 5. Map of GPS-referenced points sampled with a 0.2-m^2 quadrat in the Bedford County field (Piedmont region), Spring 2013. Each point is labeled with the ratio of parasitized alfalfa weevil/total alfalfa weevil collected at that coordinate. Black labels indicate number of larvae parasitized by *B. curculionis* obtained at locations within the field.

Imagery source: Esri et al., 2014. *http://goto.arcgisonline.com/maps/World_Imagery*.



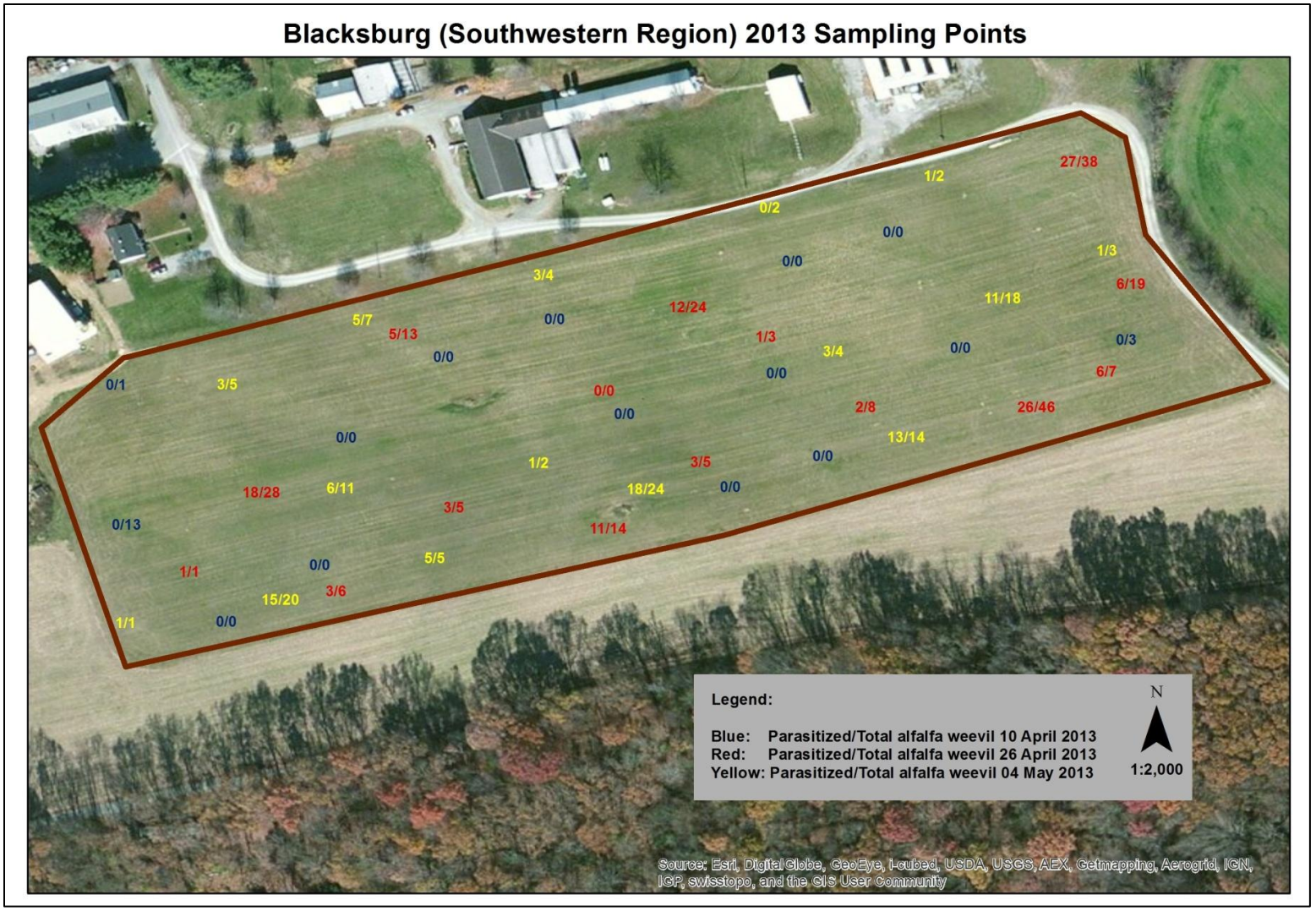
Map 6. Map of 45 GPS-referenced points sampled with a 0.2-m^2 quadrat in the Pulaski County field (southwestern region), Spring 2013. Each point is labeled with the ratio of parasitized alfalfa weevil/total alfalfa weevil collected at that coordinate.

Imagery source: Esri et al., 2014. *http://goto.arcgisonline.com/maps/World_Imagery*.



Map 7. Map of GPS-referenced points sampled with a 0.2-m^2 quadrat in the Dublin field (southwestern region), Spring 2013. Each point is labeled with the ratio of parasitized alfalfa weevil/total alfalfa weevil collected at that coordinate.

Imagery source: Esri et al., 2014. *http://goto.arcgisonline.com/maps/World_Imagery*.



Map 8. Map of GPS-referenced points sampled with a 0.2-m^2 quadrat in the Blacksburg field (southwestern region), Spring 2013. Each point is labeled with the ratio of parasitized alfalfa weevil/total alfalfa weevil collected at that coordinate.

Imagery source: Esri et al., 2014. *http://goto.arcgisonline.com/maps/World_Imagery*.