# CHARACTERIZING BILLBUG (*SPHENOPHORUS* SPP.) SEASONAL BIOLOGY USING DNA BARCODES AND A SIMPLE MORPHOMETRIC ANALYSIS

by

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*Dedicated to my family, Melba L. Soto Montañez, Melba G. Rodriguez, and Nery M. Rodriguez; three women without whom I wouldn't be here today.*

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# TABLE OF CONTENTS

# LIST OF TABLES



**Supplementary Tables**



**Appendix Table**

# LIST OF FIGURES

# ABSTRACT

Insect species complexes challenge entomologists in a variety of ways ranging from quarantine protection to pest management. Billbugs (Coleoptera: Curculionidae: *Sphenophorus* spp. Schönherr) represent one such species complex that has been problematic from a pest management perspective. These grass-feeding weevils reduce the aesthetic and functional qualities of turfgrass. Sixty-four species of billbugs are native to North America, and at least ten are associated with damage to turfgrass. Billbug species are sympatric in distribution and their species composition and seasonal biology varies regionally. Since their management relies heavily on proper choice of insecticide active ingredients and timing of insecticide applications that target specific life stages, understanding billbug seasonal biology underpins the development of efficient management programs. However, billbug seasonal biology investigations are currently hindered by our inability to identify the damaging larval stage to species level. DNA barcoding, which involves the use of short DNA sequences that are unique for each species, represents one potential tool that can aid these efforts. By combining DNA-based species identification with morphometric measures capable of serving as a proxy of larval development, it may be possible to gain a more holistic understanding of billbug seasonal biology. In this study, we developed a DNA barcoding reference library using cytochrome oxidase subunit 1 (COI) sequences from morphologically identified adult billbugs collected across Indiana, Missouri, Arizona, and Utah. Next, we applied our reference library for comparison and identification of unknown larval specimens collected across the growing season in Utah and Indiana. We then used a combination of DNA barcoding and larval head capsule diameters acquired from samples collected across a short span of the growing season to produce larval phenology maps. Adult billbug COI sequences varied within species, but the variation was not shaped by geography, indicating that this locus itself could resolve larval species identity. Overlaid with head capsule diameter data from specimens collected across the growing season, a better understanding of billbug species composition and seasonal biology emerged. This knowledge will provide researchers with the tools necessary to fill critical gaps in our understanding of billbug biology thereby improving turfgrass pest management. Using this approach researchers will be able to support efforts to provide growers with the information necessary to develop more prescriptive, location-based management programs and reduce the ecological footprint of turfgrass pest management.

# CHAPTER 1. DNA BARCODING OF BILLBUGS (*Sphenophorus* SPP.) TO TRACK THEIR SEASONAL BIOLOGY

## 1.1 Species Complexes

Insect species complexes present a myriad of challenges in entomology ranging from quarantine protection (Kunprom and Pramual 2019) to conservation of ecological diversity (Milić et al. 2018) and pest management (Ahrens et al. 2007; Hackett et al. 2000; Hassanzadeh-Avval et al. 2020; Li et al. 2020; Tyagi et al. 2017; Wang et al. 2019). Misidentifications stemming from the difficulty of distinguishing between closely related species can lead to accidental introductions and unmitigated spread of insect pests across borders, posing a serious risk for agriculture (Kunprom and Pramual 2019). Likewise, misidentifications can lead to the underestimation of insect diversity (Milić et al. 2018), creating uncertainty in conservation efforts aimed at protecting ecological diversity. In insect pest management, misidentification can stymie biological control efforts (Hassanzadeh-Avval et al. 2020) and reduce the effectiveness of management programs targeting plant and human diseases vectors (Hackett et al. 2000). The accurate distinction between pest species allows growers to more efficiently manage vectors of plant viruses using selective, species-targeted approaches (Tyagi et al. 2017) and may be vital in determining the risk of transferring insecticide resistance pathways across populations (Wang et al. 2019). Our ability to differentiate among closely related pest species underpins our understanding of host-plant associations (Li et al. 2020), supports the development of efficient IPM programs (Duffy et al. 2018) and may reduce prophylactic insecticide use by allowing growers to use more efficient, species-specific approaches that are facilitated through improved understanding of pest biology (De Meyer et al. 2015).

The challenges that emerge from misidentification of insect species in a species complex result from morphologically indistinguishable individuals. For example, adults of the virus-transmitting, plant-feeding wheat curl mite (*Aceria tosichella* Keifer) are morphologically identical but have enough genetic variation to be separated into distinct lineages (Skoracka et al. 2018). Some genetic lineages of *A. tosichella* are associated with unique host plant species and others show variation in the ability to transmit plant pathogens (Skoracka et al. 2018). Comprehending wheat curl mite-host associations is needed to provide a better assessment of the risk that this herbivorous pest poses to cereal crops worldwide. Similarly, the whitefly *Bemicia*

*tabaci* Gennadius is considered an insect species complex, and different lineage are separated by their geographic origin (Firdaus et al. 2013). Fruit fly species (*Ceratitis* spp.) from disjunct distributions show evidence of cryptic speciation (morphologically identical but distinct genetic species) in adults. Additionally, in this genus larval identification is challenging due to the high morphological intraspecific variation in this life stage (De Meyer et al. 2015).

To make the situation more complex, many pest species groups have indistinguishable larval stages (Doskocil et al. 2008; Duffy et al. 2018). For example, even though adult June beetles (*Phyllophaga* spp.) can be readily identified based on morphology, the morphological characters used to differentiate their soil-feeding larvae are relatively ambiguous (Doskocil et al. 2008). Likewise, beetle species in the genus *Cerambix* have wood-boring larvae that are morphologically indistinguishable (Torres-Vila et al. 2019). The ability to differentiate the damaging larval stage of these pests could provide crucial insights into their seasonal biology and distribution that advance the development of monitoring and management strategies.

## 1.2 Billbug Species Complex

Billbugs (Coleoptera: Curculionidae: *Sphenophoru*s spp. Schönherr) are a complex of grass-feeding weevils that have a completely indistinguishable larval stage (Duffy et al. 2018). Literature on the wide diversity of billbug species is limited, with only one revision of the genus done in 1951 (Vaurie 1951). There are approximately sixty-four native billbug species in North America (Dupuy and Ramirez 2016), associated with a variety of wild and cultured grasses and sedges. Billbug species collected in wild grasses include *S. pertinax* Olivier, *S. ludovicianus* Chittenden (Vaurie 1974), and *S. minimus* Hart (Smith and Golladay 2014). *S. maidis* Chittenden (Maas et al. 2003) and *S. striatopunctata* Goeze (Nardon et al. 2003) have been found in grasses that are grown for forage. However, there are other members of this group that are pests of commercial crops such as corn, sugarcane, rice, and wheat. *S. callosus* Olivier (Wright et al. 1982) has historically been a pest of corn, whereas *S. levis* Vaurie (Giron-Perez et al. 2009) and *S. incurrens* Gyllenhal (Illescas-Riquelme et al. 2016) are pests of sugarcane. *S. minimus* (Bouchard et al. 2005) can be found in marshes and turfgrass fields.

There are eleven turfgrass-feeding billbugs that have been documented to date (Dupuy and Ramirez 2016). The two most common turfgrass infesting billbug species are the bluegrass billbug

(*S. parvulus* Gyllenhal) and the hunting billbug (*S. venatus* Say). The former is mostly associated with cool-season grasses in the northern regions of the United States and the latter is mostly found in association warm-season grasses in the south and southeastern regions of the country. According to Vittum (1999), the phoenician billbug or phoenix billbug (*S. phoeniciensis* Chittenden) occurs mostly in the southwestern United States, whereas the rocky mountain billbug or Denver billbug (*S. cicatristriatus* Fåhraeus) is prominent in the Rocky Mountain region. The uneven billbug (*S. inaequalis* Say) is associated with warm- and cool-season grasses in the eastern part of the United States (Dupuy and Ramirez 2016), with high populations localized in different areas of Florida (Huang and Buss 2013). Similarly, the lesser billbug (*S. minimus* Hart) is present in eastern parts of the US. The remaining species of turfgrass infesting billbugs include the southern corn billbug (*S. callosus* Olivier), *S. apicalis* LeConte, *S. coesifrons* Gyllenhal, *S. rectus* Horn, and *S. cariosus* Olivier. These species can be found occurring sympatrically in the southeastern U.S., most notably in Florida (Dupuy and Ramirez 2016).

Even though some species have disjunct distributions, several species occur sympatrically, creating multi-species assemblages that vary regionally. Since larvae lack morphological characters for species identification, our understanding of the seasonal biology of each species is weak, which has constrained efforts to develop biologically-based management programs. The most widely distributed turfgrass infesting billbugs, present in almost every region of the US are the *S. venatus* and *S. parvulus*. More specifically, in Indiana there are four billbug species that are commonly associated with turfgrass: *S. parvulus*, *S. venatus*, *S. inaequalis*, and *S. minimus* (Duffy et al. 2018). However, in western states *S. parvulus*, *S. venatus*, *S. cicatristriatus*, and *S. phoeniciensis* typically dominate these assemblages (Dupuy and Ramirez 2016). One of the regions of the US with the highest diversity of billbug species is the southeast where *S. inaequalis*, *S. minimus*, *S. parvulus*, *S. venatus*, *S. apicalis*, and *S. coesifrons* have been reported (Johnson-Cicalese et al. 1990). As previously mentioned, billbugs are pests of several grass systems, but are arguably most notable and economically important in turfgrass). The fact that larvae are morphologically indistinguishable is a problem for developing effective monitoring and management programs. It is fundamental to be able to manage the larval stage effectively since it is the main cause of turfgrass damage.

## 1.3 Billbug Damage and Management in Turfgrass

Billbugs are problematic due to the aesthetic and functional damage they cause to turfgrass. The damage inflicted by these weevils is often overlooked or confused with drought, soil compaction and diseases, resulting in billbugs being the most misdiagnosed insect-related turfgrass disorder in North America (Richmond 2016). A hunting billbug report from 1956 indicated that damage initiates as a yellowing or browning of sections of sod that can be mistaken for fertilizer burn. However, if the grass can be removed easily, this is an indicator of billbug larval presence (Kelsheimer 1956). At first, the yellowing or browning of turfgrass appears to be spotty in distribution, but as damage progresses, it forms large, irregular patches that create an open canopy that is more suitable for weed colonization (Richmond 2016). Thus, failure to properly manage billbugs can cause perennial problems in turfgrass (Chong 2015), which often leads to increasing pesticide applications. This is an issue for turfgrass growers since commercial turf managers apply ca. 35,000 tons of pesticides each year to manage turfgrass pests (Thompson and Sorving 2008). As a result, turfgrass systems have attracted a considerable amount of negative attention (Potter et al. 1991).

Efficient billbug management relies heavily on proper timing of insecticide applications that target specific life stages (Tashiro and Personius 1970; Chong 2015) and one of three management approaches are typically employed: adult preventive, larval preventive or larval curative. Since billbugs overwinter as adults in the thatch, cracks and crevices in the soil, plant debris or nearby structures (Richmond 2016), the first management approach (adult preventive) targets these overwintering adults when they become active in the spring and before they begin laying eggs. The goal of this approach is to prevent oviposition thereby reducing subsequent larval infestations. If not controlled, adult billbugs chew on thicker and heavily thatched grass blades (Dupuy and Ramirez 2016) and stems, where females lay their eggs. Newly emerged legless billbug larvae bore into and feed within stems (Chong 2015), roots and crowns of cool- and warm-season grasses. Larvae spend part of their life cycle inside the stem and work their way down to the plant crown where they can kill the plant. The second approach (larval preventive) targets the larvae while they are feeding within the stem with the goal of this strategy being to reduce larval populations feeding within the plant before they cause visible damage. Once they reach the crown, billbug larvae separate the above-ground plant material from the roots causing desiccation and eventually plant death (Chong 2015). The larvae leave a fine sawdust-like excrement where they have fed heavily

(Vittum et al. 1999). Larvae then travel to the soil where they continue feeding on plant roots, eventually pupating and emerging as adults. The third approach (larval curative) targets the larvae during the relatively short period of time between reaching the soil and pupation with the goal of halting further damage and allowing the turfgrass to recover.

Having a sound understanding of seasonal biology is important for management, but like other insects, the seasonal biology of billbugs varies regionally due to environmental factors. *S. venatus*, which can overwinter as adults and larvae, in Indiana, South Carolina and North Carolina, has two adult activity peaks, while in Oregon it has one, and in Florida up to six (Chong 2015; Doskocil et al. 2012; Duffy et al. 2018; Huang and Buss 2009; Umble et al. 2005). In northeast Oregon, *S. cicatristriatus* reportedly has one generation per year, although adults and larvae are present during the entire year (Dupuy and Ramirez 2016). It is important to note that adult activity may not accurately portray seasonal biology since adult trapping only yields activity density, which can be subject to variation in local weather patterns. Although adult monitoring can lead to erroneous interpretations of seasonal biology, only a single study on the seasonal biology of this group of insects incorporated tracking of larval phenology throughout the growing season (Duffy et al. 2018). Thus, the seasonal biology of several billbug species has never been examined across wide swaths of North America.

This lack of knowledge on the seasonal biology of the billbug species complex in North America is due to our inability to identify the damaging larval stage to species level. To date, there are no morphological characters to differentiate billbug larvae. Without a dependable way to identify the larvae to species level, elucidation of seasonal biology is not possible. This is likely the main reason so many studies rely on measures of adult activity density instead of larval development. However, if employed correctly, modern molecular tools such as DNA barcoding could be leveraged to fill key gaps in our understanding of billbug seasonal biology.

### 1.4 Using DNA Barcoding to Resolve Species Complexes and Improve Pest Management

DNA barcoding is a tool that can be employed to assist in the identification of insect species complexes; it involves the use of short DNA sequences called barcodes that are unique for each species. The DNA sequence variation or nucleotide sequence differences within a species (intraspecific variation) and the variation among species (interspecific variation) are assessed in

order to assign each specimen to a known taxon. Successful species identification is achieved when there is minimal intraspecific variation, but comparatively high interspecific divergence, creating a "barcoding gap" (Jinbo et al. 2011; Hebert et al. 2004). A barcoding region ubiquitously used among scientific researchers for identification of animal species, including insects (Jinbo et al. 2011), is a 648-bp segment near the 5′ terminus of the mitochondrial cytochrome c oxidase subunit I (mtCOI) gene (Tyagi et al. 2017). The mtCOI gene is an effective barcoding region because it contains enough variation for accurate species delineations due to its high mutation rate that results from a reduced ability to repair errors efficiently during replication or DNA damage (i.e., evolves faster than other highly conserved DNA regions or genes) (Hoy 2013). To our advantage, mitochondrial DNA (mtDNA) also lacks introns (i.e. all the information is transcribed), has a limited exposure to recombination, and has a haploid mode of inheritance (Hebert et al. 2003) making it a relatively conserved region. In addition, mtCOI is also readily available in cells making it easier to amplify during polymerase chain reaction (PCR).

The mtCOI gene does however have several disadvantages for species identification, and in some cases may not be ideal for DNA barcoding (e.g. fungi and plants). One of the disadvantages of using mtCOI for identification that becomes relevant in sympatric species is introgression (transfer of genetic material from one species to another). Other challenges of using mtCOI are lack of recombination, its mode of maternal inheritance, inconsistent mutation rate, heteroplasmy (more than one type of mitochondrial DNA inherited that can cause discrepancies when sequencing), and the presence of nuclear mitochondrial pseudogenes (NUMTS) which are nonfunctional copies of mtDNA present in cell nuclei (Rubinoff et al. 2006). All of these drawbacks can cause a lack of resolution in identification during barcoding efforts. Due to these weaknesses in DNA barcoding, the use of mtCOI is most efficient when focusing on a limited set of species with known distributions and that have been previously identified using other criteria (Rubinoff et al. 2006; Jinbo et al. 2011).

Building a sound phylogenetic tree is sometimes difficult when approaches are focused on a single locus for species delimitation. When intraspecific variation is large, or when species have only recently evolved, members of the same species may not form a monophyletic clade (grouped together in the same clade of the tree). In such cases, additional DNA barcoding markers, often introns or intergenic/internal transcribed spacers (ITS's), or other nuclear ribosomal genes like

18s, can be used to improve resolution (Hebert et al. 2003; Fagan-Jeffries et al. 2019). However, finding suitable loci that easily amplify across a range of species/taxa can be challenging.

Even though there are some constraints in DNA barcoding, entomologists use this method to answer a wide range of questions. For example, DNA barcoding methods have been used to identify and differentiate between *Thrips tabaci* Lindeman biotypes (Farkas et al. 2020), which is important for understanding the composition of sympatric pest populations and devising management schemes. Other studies have used the technique to develop preliminary phylogenies and to study phylogeography. For example, Ros and Breeuwer (2007) found three clades in the family Tetranychidae and could not find evidence of phylogeography, but concluded other barcodes are needed in addition to COI. Moreover, Yeo et al. (2018) was able to document the biodiversity of Odonata after identifying 1123 barcodes for adults and morphologically distinct larvae utilizing next generation sequencing of COI barcodes. Barcoding techniques have also been used to elucidate parasitoid-host relationships (Zhou et al. 2014; Dong et al. 2018). DNA-based techniques are effective for the *Anopheles* mosquito complex (Scott et al. 1993, Hackett et al. 2000), the *Rhodnius* species complex (Pavan and Monteiro 2007), June beetle complex (Doskocil et al. 2008) and some billbug species (Duffy et al. 2018). Application of DNA barcoding techniques for larval identification is even more relevant when there are no morphological traits to distinguish larvae and they are difficult to rear making accurate associations between life stages challenging (Ahrens et al. 2007).

## 1.5 Understanding the Billbug Species complex using DNA Barcoding to Inform management

Insect species complexes cause challenges in entomology due to difficulties with identification that can misinform pest management. Uncertainty in identification of insects occurring in species complexes can be compounded by the fact that some insects have cryptic adult or larval stages. Billbugs are one example of a complex of multiple species that are major pests of turfgrass and possess a cryptic larval stage. Due to our inability to identify billbug larvae to species level, the seasonal biology of this group has only partially been resolved. As mentioned before, Duffy et al. (2018) assessed the seasonal biology of the billbug species complex in Indiana, but in other western and southern regions of the US, this information is unavailable. Since billbug species

composition and seasonal biology varies by region, the development of more efficient, prescriptive management programs has been stagnant. The development of a molecular tool involving DNA barcoding is critical to identify cryptic larval specimens and trace the seasonal biology of billbugs. In order for this tool to elucidate the seasonal biology of the billbug species complex, it must be flexible enough to use on a large geographic scale and robust enough to avoid some of the more common problems associated with DNA barcoding. Development of a DNA barcoding approach for billbug larval identification will therefore require a comprehensive molecular database to compare the diversity among individuals across the species range. Creating a reference database from morphologically distinct adults is a necessary first step in determining the effectiveness of using DNA barcoding in this system.

This research is aimed at unraveling the seasonal biology of billbugs species across a broad geographic range in the US. To do this we used DNA barcoding for species identification in combination with a simple morphometric measure (head capsule width). The combination of species identification with a surrogate measure of larval development should provide a clear picture of the seasonal biology of the billbug species complex that may be employed on a regional basis. Eventually, information gathered through this approach will translate to extension materials that growers can use to develop more prescriptive, location-based management programs and reduce the ecological footprint of turfgrass pest management.

## 1.6 References


**Ahrens, D., M. T. Monaghan, and A. P. Vogler. 2007.** DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). Mol. Phylogenet. Evol. 44: 436–449.

**Bouchard, P., L. Lessage, H. Goulet, N. J. Bostanian, C. Vincent, A. Zmudzinska, and J. Lasnier. 2005.** Weevil (Coleoptera: Curculionoidea) diversity and abundance in two Quebec vineyards. Ann. Entomol. Soc. Am. 98: 565–574.

**Chong, J-H. 2015.** Species richness and seasonal abundance of billbugs, *Sphenophorus* spp., from South Carolina golf courses. J. Agric. Urban Entomol. 31: 20–28.

**Girón-Pérez, K., O. Nakano, A. C. Silva, and M. Oda-Souza. 2009.** Atração de adultos de *Sphenophorus levis* vaurie (Coleoptera: Curculionidae) a fragmentos vegetais em diferentes estados de conservação. Neotrop. Entomol. 38: 842–846.

**De Meyer, M., H. Delatte, S. Ekesi, K. Jordaens, B. Kalinova, A. Manrakhan, M. Mwatawala, G. Steck, J. Van Cann, L. Vaníčková, R. Břízová, and M. Virgilio. 2015.** An integrative approach to unravel the *Ceratitis* FAR (Diptera, Tephritidae) cryptic species complex: A review. ZooKeys. 540: 405–427.

**Dong, Z., S. Liu, and Z. Zhang. 2018.** Efficacy of using DNA barcoding to identify parasitoid wasps of the melon-cotton aphid (*Aphis gossypii*) in watermelon cropping system. BioControl 63: 677–685.

**Doskocil, J. P., N. R. Walker, G. E. Bell, S. M. Marek, J. A. Reinert, and T. A. Royer. 2008.** Species composition and seasonal occurrence of *Phyllophaga* (Coleoptera: Scarabeidae) infesting intensely managed bermudagrass in Oklahoma. J. Econ. Entom. 101: 1624–1632.

**Duffy, A. G., G. S. Powell, J. M. Zaspel, and D. S. Richmond. 2018.** Billbug (Coleoptera: Dryophthoridae: *Sphenophorus* spp.) seasonal biology and DNA-based life stage association in Indiana turfgrass. J. Econ. Entomol. 111: 304–313.

**Dupuy, M., and R. A. Ramirez. 2016.** Biology and management of billbugs (Coleoptera: Curculionidae) in turfgrass. J. Integr. Pest Manag. 7: 1–10.

**Fagan-Jeffries, E.P., S. J. B. Cooper, T. M. Bradford, and A. D. Dustin. 2019.** Intragenomic internal transcribed spacer 2 variation in a genus of parasitoid wasps (Hymenoptera: Braconidae): implications for accurate species delimitation and phylogenetic analysis. Insect Mol. Biol. 28: 485–498.

**Farkas, P., Z. György, A. Tóth, A. Sojnóczki, and J. Fail. 2020.** A simple molecular identification method of the *Thrips tabaci* (Thysanoptera: Thripidae) cryptic species complex. Bull. Entomol. Res. 110: 397–405.

**Firdaus, S., B. Vosman, N. Hidayati, E. D. Jaya Supena, R. G. F. Visser and A. W. Van Heusden. 2013.** The *Bemisia tabaci* species complex: Additions from different parts of the world. Insect Sci. 20: 723–733.

**Hackett B. J., J. Gimnig, W. Guelbeogo, C. Costantini, L. L. Koekemoer, M. Coetzee, F. H. Collins, and N. J. Besansky. 2000.** Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *A. rivulorum*, and uncover a cryptic taxon. Ins. Mol. Biol. 9: 369–374.

**Hassanzadeh-Avval, M., H. Sadeghi-Namaghi, and L. Fekrat. 2020.** Molecular and morphological identification of *Anthocoris* spp. (Hemiptera: Anthocoridae) predators of three economically important psyllid species in Razavi Khorasan province, Northeastern Iran. Biologia 75: 1667–1677.

**Hebert P. D., E. H. Penton, J. M. Burns, D. H. Janzen, and W. Hallwachs. 2004.** Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc. Natl. Acad. Sci. USA 101: 14812–7.

**Hebert, P. D. N., S. Ratnasingham, and J. R. de Waard. 2003.** Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B Biol. Sci. 270: 96–S99.

**Hoy, M. 2013.** Insect molecular genetics: An introduction to principles and applications, 3rd ed. Academic Press, Waltham, MA.

**Huang, T. I., and E. A. Buss**. **2013**. Abundance and population dynamics of adult *Sphenophorus inaequalis* (Coleoptera: Curculionidae) in Florida. Florida Entomol. 96: 1609–1611.

**Huang, T., and E. A. Buss. 2009.** Billbug (Coleoptera: Curculionidae) species composition, abundance, seasonal activity, and developmental time in Florida. J. Econ. Entomol. 102: 309–314.

**Illescas-Riquelme, C. P., C. Llanderal-Cázares, C. Ruiz-Montiel, H. González-Hernández, R. Alotarre-Rosas, L. Cruz-Lopez, and J. C. Rojas. 2016.** Evidence for male-produced aggregation pheromone in *Sphenophorus incurrens* (Coleoptera: Curculionidae). Fla. Entomol. 99: 522–527.

**Jinbo, U., T. Kato, and M. Ito. 2011.** Current progress in DNA barcoding and future implications for entomology. Entomol. Sci. 14: 107–124.

**Johnson-Cicalese, J. M., G. W. Wolfe, and C. R. Funk. 1990.** Biology, distribution, and taxonomy of billbug turf pests (Coleoptera: Curculionidae). Entomol. Soc. Am. 19: 1037–1046.

**Kelsheimer, E. G. 1956.** The hunting billbug a serious pest of zoysiza. Proc. Annu. Meet. Fla. State Hortic. Soc. 415–418.

**Kunprom, C., and P. Pramual. 2019.** DNA barcoding of fruit flies (Diptera: Tephritidae) in Thailand: ambiguity, misidentification and cryptic diversity. Mitochondrial DNA A. 30: 861–873.

**Li, Q., J. Deng, C. Chen, L. Zeng, X. Lin, Z. Cheng, G. Qiao, and X. Huang. 2020.** DNA barcoding subtropical aphids and implications for population differentiation. Insects. 11: 11–28.

**Maas, D. L., T. L. Springer, and D. C. Arnold. 2003.** Occurrence of the maize billbug, *Sphenophorus maidis* in eastern gamagrass. Southwest. Entomol. 28: 151–152.

**Meyer, C. P., and G. Paulay. 2005.** DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 3: e422.

**Milić, D., S. Radenković, J. Ačanski, and A. Vujić. 2018.** The importance of hidden diversity for insect conservation: a case study in hoverflies (the *Merodon atratus* complex, Syrphidae, Diptera). J. Insect. Conserv. 23: 29–44.

**Nardon, P., H. Charles, B. Delobel, C. Lefevre, and A. Heddi. 2003.** Symbiosis in the Dryophthoridae weevils (Coleoptera, Curculionoidea): Morphological variability of symbiotic intracellular bacteria. Symbiosis. 34: 231–251.

**Pavan, M. G., and F. A. Monteiro. 2007.** A multiplex PCR assay that separates *Rhodnius prolixus* from members of the *Rhodnius robustus* cryptic species complex (Hemiptera: Reduviidae). Trop. Med. Int. Health. 12: 751–758.

**Potter, D. A., and S. K. Braman. 1991.** Ecology and management of turfgrass insects. Annu. Rev. of Entomol. 36: 383–406.

**Ros, V. I. D., and J. A. J. Breeuwer. 2007.** Spider mite (Acari: Tetranychidae) mitochondrial COI phylogeny reviewed: Host plant relationships, phylogeography, reproductive parasites and barcoding. Exp. Appl. Acarol. 42: 239–262.

**Richmond, D. S. 2016.** Managing billbugs in turfgrass. https://extension.entm.purdue.edu/publications/E-266/E-266.html.

**Rubinoff, D., S. Cameron, and K. Will. 2006.** A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. J. Hered. 97: 581–594.

**Scott, J. A., W. G Brogdon, and F. H. Collins. 1993.** Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am. J. Trop Med. Hyg 49: 520–529.

**Skoracka, A. L. F. Lopes, M. J. Alves, A. Miller, M. Lewandowski, W. Szydlo, A. Majer, E. Rozanska and L. Kuczynski. 2018.** Genetics of lineage diversification and the evolution of host usage in the economically important wheat curl mite, *Aceria tosichella* Keifer, 1969. BMC Evol. Biol. 18: 122–136.

**Smith, N., and S. Golloday. 2014.** Curculionidae (Coleoptera) species within geographically isolated wetlands of the Gulf Coastal Plain in Southwestern Georgia. T. Am. Entomol. Soc. 140: 237–244.

**Tashiro, H., and K. E. Personius. 1970.** Current status of the bluegrass billbug and its control in Western New York home lawns. J. Econ. Entomol. 63: 23–29.

**Thompson, J. W., and K. Sorving. 2008.** Sustainable landscape construction. 2nd ed. Island Press, DC.

**Torres-Vila, L. M., and R. Bonal. 2019.** DNA barcoding of large oak-living cerambycids: diagnostic tool, phylogenetic insights and natural hybridization between *Cerambyx cerdo* and *Cerambyx welensii* (Coleoptera: Cerambycidae). Bull. Entomol. Res. 109: 583–594.

**Tyagi, K., V. Kumar, D. Singha, K. Chandra, B. A. Laskar, R. Chakraborty, and S. Chatterjee. 2017.** DNA barcoding studies on thrips in India: Cryptic species and species complexes. Sci. Rep. 7: 4898.

**Umble, J., G. Fisher, and S. Rao. 2005.** Sampling methods and seasonal phenology of *Sphenophorus venatus* confluens Chittenden (Coleoptera: Curculionidae) in orchardgrass (*Dactylis glomerata* L.). J. Agr. Urban Entomol. 22: 79–85.

**Vaurie, P. 1951.** Revision of the genus *Calendra* (formerly *Sphenophorus*) in the United States and Mexico (Coleoptera: Curculionidae). Bull. Am. Mus. Nat. Hist. 93: 33–186.

**Vaurie P. 1974.** Synonymy in *Sphenophorus pertinax* Olivier (Coleoptera, Curculionidae, Rhynchophorinae). J. N. Y. Entomol. Soc. 82: 14.

**Vittum P., M. Villani, and H. Tashiro. 1999.** Turfgrass insects of the United States and Canada. Cornell University Press, Ithaca, NY.

**Wang, Y., C. Huang, B. Hu, Y. Liu, G. H. Walter, and J. P. Hereward. 2019.** Gene flow across host-associated populations of the ricestem borer *Chilo suppressalis* Walker (Lepidoptera; Crambidae): implications for Bt resistance management in rice. Pest Manag Sci. 76: 695–703.

**Wright R. J., J. W. Van Duyn, and J. R. Bradley, Jr. 1982.** Host range of southern corn billbug (Coleoptera: Curculionidae) adults and larvae. Environ. Entomol. 11: 954–957.

**Yeo, D., J. Puniamoorthy, R. W. J. Ngiam, and R. Meier. 2018.** Towards holomorphology in entomology: rapid and cost-effective adult-larva matching using NGS barcodes. Syst. Entomol. 43: 678–691.

**Zhou, Q., Y. Xi, F. Yu, X. Zhang, X. Li, C. Liu, Z. Niu, C. Zhu, G. Qiao, and Y. Zhang. 2014.** Application of DNA barcoding to the identification of Hymenoptera parasitoids from the soybean aphid (*Aphis glycines*) in China. Insect Science. 21: 363–373.

# CHAPTER 2. CHARACTERIZING BILLBUG (*Sphenophorus* SPP.) SEASONAL BIOLOGY USING DNA BARCODES AND A SIMPLE MORPHOMETRIC ANALYSIS

## 2.1 Abstract

Billbugs (*Sphenophorus* spp.) are a complex of grass-feeding weevil species that reduce the aesthetic and functional qualities of turfgrass. Effective billbug monitoring and management programs rely on a clear understanding of their seasonal biology. However, our limited understanding of regional variation in the species compositions and seasonal biology of billbugs, stemming primarily from our inability to identify the damaging larval stage to species level, has hindered efforts to articulate efficient IPM strategies to growers. We used a combination of DNA barcoding methods and morphometric measures to begin filling critical gaps in our understanding of the seasonal biology of the billbug species complex across a broad geographic range. First, we developed a DNA barcoding reference library using cytochrome oxidase subunit 1 (COI) sequences from morphologically identified adult billbugs collected across Indiana, Missouri, Utah and Arizona. Next, we used our reference library for comparison and identification of unknown larval specimens collected across the growing season in Utah and Indiana. Finally, we combined our DNA barcoding approach with larval head capsule diameter, a proxy for developmental instar, to develop larval phenology maps. Adult COI sequences varied among billbug species, but variation was not influenced by geography, indicating that this locus alone was useful for resolving larval species identity. Overlaid with head capsule diameter data from specimens collected across the growing season, a better visualization of billbug species composition and seasonal biology emerged. This approach will provide researchers with the tools necessary to fill critical gaps in our understanding of billbug biology thereby improving the development of turfgrass pest management programs.

## 2.2 Introduction

In applied entomology, delimiting species complexes derived from different types of speciation (i.e., sympatric or cryptic) is fundamental to insect biological research (Feder and Filchak 1999; Berlocher and Feder 2002; Farkas et al 2020). Species complexes, composed of a

group of closely related species, often lack morphological characters for species identification, which can lead to shortcomings in our understanding of a specific insect's biology and impede the development of new strategies to manage pests (Ahrens et al. 2007; Hackett et al. 2000; Hassanzadeh-Avval et al. 2020; Li et al. 2020; Tyagi et al. 2017; Wang et al. 2019). Misidentification of pest species can reduce the effectiveness of management programs targeting plant and human diseases vectors (Hackett et al. 2000; Tyagi et al. 2017), slow the advancement of biological control efforts (Hassanzadeh-Avval et al. 2020) and potentially increase grower dependency on the prophylactic use of insecticides. Conversely, proper identification of pests that occur in species complexes can facilitate the understanding of pest biology (De Meyer et al. 2015) necessary to optimize management efforts (Ahrens et al. 2007) and allow growers to implement more judicious insecticide use (Doskocil et al. 2008).

One such species complex, whose biology is sporadically understood across broad swaths of the United States, is the billbug complex, a group of grass feeding weevils (Coleoptera: Curculionidae: *Sphenophorus* spp. Schönherr). Sixty-four described billbug species are native to North America, and although adults can be identified based on morphological characteristics (Johnson-Cicalese et al. 1990), the larvae are morphologically indistinguishable. Our inability to accurately identify the cryptic, soil-dwelling larval stage of these weevils has limited our capacity to characterize the seasonal biology of the eleven turfgrass feeding species. Because the seasonal biology of most species is poorly understood, our ability to effectively manage these pests on golf courses, athletic fields, home lawns and other turfgrass ecosystems, has been problematic. Billbug larvae feed within the stems, roots and crowns of turfgrasses causing desiccation and plant death, markedly reducing the aesthetic and functional quality of managed turfgrass (Chong 2015). Currently, growers are faced with the challenge of managing these pests without a sound understanding of regional variation in pest species composition and seasonal biology. As a result, they often resort to prophylactic use of insecticides, with variable outcomes.

Efficient billbug management relies heavily on proper timing of synthetic insecticides or biological control approaches that often vary in their efficacy against specific life stages (Georgis et al. 2006, Richmond 2016). These approaches, and the number of synthetic insecticide applications necessary for satisfactory control also vary depending on the species present and the seasonal biology of each within a particular region. Because of the sympatric distribution of several billbug species, our patchy understanding of their seasonal biology can make satisfactory

management difficult to achieve in many regions. In Indiana there are four billbug species that are commonly associated with turfgrass (*S. parvulus* Gyllenhal, *S. venatus* Say, *S. minimus* Hart, and *S. inaequalis* Say), with the two most common species (*S. parvulus* and *S. venatus*) having very different life history strategies. While *S. parvulus* overwinters in Indiana in the adult stage, *S. venatus* overwinters in both the adult and larval stage (Duffy et al. 2018). In Utah, three species routinely infest turfgrass (*S. parvulus*, *S. venatus* and *S. cicatristriatu*s Fåhraeus) and although the adults of each species are active from February to October, their larval phenology has not been documented in this region (Dupuy and Ramirez 2016). Further, the most widely studied species, *S. venatus*, displays a highly flexible seasonal biology that appears to vary regionally in the number of generations produced each year (1-6) and potentially the life stage structure or demographics of the overwintering cohort (Chong 2015; Doskocil and Brandenburg 2012; Duffy et al. 2018; Huang and Buss 2009; Umble et al. 2005). As such, holistic, regionally appropriate management strategies have been difficult to articulate. The development of a reliable approach for distinguishing billbug larval species identity would assist regional efforts to characterize billbug seasonal biology and support grower's efforts to develop more prescriptive management programs.

One potential avenue for reliably identifying billbug larvae is DNA barcoding, which involves the use of specific genes or genomic regions for species identification. DNA barcoding between species with cryptic developmental stages (Ahrens et al. 2007; Doskocil et al. 2008; Etzler et al. 2014; Pramual and Wongpakam 2014). In particular, the cytochrome oxidase subunit I (COI) gene is commonly targeted for animals, and COI has also been widely applied in insect identification studies (Jinbo et al. 2011) that range from biodiversity (Brasier et al. 2016) to food safety (Tagliavia et al. 2016), and community ecology (Pompanon et al. 2012). COI typically exhibits limited intraspecific variation, allowing researchers to reliably group members of the same species together, but demonstrates enough interspecific variation to separate different species, even if has been used previously to improve understanding of pest species complexes and differentiate they are closely related (Jinbo et al. 2011). DNA barcoding has been widely used to support the development of more efficient and sustainable insect management programs, and there are at least two previous examples specific to turfgrass. By using DNA barcoding techniques to identify turfgrass-infesting *Phyllophaga* spp larvae, Doskocil et al. (2008) laid the groundwork for later efforts aimed at characterizing the temporal and spatial distribution of these insects in Oklahoma (Graf et al. 2017). Similar techniques were used by Duffy et al. (2018), to identify the larval stage

of several billbug species and clarify their seasonal phenology in Indiana. Biological information emerging from such studies may translate directly into extension programming that supports growers' ability to develop and implement regionally tailored IPM strategies.

The findings of Duffy et al. (2018) demonstrated that DNA barcoding can provide critical biological insights in support of billbug IPM goals on a local scale. In their study, Duffy et al. (2018) included four species present in Indiana, *S. venatus*, *S. parvulus*, *S. inaequalis*, and *S. minimus*, and amplified three barcoding genes; COI (mitochondrial), 18S (nuclear ribosomal), and ITS2 (nuclear). Using this multi-gene approach, the researchers were able to detect that *S. venatus* overwinters in both the adult and larval stage, resulting in two distinct cohorts capable of damaging turfgrass during different times of the growing season. Although Duffy et al. (2018) assessed the seasonal biology of the billbug species complex within one geographically defined area (Indiana), our ability to apply this technique across a larger geographic area, where intraspecific genetic variation may be much higher, remains unclear.

Since geographically driven genetic variation could influence the utility of DNA barcoding genes for resolving species identity (Rubinoff et al. 2006), we assessed the utility of a DNA barcoding approach using three different genes (i.e., COI, ITS2, 18S). First, we hypothesized that by using a combination of three genetic loci we could characterize the intraspecific variation and interspecific divergence of billbug species across several states located in different regions of the U.S. (Indiana, Missouri, Utah, and Arizona). Secondly, as proof of concept, we hypothesized that by employing intensive billbug larval sampling and a simple morphometric measure (head capsule diameter) in conjunction with DNA barcoding, we could characterize the seasonal biology of the billbug complex across geographically disparate U.S. states. These aims will provide insights into billbug biology that could be used to develop regionally relevant, prescriptive monitoring and management programs for billbug pests in turfgrass systems.

## 2.3 Materials and Methods

### 2.3.1 Adult DNA Barcoding Reference Database

The first step towards assessing the utility of a DNA barcoding approach to differentiate cryptic billbug larval species was to create a reference database of adult billbug barcoding sequences. This reference database was used to characterize intraspecific variation and

interspecific divergence of billbug species, and for comparison with larval sequences for species identification.

*Adult Sample Collection*

In order to include representation of the most common billbug species from different regions, billbug adults were collected from Indiana, Utah, Missouri, and Arizona (Table 2.1). Adult specimens were identified to species level based on morphological characters, placed into glass vials containing 90% ethanol and stored at -20℃ until further processing. Each specimen was assigned a number and the corresponding species identity, collection location, and collection date were entered into the database.

*DNA Extraction and PCR Amplification*

The thorax and abdomen of each adult specimen were homogenized using a pestle, and DNA was extracted using the Qiagen® DNAeasy Blood and Tissue kit following the standard protocol established by the manufacturer. The optimal incubation period for adult specimens was 3h at 56°C. DNA quality was assessed by visualizing genomic DNA on a 1% agarose gel. DNA concentration was measured using the Thermo Scientific™ NanoDrop™ one Microvolume UV-Vis Spectrophotometer. Samples with DNA concentrations above 50ng/μL were diluted to reach concentrations between 20ng/μL–50ng/μL for optimal polymerase chain reaction (PCR).

To assess the effectiveness of different genes to differentiate between billbug species, we amplified three commonly used barcoding genes: cytochrome oxidase subunit I (COI, mtDNA), internal transcribed spacer region 2 (ITS2, nrDNA), and 18S rRNA (nrDNA). Primer sequences and PCR conditions were established following the protocols of Duffy et al. (2018) (see supplemental material, Table S.1 and S.2). Gel electrophoresis at 1% agarose in 1X TAE buffer was used to confirm amplification of PCR products from adult billbugs. The expected length of PCR products for each gene were 750 bp - COI, 650 bp - 18S, and 250 bp – ITS2 based-on data from Duffy et al. (2018). Amplified products then were cleaned using the Exo SAP-IT PCR Product Cleanup Reagent™ following manufacturer protocols. After cleanup, samples were sent for Sanger Sequencing to the Purdue Genomics Core facility or to Genewiz (South Plainfield, New Jersey).

*DNA Sequence Analysis*

Resulting forward and reverse sequences for each of the three barcoding genes were processed using the Aliview (Larsson 2014) alignment and editing software. The quality of nucleotide sequence was determined by examining individual chromatograms using the 4peaks software (Griekspoor and Groothuis 2006). To create a consensus sequence or contig, reverse sequences were reverse complemented and then aligned with the forward sequence. Primer sequences and low-quality base pairs were trimmed from the ends of the aligned sequences and then forward and reverse alignment were merged creating the consensus contig. The resulting length of consensus sequences used for further analysis were 640bp (COI), 350bp (18S), and 202-520bp (ITS2).

Consensus sequence alignment was performed using the MUSCLE algorithm (Edgar 2004) and included existing billbug sequences from Duffy et al. (2018). A phylogenetic analysis for non-coding proteins was done using MEGA software (Kumar et al. 2018). The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model in MEGA software with nodal branch support of 1,000 bootstrap replicates. Initial tree(s) were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a pairwise distance matrix estimated using the Maximum Composite Likelihood (MCL) approach. Log Likelihood values were then used to select the trees with superior topology. A discrete Gamma distribution was used to model evolutionary rate differences among sites (each base pair). These initial trees were then edited using Mesquite software (Maddison and Maddison 2019) and Inkscape (Inkscape Project 2020) to improve their aesthetic quality for publication. A final phylogenetic tree following this pipeline was produced for each of the three sequenced genes separately (COI, 18S, and ITS2).

Due to the potential intraspecific variation in the DNA sequences of the species widely distributed through North America (*S. parvulus* and *S. venatus*), we assessed whether this variation could result in discrepancies in identification. A distance matrix of percent differences was assembled and translated into percent sequence similarity to compare sequences within *S. parvulus* and *S. venatus* to every other species. Graphs depicting sequence variation were constructed using the ggplot2 (Wickham 2016) package in R.

### 2.3.2 Larval Species Identification

*Larval Collection*

To test the effectiveness of DNA barcoding for larval species identification and elucidate billbug seasonal biology, billbug larval sampling was performed throughout a portion of the growing season in Utah (May 21st to July 26th, 2018) and Indiana (June 3rd to August 5th, 2020). Soil cores were collected using a standard golf course cup-cutter (10.8 cm diameter) to a depth of 10 cm, and cores were carefully broken apart while searching for larvae. Larvae from Utah were collected from three different locations (Logan Country Club -LCC, Greenville Research Farm-GRF, and Utah State University greenhouse-USUG), planted with *Poa pratensis* L. (Kentucky bluegrass) growing in silty loam soil. Indiana larvae were collected from two locations: Bimmel Practice Complex (BPC) and William H. Daniel Turfgrass Research and Diagnostic Center (DTRC). Turfgrass cover at BPC consisted of *Cynodon dactylon* L. Pers. (Bermudagrass 'Patriot') growing in a sandy clay loam soil. Turfgrass cover at DTRC consisted of either *P. pratensis* (Kentucky Bluegrass 'Park') or *Zoyzia japonica* Steud. (Zoyziagrass 'Meyer') growing in a silty clay loam soil. Larvae were placed in 90-95% ethanol and stored at -20°C for further processing. Each larval specimen was numbered, and the corresponding collection date and location were entered into a larval database.

To track larval development across the growing season, larval head capsule width was measured and recorded for all larvae and entered into the corresponding database. Larvae were dorsally imaged using a Leica DFC450 camera mounted onto a MC165C stereomicroscope and head capsule widths were measured using the Leica Application Suite version 4.2.0 (Leica Microsystems). After head capsule measurements were taken, DNA barcoding of the larval specimens was performed following the same protocol used for adults, including DNA extraction, PCR amplification, Sanger sequencing and processing of consensus sequences for each individual. Only the COI gene was sequenced for larval specimens since it provided the optimal combination of species resolution and sequencing success for adults.

*Larval Species Identification*

We attempted to identify morphologically cryptic billbug larvae to species level using two methods based on DNA barcoding with the COI gene. First, we built a phylogenetic tree that included all larval and adult sequences following the same maximum likelihood approach

previously described (see DNA Sequence Analysis section above). Phylogenetic tree-based identification was employed by observing where the larvae were located within well supported clades (bootstrap value >70%) that included adult specimens. The second approach we used to identify larval specimens and assess intra- and inter-specific variation involved measuring average percent sequence similarity, which was done by comparing each larval sequence to all other larval and adult COI sequences. The length of the gene region used for sequence comparison for COI was 640bp. Graphs depicting sequence variation were constructed using the R package ggplot2 and species identification was assessed by looking at the position of each data point within the graph. A threshold of 90% sequence similarity was used for larval species differentiation. This percentage was chosen based on observed COI sequence variation for *S. parvulus* larvae, the species showing the highest level of intraspecific variation.

### 2.3.3 Larval Seasonal Phenology Maps

*Plotting Seasonal Phenology*

As proof of concept, we developed phenology maps based on a combination of morphometric measures (larval head capsule width) in conjunction with larval DNA barcoding to characterize the partial seasonal biology of the billbug species complex from Indiana and Utah. Larval phenology maps were created by including the head capsule width data, collection location, date of collection, and larval species identification based on our DNA barcoding methods using the COI gene. Two phenology maps were created, one each from Utah (2018) and Indiana (2020). Statistica 13.3.0 (TIBCO Software Inc. 2017) was used to develop the maps by plotting head capsule diameter (Y-axis) against day of year (X-axis) for each species present at a given location. Head capsule width measurements were used as a proxy for larval development, a method commonly used in Entomology (Godin et al. 2002). Although billbug larval development proceeds through the course of five instars (Dupuy and Ramirez 2016), we adopted the approach of previous studies (Doskocil and Brandenburg 2012; Duffy et al. 2018) and binned larvae as small (head capsule width < 1.0mm), medium (1.0-1.7mm), or large (above 1.7mm).

## 2.4 Results

### 2.4.1 Adult DNA Barcoding Reference Database

As the first step towards developing a DNA barcoding method to identify morphologically indistinguishable billbug larva, we created a reference database of morphologically identified adult billbug species sequences. Then we assessed the utility of three potential barcoding genes: COI, ITS2 and 18S. The adult DNA reference database included sequences from a wide variety of turfgrass feeding billbug species collected from different geographic locations. A total of ninety-seven adult sequences were obtained across all three potential barcoding genes (Table 2.1) and thirteen sequences were retrieved from the database created by Duffy et al. (2018) (Table 2.1). COI produced the highest percentage of success in obtaining high-quality sequences across specimens (Table 2.1). The phylogenetic tree constructed using adult COI sequences shows strong support for monophyly of every billbug species except *S. parvulus*, but within *S. parvulus* there was strong support for two subclades with bootstraps = 100% for both (Figure 2.1). ITS2 provided lower single gene resolution than COI (Figure 2.2) with strong support for *S. inaequalis* and *S. cicatristriatus* monophyletic clades (bootstrap values ≥ 95%) but weak support for *S. parvulus* and *S. venatus* even though they were grouped into monophyletic clades (bootstrap values ≥ 49%). ITS2 failed to group *S. minimus* into a monophyletic clade. This gene also showed inconsistent amplification compared to COI and produced only twenty-two usable sequences (Table 2.1). Finally, 18S provided almost no resolution, did not reliable identify billbug species, and failed to group species into monophyletic clades (Figure 2.3; bootstrap values ≥ 0%).

Overall, our results indicate the COI barcoding region reliably differentiated between species regardless of the geographic location from which they were collected (Figure 2.1). Each species (*S. parvulus* and *S. venatus*) formed well-supported (bootstrap values of 66 and 100% respectively), monophyletic clades that were not separated by location (Figure 2.1). When comparing the average percent sequence similarity of each adult specimen with all others within the same species group, *S. parvulus* contained more intraspecific sequence variation (92.5-99% sequence similarity) than *S. venatus* (95-99% sequence similarity) (Figure 2.4). However, in both species, sequence similarity was above 90% (Figures 2.4), suggesting intraspecific variation did not prevent use of the COI gene for species identification using our DNA barcoding method. Due to the usefulness of average percent sequence similarity value of 90% observed in the adult

specimen's analysis, this percentage was used as a threshold for larval identification in further analyses.

### 2.4.2 Larval Species Identification

Using our adult billbug DNA reference database of COI sequences, we were able to identify morphologically cryptic billbug larvae collected from Indiana and Utah to species level (Figures 2.5 & 2.6; Table A.1). Using a phylogenetic approach, larval COI sequences formed monophyletic clades that aligned with the adult billbug sequences (bootstrap values above 99%) (Figure 2.5). In addition, average percent sequence similarity confirmed that our DNA barcoding approach could reliably identify billbug larvae given existing intraspecific variation (>91.25-99% average sequence similarity) (Figure 2.6). Intraspecific variation in larval COI sequences ranged across species; *S. parvulus* (>91.25 average sequence similarity), *S. venatus* (>97.5 average sequence similarity) and *S. minimus* (>99% average sequence similarity) (Figure 2.6). However, these differences in intraspecific variation may be due to differences in the number of specimens collected for each billbug species.

### 2.4.3 Larval Seasonal Phenology Maps

As proof of concept that elucidating the seasonal biology of billbugs could be achieved by combining our DNA barcoding approach with head capsule width data, we developed larval seasonal phenology maps for Indiana and Utah. The resulting maps allowed us to visualize differences in species composition and how larval development of different billbug species proceeded across a portion of the growing season in these two different regions of the U.S. (Figure 2.7). Larval specimens collected from Utah during the summer of 2018 consisted of, *S. venatus* and *S. parvulus*, whereas larval specimens collected from Indiana included *S. venatus*, *S. parvulus*, and *S. minimus* (Table 2.1). All larval specimens from Utah were collected from stands of Kentucky bluegrass (cool-season grass) while in Indiana they were collected from warm- and cool-season grasses. In Indiana, 68% of the specimens sequenced in cool-season grasses were identified as *S. parvulus*, 12% as *S. venatus*, and 20% as *S. minimus* (Table 2.2). In warm-season grasses 92% were identified as *S. parvulus*, 6% as *S. venatus*, and 2% as *S. minimus* (Table 2.2).

## 2.5 Discussion

Turfgrass infesting billbugs represent an economically significant species complex consisting of no fewer than eleven different North American species (Held and Potter 2012; Dupuy and Ramirez 2016; Vittum 2020). The composition of the billbug species complex varies regionally (Johnson-Cicalese et al. 1990), with the geographic distribution of several species overlapping over large portions of their range. Although the seasonal biology of many species has not been studied in detail, the seasonal biology of the most well-studied species also appears to vary geographically. Additionally, it is currently impossible to accurately identify the larval stage to species level based on morphological characters. As a result, efforts to disentangle the seasonal biology and life history of these insects have been impossible to achieve in areas where mixed-species populations are common. Our study aimed to provide a molecular tool that will allow researchers across the U.S. to accurately identify the cryptic, soil-dwelling, larval stage and apply that tool to better understand billbug seasonal biology— a prerequisite for developing sound monitoring and management strategies.

### 2.5.1 Development of a reliable DNA Barcoding Tool for *Sphenophorus* in the U.S.

In order to precisely identify unknown specimens, a robust reference database is essential in DNA barcoding (Yusseff-Vanegas and Agnarsson 2017). It requires expanding sample sizes beyond what is available in public databases such as GenBank and the Barcode of Life Data Systems (BOLD) to avoid ambiguous results (Dong et al. 2018). Previous work by Duffy et al. (2018) demonstrated that a combination of three different barcoding genes (COI, ITS2, and 18S) could be used collectively to identify the morphologically indistinguishable larvae of four different billbug species in Tippecanoe County, IN. However, we suspected that our goal of producing a robust, well-supported and broadly applicable phylogenetic identification tool would require a broader, more geographically diverse sampling of *Sphenophorus* taxa. For this reason, our reference database of adult billbug sequences included six species, four of which are regionally dominant in the U.S.: *S. parvulus* (North), *S. venatus* (Southeast), *S. phoeniciensis* (Southwest), and *S. cicatristriatus* (Rocky Mountain) (Vittum 2020). Sequenced specimens also originated from four, geographically diverse states (Utah, Indiana, Arizona, and Missouri), with the two most

widely distributed species (*S. venatus* and *S. parvulus*) providing sequences from three of these states (Utah, Missouri, and Indiana).

Based on earlier reports (Duffy et al. 2018), we hypothesized that a combination of three barcoding genes (COI, 18S, ITS2) may be required to accurately characterize intraspecific variation and interspecific divergence of billbug species across the U.S. regions included in the current study. The limited dispersal capabilities of billbug adults (Dupuy et al. 2016) suggests that Midwestern billbugs could be genetically dissimilar to those inhabiting other parts of the U.S., potentially creating a variable geographic signature that could limit the utility of any single barcoding gene. However, contrary to our prediction, COI alone provided the highest, single gene resolution, and was able to consistently separate all six billbug species into monophyletic clades, regardless of geographic variation. These findings differ from those of other researchers who have reported a lack of success in using COI as a single barcoding gene in other insect groups and postulated the need to include additional genes (Jordal and Kambestad 2014; Yusseff-Venegas and Agnarsson 2017; Rubinoff et al. 2006). The use of a single gene in DNA barcoding studies has been contested in the past due to known limitations that include multiple mitochondrial gene haplotypes (heteroplasmy) and nuclear pseudogenes of the mitochondria genome (NUMT) (Jinbo et al. 2011). However, Rubinoff et al. (2006) demonstrated that as long as COI is being used in a well-studied group of insects with known characteristics for adult species differentiation, this gene alone may provide the resolution required to effectively distinguishes species. The current study complies with the general guidelines set forth by Rubinoff et al. (2006) and supports the idea that COI alone may provide the resolution necessary for species differentiation among *Sphenophorus* taxa across broad swaths of the continental U.S. Further, these results indicated that COI could be used to identify morphologically indistinguishable billbug larvae, including widely distributed species, potentially strengthening the utility of this single gene approach for clarifying regional differences in seasonal biology.

Although results from the current study demonstrate that COI alone works well for billbug species identification, we also considered the utility of additional genes. In addition to COI, we individually assessed the utility of 18S and ITS2 as barcoding genes for *Sphenophorus*. Even though in some cases the use of 18S has been plagued with a low success rate in PCR amplification, it did provide resolution of scale insects when amplification was successful (Sethusa et al. 2014) and in ticks at the genera level (Lv et al. 2014). Likewise, ITS2 has provided adequate resolution

for species differentiation in other groups of insects such as *Anopheles* spp. (Hackett et al. 2000), calliphorids (Yusseff-Venegas and Agnarsson 2017), braconids (Fagan-Jeffries et al. 2019), among others. However, ITS2 presents some documented difficulties, such as indels, that may affect alignment (Brown et al. 2012), and intragenomic variants capable of complicating the Sanger sequencing reaction (Fagan-Jeffries et al. 2019).

In the current study, 18S did not provide the resolution necessary to differentiate billbug species due to low interspecific variation. Both 18S and ITS2 suffered from lower PCR success rates compared to COI. Similarly, ITS2 fell short of the single gene resolution provided by COI, grouping *S. parvulus* and *S. venatus* sequences into separate clades, but with lower bootstraps values and failing to group together members of *S. minimus*. Although problems articulated with the use of ITS2 could potentially be addressed through the use of next-generation sequencing techniques (Fagan-Jeffries et al. 2019), difficulties in using 18S may be more challenging to manage. As such, neither 18S nor ITS2 alone appear to be viable candidates for the development of a robust, well-supported and broadly applicable phylogenetic identification tool for *Sphenophorus*.

### 2.5.2 DNA Barcoding tool provides insight into Billbug biology

Because of the resolution provided by COI, we used this barcoding gene in conjunction with billbug larval sampling and head capsule data to characterize a portion of the seasonal biology of the billbug complex in two different geographic regions of the U.S. Using this approach, we were able to visualize species composition and characterize larval development for several billbug species across a portion of the growing season in Utah and Indiana. The resulting seasonal phenology maps support the idea that the life history of turfgrass-inhabiting billbugs, including the cryptic, soil dwelling larvae, can be brought into focus across different U.S. regions using this methodology. Although the data presented herein (May-August) represent only a portion of the entire growing season (March-November), we could clarify the species composition and variation in the development of each species' destructive, soil-dwelling larval stage. This information is crucial for developing efficient pest management programs because the effectiveness of different chemical and biological management approaches hinges on knowledge of the target stage.

As evidence for how this new approach may reveal important insights into billbug biology, we catalogued two somewhat unexpected findings. First, we were able to identify *S. parvulus* as the primary species infesting warm-season (C4) grasses at the Indiana location, despite the presence of *S. venatus* within the same turf stand. *S. parvulus*' distribution is closely linked to areas where Kentucky bluegrass is grown which has resulted in the general working assumption that *S. parvulus* is primarily associated with cool-season grasses (Vittum 2020). Second, our larval sampling efforts revealed *S. venatus* as a secondary pest species infesting cool-season grasses (Kentucky bluegrass) at both locations, despite its common association with, and documented damage in Zoysiagrass and Bermudagrass (Vittum 2020). Moreover, *S. parvulus*, *S. venatus*, and *S. minimus* larvae were collected from the same stand of Kentucky bluegrass at the Indiana DTRC location. These findings support the utility of DNA barcoding as a larval identification tool and underscore that common billbug-host associations, or the mere presence of morphologically identifiable adults may not translate directly to soil-dwelling larval populations that are responsible for the majority of turfgrass damage.

Our finding supports the idea that billbug management should be anchored in biology and that species composition and seasonal biology investigations are essential for effective billbug management. Billbug control strategies rely heavily on the proper matching and timing of synthetic insecticides or biological control approaches targeting a particular billbug life stage, with active ingredients, application timing and number of applications required to provide satisfactory control varying depending on the seasonal biology of the target species. In the Midwest, the application of DNA barcoding revealed that the seasonal biology and population dynamics of the two most common billbug species differ in ways that required fundamentally different approaches toward monitoring and management (Duffy et al. 2018). In regions where billbug species composition and seasonal biology is still unknown, our DNA barcoding tool will be useful for disentangling species identity and clarifying seasonal population dynamics thereby supporting ongoing efforts to develop efficient management strategies.

## 2.6 Conclusion

The current study advances our ability to accurately identify the destructive, soil-dwelling larval stage of *Sphenophorous* taxa, even in cases where geographically-driven genetic variation may be expected. With a more robust DNA based larval identification tool in place, this research

may be leveraged to close important gaps in our understanding of billbug seasonal biology and species composition throughout the continental U.S. Since efforts to create effective and efficient management strategies are undermined by making "common sense" associations between presence of particular adult species and their favored host plants, these findings emphasize the importance of identifying the damaging larval stage. The COI gene alone was able to differentiate between billbug species regardless of where they were collected, and we were able to confidently identify billbug larvae using this single mitochondrial gene. By combining larval identification, collection dates, and morphometric data (head capsule diameter), the regionally variable life history of turfgrass-inhabiting billbugs can be clarified and used to anchor management programs. Future efforts are still needed to test the robustness of COI across additional species and regions that were not included in the current study.

## 2.7 Figures and Tables

Table 2.1. Summary of DNA barcoding results used to create a reference sequence database from adult billbug (*Sphenophorus* spp.) species collected in Indiana, Utah, Missouri, Arizona, and two outgroup species. The total number of DNA extractions performed, successful sequencing of PCR products (% Success) and total number of sequences included in the reference database are shown for each barcoding gene (COI, 18S and ITS2).

| Species | DNA Extractions | COI | % Success | 18S | % Success | ITS2 | % Success |
|---|---|---|---|---|---|---|---|
| *S. parvulus* | | | | | | | |
| Utah | 14 | 10 | 71.4 | 7 | 50.0 | 7 | 50.0 |
| Missouri | 12 | 5 | 41.6 | 5 | 41.6 | 4 | 33.3 |
| Indiana | 0 | 3* | | 0 | | 3* | |
| *S. venatus* | | | | | | | |
| Utah | 15 | 12 | 80.0 | 7 | 46.6 | 4 | 26.6 |
| Missouri | 16 | 6 | 37.5 | 5 | 31.2 | 2 | 12.5 |
| Indiana | 0 | 3* | | 0 | | 3* | |
| *S. minimus* | | | | | | | |
| Indiana | 0 | 3* | | 0 | | 3* | |
| *S. inaequalis* | | | | | | | |
| Indiana | 0 | 3* | | 0 | | 3* | |
| *S. cicatristriatus* | | | | | | | |
| Utah | 17 | 10 | 58.8 | 4 | 23.5 | 4 | 23.5 |
| *S. phoeniciensis* | | | | | | | |
| Arizona | 2 | 2 | 100.0 | 2 | 100.0 | 1 | 50.0 |
| *Listronotus maculicollis** | 0 | 1* | | 1* | | 1* | |
| *Donus zoilus** | 0 | 1* | | 1* | | 1* | |
| Total sequences | | 57 | | 30 | | 23 | |
| Avg % | | | 64.8 | | 48.82 | | 32.6 |

*Sequences obtained from previous study by Duffy et al (2018).

Table 2.2. Summary of larval specimens collected at Utah 2018 and Indiana 2020 in cool- and warm-season turfgrass. The total number of each species identified at each location and type of grass (*cool- or °warm-season) is included.

| | | | | | Total of Larvae Identified per species | | |
|---|---|---|---|---|---|---|---|
| Location | Year | Grass Type | Total of Larvae Collected | Total of Larvae Sequenced | *S. parvulus* | *S. venatus* | *S. minimus* |
| Utah | 2018 | | | | | | |
| | | Cool | 24 | 17 | 15 | 2 | 0 |
| | | Warm | 0 | 0 | 0 | 0 | 0 |
| Indiana | 2020 | | | | | | |
| | | Cool | 63 | 41 | 28 | 5 | 8 |
| | | Warm | 51 | 36 | 33 | 2 | 1 |
| Total | | | 138 | 94 | 76 | 9 | 9 |

**Poa pratensis*

°*Cynodon dactylon* 'Patriot' and *Zoyzia japonica* 'Meyer'

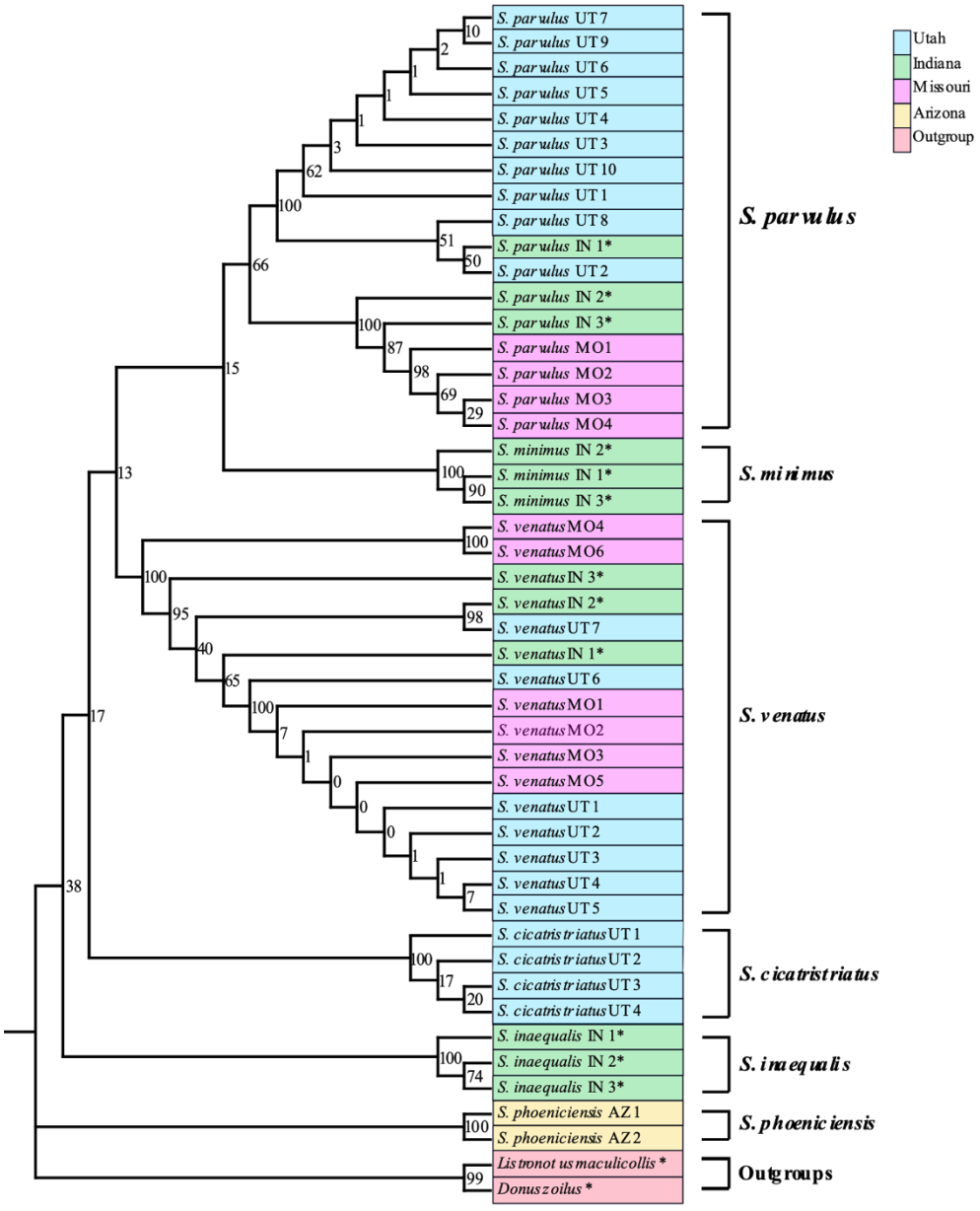



Figure 2.1. Maximum likelihood tree of COI sequences from *Sphenophorus parvulus*, *S. venatus*, *S. minimus*, *S. inaequalis*, *S. cicatristriatus*, and *S. phoeniciensis* adults. Collection location is represented by color blocks; Utah (blue), Indiana (green), Missouri (purple), Arizona (yellow), outgroups (red). Replicate numbers are indicated to the right of the scientific name and collection state (Utah=UT, Indiana=IN, Missouri=MO, Arizona-AZ). Numbers at nodes are bootstraps values (1,000 bootstrap replicates as percentages). * indicates sequences obtained from Duffy et al. (2018).

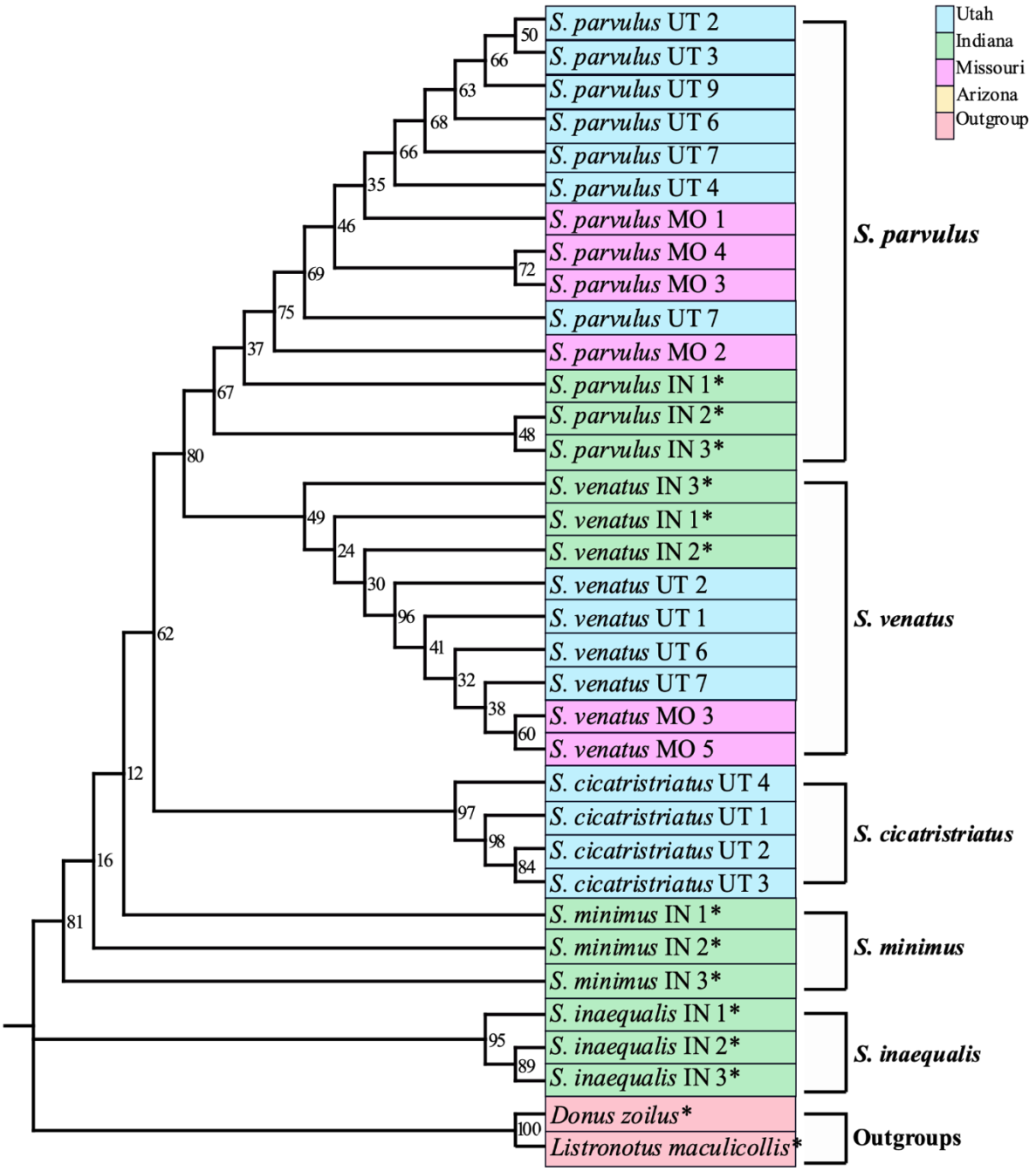



Figure 2.2. Maximum likelihood tree of ITS2 sequences from *Sphenophorus parvulus*, *S. venatus*, *S. minimus*, *S. inaequalis*, and *S. cicatristriatus*. Collection location is represented by color blocks; Utah (blue), Indiana (green), Missouri (purple), Arizona (yellow), outgroups (red). Replicate numbers are indicated to the right of the scientific name and collection state (Utah=UT, Indiana=IN, Missouri=MO, Arizona-AZ). Numbers at nodes are bootstraps values (1,000 bootstrap replicates as percentages). * indicates sequences obtained from Duffy et al. (2018).

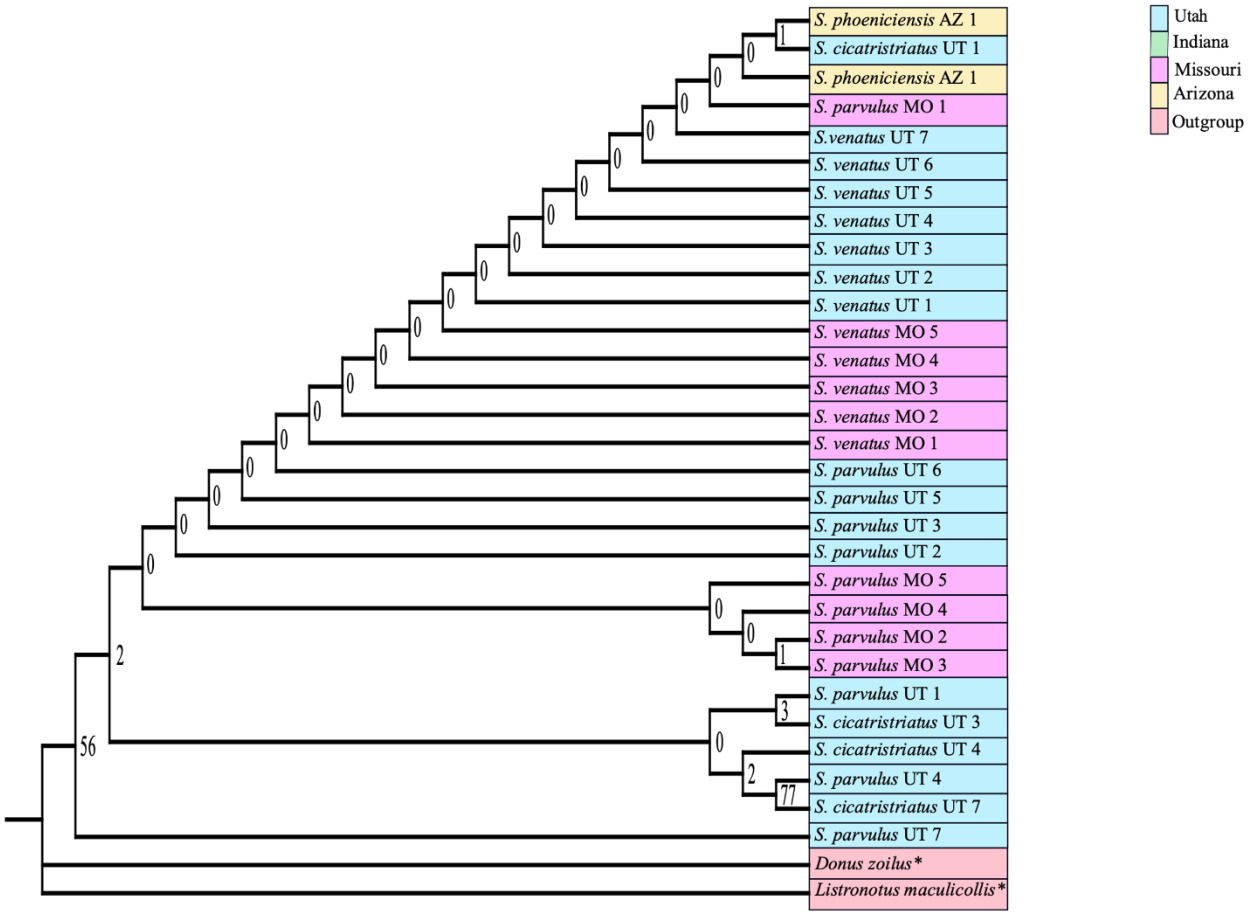



Figure 2.3. Maximum likelihood tree of 18S sequences from *Sphenophorus parvulus*, *S. venatus*, *S. inaequalis*, and *S. cicatristriatus*. Collection location is represented by color blocks; Utah (blue), Indiana (green), Missouri (purple), Arizona (yellow), outgroups (red). Replicate numbers are indicated to the right of the scientific name and collection state (Utah=UT, Indiana=IN, Missouri=MO, Arizona-AZ). Numbers at nodes are bootstraps values (1,000 bootstrap replicates as percentages). * indicates sequences obtained from Duffy et al. (2018).

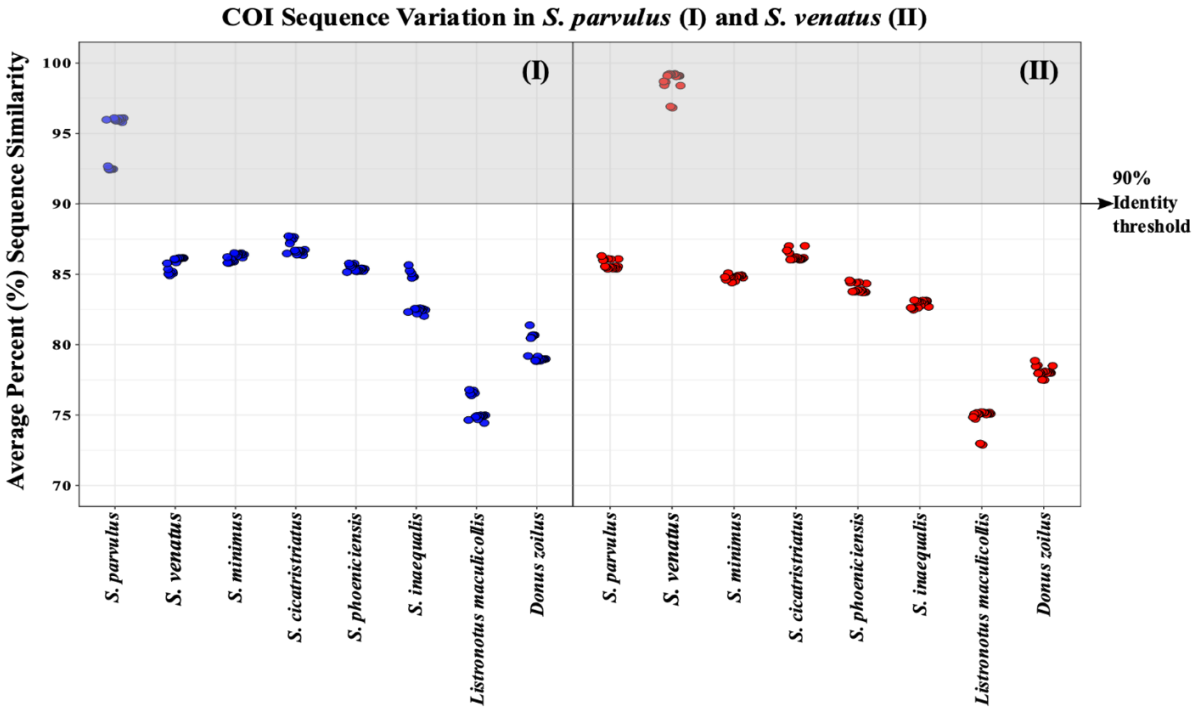



Figure 2.4. Pairwise distances between adults measured as average percent sequence similarity of the COI gene (640bp). Graph (I) depicts *Sphenophorus parvulus* specimens collected from several locations (blue dots) and graph (II), *S. venatus* specimens collected in several locations (red dots). Area highlighted in gray indicates 90% average sequence similarity threshold. *S. parvulus* (I) and *S. venatus* (II) sequences compared to all other sequences of each species included in the reference database: *S. parvulus*, *S. venatus*, *S. minimus*, *S. cicatristriatus*, *S. phoeniciensis*, *S. inaequalis*, *Listronotus maculicollis*, *Donus zoilus*.

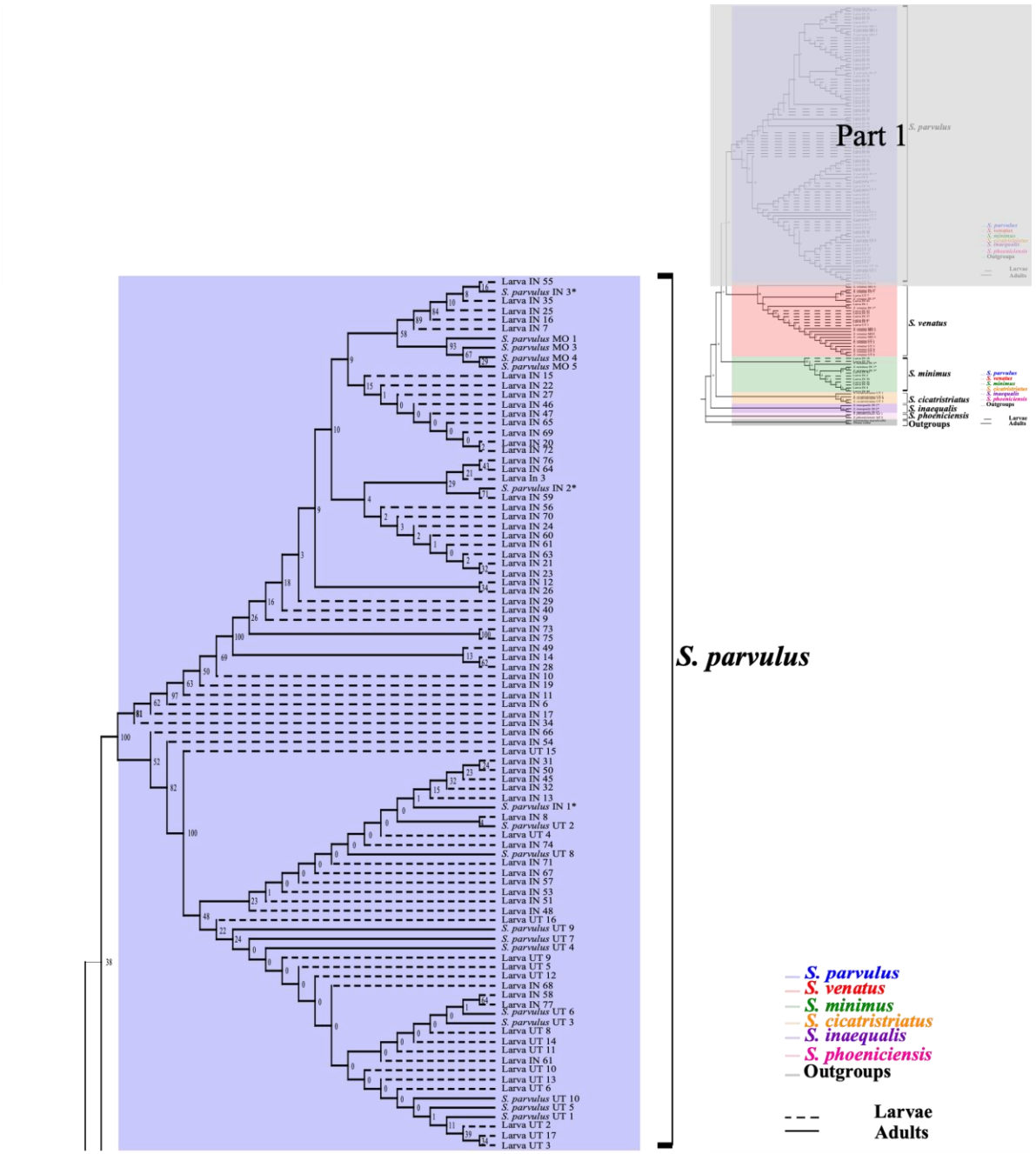
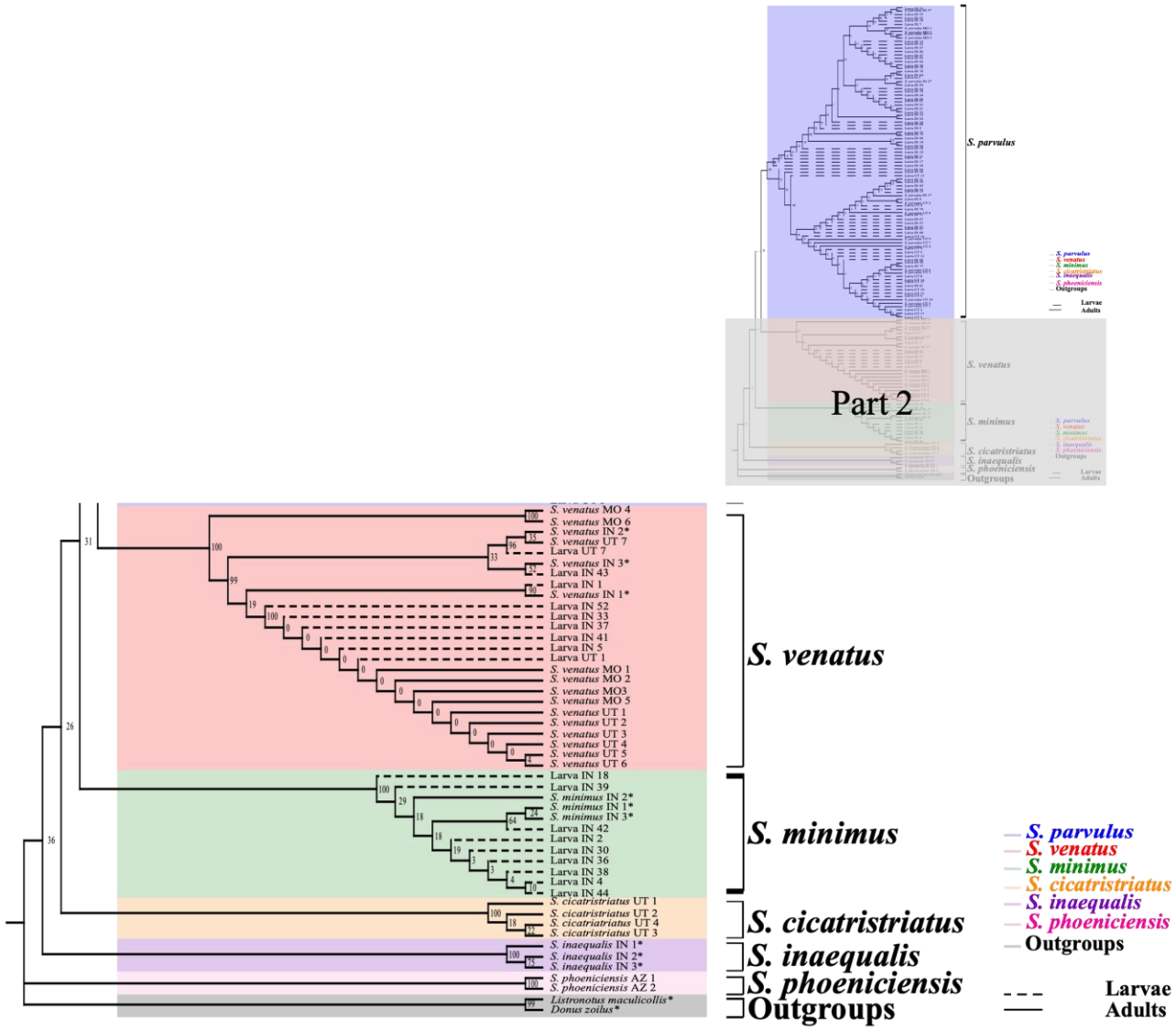



Figure 2.5. Maximum likelihood tree of COI sequences from adults and larvae. Larvae are represented as dashed lines and were identified based on their position in the tree with bootstraps values ≥50%. Larvae were successfully grouped with adult species included in the reference database, species groupings are represented by color blocks: *Sphenophorus parvulus* (blue), *S. venatus* (red), *S. minimus* (green), *S. inaequalis* (purple), *S. cicatristriatus* (orange), and *S. phoeniciencis* (pink). Replicate numbers are indicated to the right of the scientific name and collection state (Utah=UT, Indiana=IN, Missouri=MO, Arizona-AZ). Numbers at nodes are bootstraps values (1,000 bootstrap replicates as percentages). * indicates sequences obtained from Duffy et al. (2018).

Figure 2.5 continued



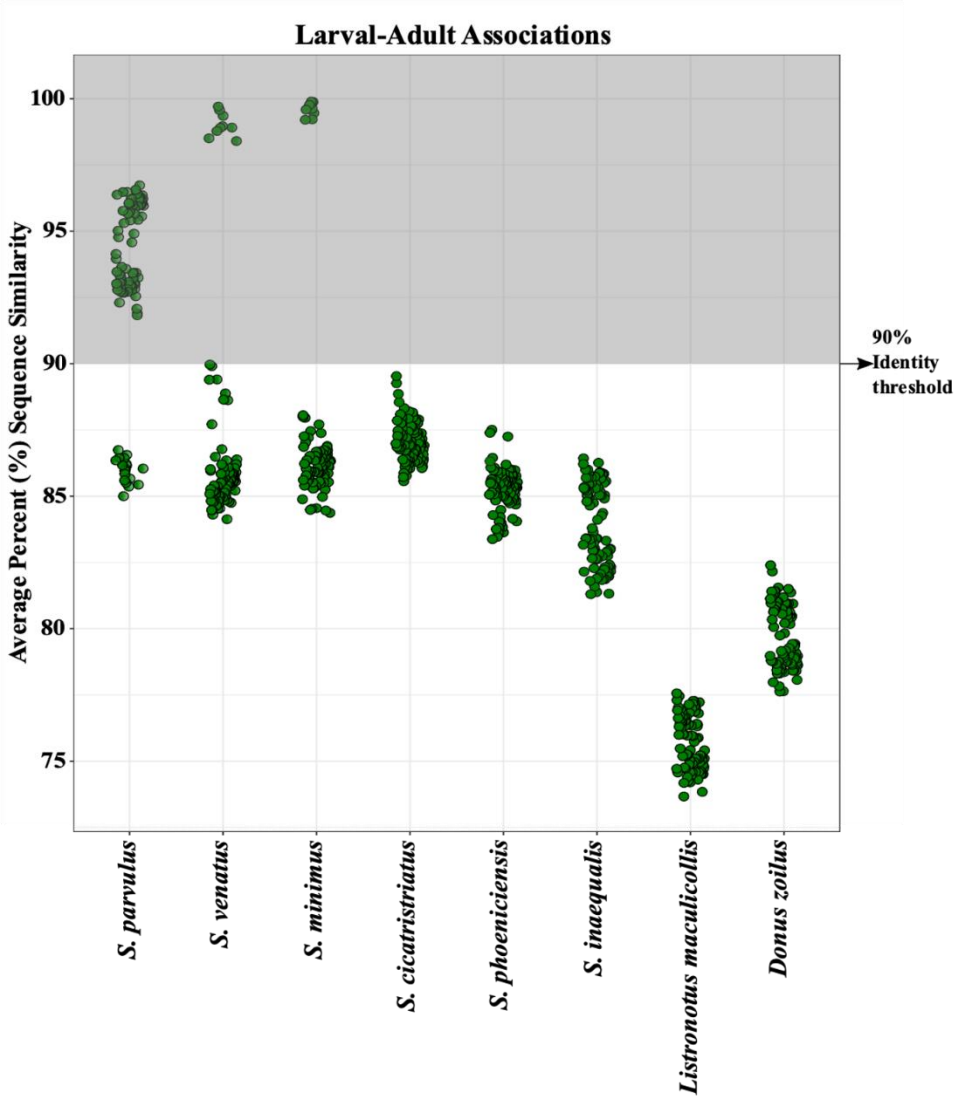



Figure 2.6. Pairwise distances between larvae and adults (Utah 2018 and Indiana 2020) measured as average percent sequence similarity of the COI gene (640bp). Each green dot represents a larval sequence matched against the adult reference database sequences (i.e., above the 90%; in grey area of figure). Larval sequences are compared to all sequences of each species included in the reference database: *Sphenophorus parvulus*, *S. venatus*, *S. minimus*, *S. cicatristriatus*, *S. phoeniciensis*, *S. inaequalis*, *Listronotus maculicollis*, *Donus zoilus*.

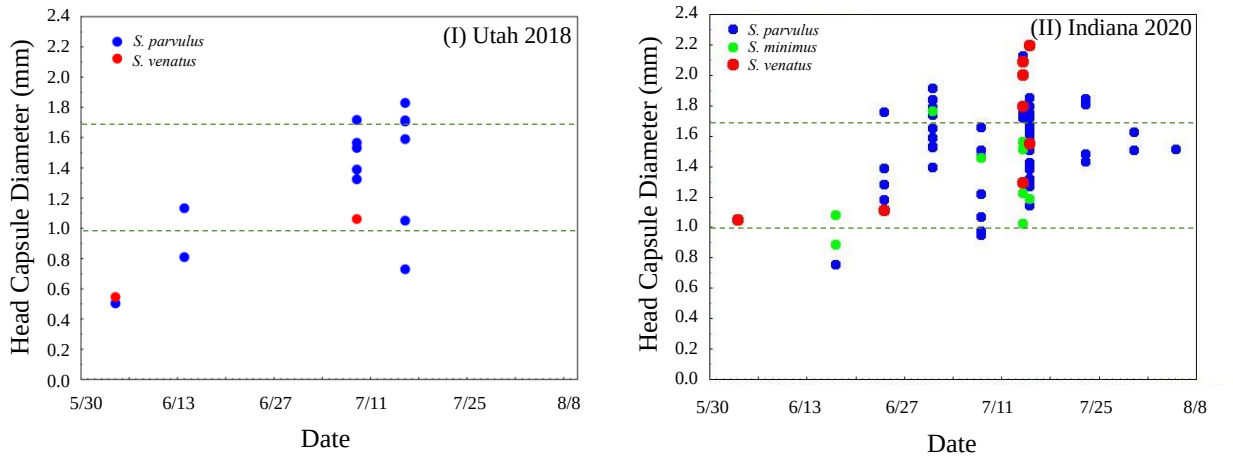



Figure 2.7. Seasonal phenology maps of billbug larvae collected from (I) Utah 2018 and (II) Indiana 2020. We adopted the approach of previous studies (Doskocil and Brandenburg 2012; Duffy et al. 2018) and binned larvae as small (head capsule diameter<1.0mm of), medium (1.0-1.7mm), or large (above 1.7mm).

## 2.8 References


**Ahrens, D., M. T. Monaghan, and A. P. Vogler. 2007.** DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). Mol. Phylogenet. Evol. 44: 436–449.

**Berlocher, S. H., and J. L. Feder. 2002.** Sympatric speciation in phytophagous insects: moving beyond controversy? Annu. Rev. Entomol. 47: 773–815.

**Brasier, M. J., H. Wiklund, L. Neal, R. Jeffreys, K. Linse, H. Ruhl, and A. G. Glover. 2016.** DNA barcoding uncovers cryptic diversity in 50 % of deep-sea Antarctic polychaetes. R. Soc. Open. Sci. 3: 160432.

**Brown, S. D. J., K. F. Armstrong, and R. H. Cruickshank. 2012.** Molecular phylogenetics of a South Pacific sap beetle species complex (*Carpophilus* spp., Coleoptera: Nitidulidae). Mol. Phylogenet. Evol. 64: 428–440.

**Chong, J-H. 2015.** Species richness and seasonal abundance of billbugs, *Sphenophorus* spp., from South Carolina golf courses. J. Agric. Urban Entomol. 31: 20–28.

**De Meyer, M., H. Delatte, S. Ekesi, K. Jordaens, B. Kalinova, A. Manrakhan, M. Mwatawala, G. Steck, J. Van Cann, L. Vaníčková, R. Břízová, and M. Virgilio. 2015.** An integrative approach to unravel the *Ceratitis* FAR (Diptera, Tephritidae) cryptic species complex: A review. ZooKeys. 540: 405–427.

**Dong, Z., S. Liu, Z. Zhang. 2018.** Efficacy of using DNA barcoding to identify parasitoid wasps of the melon-cotton aphid (*Aphis gossypii*) in watermelon cropping system. BioControl. 63: 677–685.

**Doskocil, J. P., N. R. Walker, G. E. Bell, S. M. Marek, J. A. Reinert, and T. A. Royer. 2008.** Species composition and seasonal occurrence of *Phyllophaga* (Coleoptera: Scarabaeidae) infesting intensely managed bermudagrass in Oklahoma. J. Econ. Entomol. 101: 1624–1632.

**Doskocil, J. P., and R. L. Brandenburg. 2012.** Hunting billbug (Coleoptera: Curculionidae) Life Cycle and Damaging Life Stage in North Carolina, With Notes on Other Billbug Species Abundance. J. Econ. Entomol. 105: 2045–2051.

**Duffy, A. G., G. S. Powell, J. M. Zaspel, and D. S. Richmond. 2018.** Billbug (Coleoptera: Dryophthoridae: *Sphenophorus* spp.) seasonal biology and DNA-based life stage association in Indiana turfgrass. J. Econ. Entomol. 111: 304–313.

**Dupuy, M. and R. A. Ramirez. 2016.** Biology and management of billbugs (Coleoptera: Curculionidae) in turfgrass. J. Integr. Pest Manag. 7: 1–10.

**Edgar, R. C. 2004.** MUSCLE: A multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 5: 1–19.

**Etzler, F. E., K. W. Wanner, A. Morales-Rodriguez, and M. A. Ivie. 2014.** DNA barcoding to improve the species-level management of wireworms (Coleoptera: Elateridae). J. Econ. Entomol. 107: 1476–1485.

**Fagan-Jeffries, E.P., S. J. B. Cooper, T. M. Bradford, and A. D. Dustin. 2019.** Intragenomic internal transcribed spacer 2 variation in a genus of parasitoid wasps (Hymenoptera: Braconidae): implications for accurate species delimitation and phylogenetic analysis. Insect Mol. Biol. 28: 485–498.

**Farkas, P., Z. György, A. Tóth, A. Sojnóczki, and J. Fail. 2020.** A simple molecular identification method of the *Thrips tabaci* (Thysanoptera: Thripidae) cryptic species complex. Bull. Entomol. Res. 110: 397–405.

**Feder, J. L., and K. E. Filchak. 1999.** It's about time: The evidence for host plant-mediated selection in the apple maggot fly, *Rhagoletis pomonella*, and its implications for fitness trade-offs in phytophagous insects. Entomol. Exp. Appl. 91: 211–225.

**Georgis, R., A. M. Koppenhofer, L. A. Lacey, G. Belair, and L. W. Duncan. 2006.** Successes and failures of entomopathogenic nematodes. Biological Control 38: 103–123.

**Godin, J., P. Maltais, and S. Gaudet. 2002.** Head capsule width as an instar indicator for larvae of the cranberry fruitworm (Lepidoptera: Pyralidae) in southeastern New Brunswick. J. Econ. Entomol. 95: 1308–1313.

**Graf, N., E. Rebek, T. Troyer, and N. Walker. 2017.** Spatial and temporal distribution of *Phyllophage* spp. infesting bermudagrass in Oklahoma. I. T. S. R. J. 13: 489–496.

**Griekspoor, A., and T. Groothuis. 2006.** 4peaks, version 1.8. https://www.nucleobytes.com/4peaks/index.html

**Hackett B. J., J. Gimnig, W. Guelbeogo, C. Costantini, L. L. Koekemoer, M. Coetzee, F. H. Collins, and N. J. Besansky. 2000.** Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *A. rivulorum*, and uncover a cryptic taxon. Ins. Mol. Biol. 9: 369–374.

**Hassanzadeh-Avval, M., H. Sadeghi-Namaghi, and L. Fekrat. 2020.** Molecular and morphological identification of *Anthocoris* spp. (Hemiptera: Anthocoridae) predators of three economically important psyllid species in Razavi Khorasan province, Northeastern Iran. Biologia 75: 1667–1677.

**Held, D. W., and D. A. Potter. 2012.** Prospects for managing turfgrass pests with reduced chemical inputs. Annu. Rev. Entomol. 57: 329–354.

**Huang, T., and E. A. Buss**. **2009**. Billbug (Coleoptera: Curculionidae) species composition, abundance, seasonal activity, and developmental time in Florida. J. Econ. Entomol. 102: 309–314.

**Inkscape Project. 2020.** Inkscape. https://inkscape.org.

**Jinbo, U., T. Kato, and M. Ito. 2011.** Current progress in DNA barcoding and future implications for entomology. Entomol. Sci. 14: 107–124.

**Johnson-Cicalese, J. M., G. W. Wolfe, and C. R. Funk. 1990.** Biology, distribution, and taxonomy of billbug turf pests (Coleoptera: Curculionidae). Entomol. Soc. Am. 19: 1037–1046.

**Jordal, B. H., and M. Kambestad. 2014.** DNA barcoding of bark and ambrosia beetles reveals excessive NUMTs and consistent east-west divergence across Palearctic forests. Mol. Ecol. Resour. 14: 7–17.

**Kumar S., G. Stecher, M. Li, C. Knyaz, and K. Tamura. 2018.** MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549.

**Larsson, A. 2014.** AliView: A fast and lightweight alignment viewer and editor for large datasets. Bioinformatics. 30: 3276–3278.

**Li, Q., J. Deng, C. Chen, L. Zeng, X. Lin, Z. Cheng, G. Qiao, and X. Huang. 2020.** DNA barcoding subtropical aphids and implications for population differentiation. Insects. 11: 1–17.

**Lv, J., S. Wu, Y. Zhang, T. Zhang, C. Feng, G. Jia, and X. Lin. 2014.** Development of a DNA barcoding system for the Ixodida (Acari: Ixodida). Mitochondrial DNA. 25: 142–149.

**Maddison, W. P., and D. R. Maddison. 2019.** Mesquite: a modular system for evolutionary analysis. Version 3.61. http://www.mesquiteproject.org

**Pompanon, F., B. E. Deagle, W. O. C. Symondson, D. S. Brown, S. N. Jarman, and P. Taberlet. 2012.** Who is eating what: Diet assessment using next generation sequencing. Mol. Ecol. 21: 1931–1950.

**Pramual, P., and K. Wongpakam. 2014.** Association of black fly (Diptera: Simuliidae) life stages using DNA barcode. J. Asia. Pac. Entomol. 17: 549–554.

**Richmond, D. S. 2016.** Managing billbugs in turfgrass. https://extension.entm.purdue.edu/publications/E-266/E-266.html.

**Rubinoff, D., S. Cameron, and K. Will. 2006.** A genomic perspective on the shortcomings of mitochondrial DNA for “barcoding” identification. J. Hered. 97: 581–594.

**Sethusa, M. T., I. M. Millar, K. Yessoufou, A. Jacobs, M. Van Der Bank, and H. Van Der Bank. 2014.** DNA barcode efficacy for the identification of economically important scale insects (Hemiptera: Coccoidea) in South Africa. African Entomol. 22: 257–266.

**Tagliavia, M., A. Nicosia, M. Salamone, G. Biondo, C. D. Bennici, S. Mazzola, and A. Cuttitta. 2016.** Development of a fast DNA extraction method for sea food and marine species identification. Food Chem. 203: 375–378.

**TIBCO Software Inc. 2017.** TIBCO Statistica® User's Guide, version 13.3.0. https://docs.tibco.com/products/tibco-statistica-13-3-0

**Tyagi, K., V. Kumar, D. Singha, K. Chandra, B. A. Laskar, R. Chakraborty, and S. Chatterjee. 2017.** DNA barcoding studies on Thrips in India: Cryptic species and species complexes. 17: 4898.

**Umble, J., G. Fisher, and S. Rao. 2005.** Sampling methods and seasonal phenology of *Sphenophorus venatus* confluens Chittenden (Coleoptera: Curculionidae) in orchardgrass (*Dactylis glomerata* L.). J. Agr. Urban Entomol. 22: 79–85.

**Vittum, P. J. 2020.** Turfgrass insects of the United States and Canada. 3rd ed. Cornell University Press, Ithaca, NY.

**Wang, Y., C. Huang, B. Hu, Y. Liu, G. H. Walter, and J. P. Hereward. 2019.** Gene flow across host-associated populations of the ricestem borer *Chilo suppressalis* Walker (Lepidoptera; Crambidae): implications for Bt resistance management in rice. Pest Manag Sci. 76: 695–703.

**Wickham, H. 2016.** ggplot2: Elegant Graphics for Data Analysis. https://ggplot2.tidyverse.org

**Yusseff-Vanegas, S. Z., and I. Agnarsson. 2017.** DNA-barcoding of forensically important blow flies (Diptera: Calliphoridae) in the Caribbean region. PeerJ. 5: e3516.

## 2.9 Supplemental Material

Table S.1. COI, 18S, and ITS2 primer sequences for PCR reactions for *Sphenophorus* spp. Schönherr adults and larvae associated with turfgrass following Duffy et al. (2018).

| Gene | Primer Sequence |
|---|---|
| CO1 | (F)TAATACGACTCACTATAGGGCAACATTTATTTTGATTTTTTGG<br>(R)ATTAACCCTCACTAAAGTCCAATGCACTAATCTGCCATATTA |
| 18S | (F)TACCTGGTTGATCCTGCCAGTAG<br>(R)GACGGTCCAACAATTTCACC |
| ITS2 | (F)AATACGACTCACTATAGGGTGAACATCGACATTTYGAACGCACA<br>(R)TTAACCCTCACTAAAGTTCTTTTCCSCTTAYTRATATGCTTAA |

Table S.2. COI, 18S, and ITS2 primer amplification conditions for PCR reactions for *Sphenophorus* spp. Schönherr adults and larvae associated with turfgrass following Duffy et al. (2018).

| Gene | Hot Start ◦C (min) | Denature ◦C (min) | Anneal ◦C (min) | Extend ◦C (min) | Final extend ◦C (min) | No. cycles |
|---|---|---|---|---|---|---|
| CO1 | 94 (2:00) | 94 (1:00) | 48 (1:00) | 72 (1:00) | 72 (12:00) | 40 |
| 18S | 95 (10:00) | 94 (0:30) | 50–55(0:30) | 72 (1:30) | 72 (10:00) | 41 |
| ITS2 | 95 (5:00) | 95 (1:00) | 57– 60 (0:30) | 72 (1:00) | 72 (7:00) | 33 |

# APPENDIX

Table A.1. Association of each larval specimen with its respective species identification (*Shenophorus parvulus*, *S. venatus*, and *S. minimus*), state (Utah and Indiana), collection location (DTRC=William H. Daniel Turfgrass Research and Diagnostic Center, GRF=Greenville Research Farm, LCC=Logan Country Club, BPC=Bimmel Practice Complex), grass type (*cool- or °warm-season), collection date, and head capsule width diameter (mm).

| Larval Specimen | Species | State | Collection Location | Grass Type | Collection Date | Head capsule width diameter (mm) |
|---|---|---|---|---|---|---|
| Larva UT 1 | *S. parvulus* | Utah | GRF | Cool | 6/4/18 | 0.504 |
| Larva UT 2 | *S. venatus* | Utah | GRF | Cool | 6/4/18 | 0.546 |
| Larva UT 3 | *S. parvulus* | Utah | LCC | Cool | 6/14/18 | 0.81 |
| Larva UT 4 | *S. parvulus* | Utah | LCC | Cool | 6/14/18 | 1.133 |
| Larva UT 5 | *S. parvulus* | Utah | LCC | Cool | 7/9/18 | 1.532 |
| Larva UT 6 | *S. parvulus* | Utah | LCC | Cool | 7/9/18 | 1.39 |
| Larva UT 7 | *S. parvulus* | Utah | LCC | Cool | 7/9/18 | 1.718 |
| Larva UT 8 | *S. parvulus* | Utah | LCC | Cool | 7/9/18 | 1.325 |
| Larva UT 9 | *S. venatus* | Utah | LCC | Cool | 7/9/18 | 1.062 |
| Larva UT 10 | *S. parvulus* | Utah | LCC | Cool | 7/9/18 | 1.567 |
| Larva UT 11 | *S. parvulus* | Utah | GRF | Cool | 7/16/18 | 1.715 |
| Larva UT 12 | *S. parvulus* | Utah | GRF | Cool | 7/16/18 | 1.707 |
| Larva UT 13 | *S. parvulus* | Utah | GRF | Cool | 7/16/18 | 1.83 |
| Larva UT 14 | *S. parvulus* | Utah | GRF | Cool | 7/16/18 | 0.73 |
| Larva UT 15 | *S. parvulus* | Utah | GRF | Cool | 7/16/18 | 1.591 |
| Larva UT 16 | *S. parvulus* | Utah | GRF | Cool | 7/16/18 | 1.051 |
| Larva UT 17 | *S. parvulus* | Utah | GRF | Cool | 7/16/18 | 1.711 |
| Larva IN 1 | *S. venatus* | Indiana | BPC | Warm | 6/3/20 | 1.054 |
| Larva IN 2 | *S. minimus* | Indiana | DTRC | Cool | 6/17/20 | 0.891 |
| Larva IN 3 | *S. parvulus* | Indiana | DTRC | Cool | 6/17/20 | 0.760 |
| Larva IN 4 | *S. minimus* | Indiana | DTRC | Cool | 6/17/20 | 1.083 |
| Larva IN 5 | *S. venatus* | Indiana | DTRC | Cool | 6/24/20 | 1.113 |
| Larva IN 6 | *S. parvulus* | Indiana | DTRC | Cool | 6/24/20 | 1.287 |
| Larva IN 7 | *S. parvulus* | Indiana | DTRC | Cool | 6/24/20 | 1.762 |
| Larva IN 8 | *S. parvulus* | Indiana | DTRC | Cool | 6/24/20 | 1.184 |
| Larva IN 9 | *S. parvulus* | Indiana | DTRC | Cool | 6/24/20 | 1.393 |
| Larva IN 10 | *S. parvulus* | Indiana | BPC | Warm | 7/1/20 | 1.919 |
| Larva IN 11 | *S. parvulus* | Indiana | BPC | Warm | 7/1/20 | 1.592 |
| Larva IN 12 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.398 |
| Larva IN 13 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.841 |

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| Larva IN 14 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.743 |
| Larva IN 15 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.782 |
| Larva IN 16 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.536 |
| Larva IN 17 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.795 |
| Larva IN 18 | *S. minimus* | Indiana | DTRC | Cool | 7/1/20 | 1.769 |
| Larva IN 19 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.758 |
| Larva IN 20 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.654 |
| Larva IN 21 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.593 |
| Larva IN 22 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.529 |
| Larva IN 23 | *S. parvulus* | Indiana | DTRC | Cool | 7/1/20 | 1.748 |
| Larva IN 24 | *S. parvulus* | Indiana | DTRC | Cool | 7/8/20 | 1.512 |
| Larva IN 25 | *S. parvulus* | Indiana | DTRC | Cool | 7/8/20 | 1.466 |
| Larva IN 26 | *S. parvulus* | Indiana | DTRC | Cool | 7/8/20 | 1.224 |
| Larva IN 27 | *S. parvulus* | Indiana | DTRC | Cool | 7/8/20 | 0.953 |
| Larva IN 28 | *S. parvulus* | Indiana | DTRC | Cool | 7/8/20 | 1.660 |
| Larva IN 29 | *S. parvulus* | Indiana | DTRC | Cool | 7/8/20 | 1.659 |
| Larva IN 30 | *S. minimus* | Indiana | DTRC | Cool | 7/8/20 | 1.459 |
| Larva IN 31 | *S. parvulus* | Indiana | DTRC | Cool | 7/8/20 | 1.074 |
| Larva IN 32 | *S. parvulus* | Indiana | DTRC | Cool | 7/8/20 | 0.975 |
| Larva IN 33 | *S. venatus* | Indiana | DTRC | Cool | 7/14/20 | 1.799 |
| Larva IN 34 | *S. parvulus* | Indiana | DTRC | Cool | 7/14/20 | 2.130 |
| Larva IN 35 | *S. parvulus* | Indiana | DTRC | Cool | 7/14/20 | 1.722 |
| Larva IN 36 | *S. minimus* | Indiana | DTRC | Cool | 7/14/20 | 1.568 |
| Larva IN 37 | *S. venatus* | Indiana | DTRC | Cool | 7/14/20 | 1.300 |
| Larva IN 38 | *S. minimus* | Indiana | DTRC | Cool | 7/14/20 | 1.031 |
| Larva IN 39 | *S. minimus* | Indiana | DTRC | Cool | 7/14/20 | 1.517 |
| Larva IN 40 | *S. parvulus* | Indiana | DTRC | Cool | 7/14/20 | 1.753 |
| Larva IN 41 | *S. venatus* | Indiana | DTRC | Cool | 7/14/20 | 2.091 |
| Larva IN 42 | *S. minimus* | Indiana | DTRC | Cool | 7/14/20 | 1.227 |
| Larva IN 43 | *S. venatus* | Indiana | DTRC | Cool | 7/14/20 | 2.008 |
| Larva IN 44 | *S. minimus* | Indiana | DTRC | Warm | 7/15/20 | 1.192 |
| Larva IN 45 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.643 |
| Larva IN 46 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.323 |
| Larva IN 47 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.752 |
| Larva IN 48 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.797 |
| Larva IN 49 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.574 |
| Larva IN 50 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.668 |
| Larva IN 51 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.655 |
| Larva IN 52 | *S. venatus* | Indiana | DTRC | Warm | 7/15/20 | 1.553 |
| Larva IN 53 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.857 |

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| Larva IN 54 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.626 |
| Larva IN 55 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.302 |
| Larva IN 56 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.423 |
| Larva IN 57 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.147 |
| Larva IN 58 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.559 |
| Larva IN 59 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.650 |
| Larva IN 60 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.431 |
| Larva IN 61 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.424 |
| Larva IN 62 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.673 |
| Larva IN 63 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.645 |
| Larva IN 64 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.514 |
| Larva IN 65 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.384 |
| Larva IN 66 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.606 |
| Larva IN 67 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.273 |
| Larva IN 68 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.659 |
| Larva IN 69 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.298 |
| Larva IN 70 | *S. parvulus* | Indiana | DTRC | Warm | 7/15/20 | 1.723 |
| Larva IN 71 | *S. parvulus* | Indiana | DTRC | Warm | 7/23/20 | 1.850 |
| Larva IN 72 | *S. parvulus* | Indiana | DTRC | Warm | 7/23/20 | 1.810 |
| Larva IN 73 | *S. parvulus* | Indiana | DTRC | Warm | 7/23/20 | 1.438 |
| Larva IN 74 | *S. parvulus* | Indiana | DTRC | Warm | 7/23/20 | 1.488 |
| Larva IN 75 | *S. parvulus* | Indiana | DTRC | Warm | 7/30/20 | 1.512 |
| Larva IN 76 | *S. parvulus* | Indiana | DTRC | Warm | 7/30/20 | 1.631 |
| Larva IN 77 | *S. parvulus* | Indiana | DTRC | Warm | 8/5/20 | 1.520 |

Grass Type: [*]*Poa pratensis*, [°]*Cynodon dactylon* ‘Patriot’ and *Zoyzia japonica* ‘Meyer’