Turfgrass and Environmental Research Online

Using Science to Benefit Golf

Because the damaging larval stages of billbugs cannot be identified to species, the seasonal population dynamics of the most common billbug pests remains largely unresolved. Research at Purdue University is exploring the potential for exploiting the unique properties of the nuclear ribosomal DNA multigene family (nrDNA) to identify species-specific molecular markers that could be used in diagnostic assays for several of the most common billbug species associated with turfgrass environments.

Volume 10, Volume 22
November 15, 2011

PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 400 projects at a cost of $31 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.

Editor

Jeff Nus, Ph.D.
1032 Rogers Place
Lawrence, KS 66049
jnus@usga.org
(785) 832-2300
(785) 832-9265 (fax)

Research Director

Michael P. Kenna, Ph.D.
P.O. Box 2227
Stillwater, OK 74076
mkenna@usga.org
(405) 743-3900
(405) 743-3910 (fax)

Developing a DNA-Based Larval Identification Tool for Billbugs

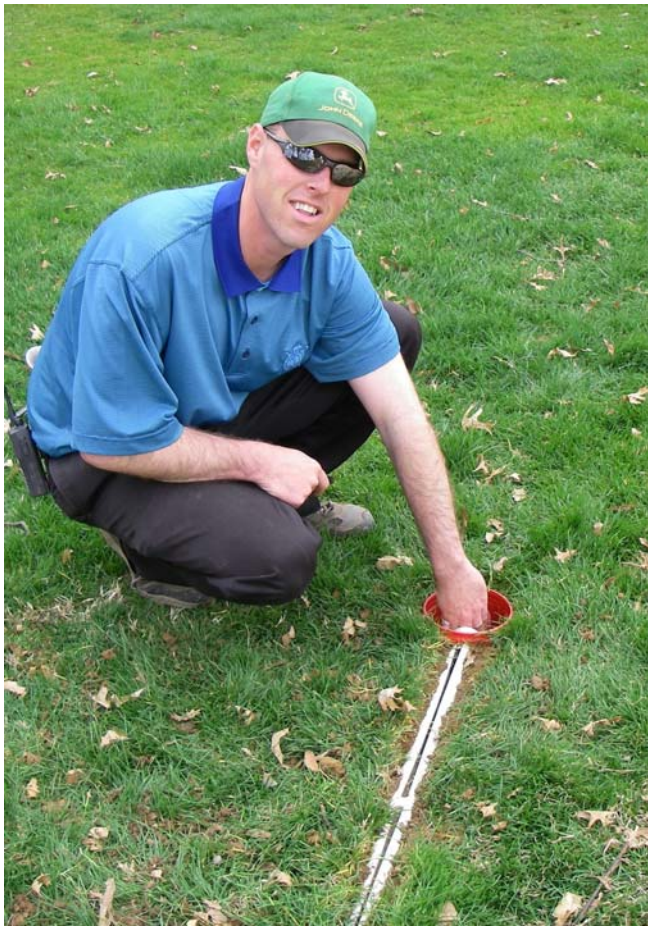
Douglas S. Richmond, Brandi J. Schemerhorn, and Mohamed G. Abdelwahab

SUMMARY

Billbugs pose a serious threat to managed turfgrass across North America. With the continuously expanding ranges of several species resulting in novel species interactions and a nation-wide collage of billbug species assemblages, satisfactory management has been difficult to achieve in many regions. Because the damaging larval stages of these insects cannot be identified to species, the seasonal population dynamics of many of the most common billbug pests remains largely unresolved. This project aimed to explore the potential for exploiting the unique properties of the nuclear ribosomal DNA multigene family (nrDNA) to identify species-specific molecular markers that can be used in diagnostic assays for several of the most common billbug species associated with turfgrass environments. Results to date include:

- Two nrDNA internal transcribed spacer regions (ITS1 and ITS2) were selected as the focus of efforts to differentiate 6 common billbug species (*Sphenophorus venatus*, *S. parvulus*, *S. minimus*, *S. inaequalis*, *S .cicatristriatus*, and *S. phoenesiensis*) associated with managed turfgrass.
- ITS1 and ITS2 each provided adequate variation in size and nucleotide sequence to allow for complete differentiation of the species examined based on either nrDNA region.
- Using these sequences, it should be possible to design specific primers that will facilitate identification of the otherwise indistinguishable larval stage of these insects based on only a single nrDNA region (ITS1 or ITS2).
- These findings could greatly facilitate the collection of baseline information on billbug seasonal ecology that is needed in order to formulate and improve management strategies and clarify new management opportunities.

Billbugs are increasingly being recognized as a serious threat to managed turfgrass across North America. The larvae of this group of insects (the billbug species complex) cause damage by feeding on or inside the stems, crowns, roots, stolons, and rhizomes of cool- and warm-season turfgrasses. The continuously expanding ranges of several billbug species, possibly driven by increasing interstate movement of turfgrass sod or changes in climatic conditions, have resulted in novel species interactions and a nation-wide collage of billbug species assemblages that has made satisfactory management difficult to achieve in many regions. Unfortunately, most of our current knowledge about billbug biology is based on a relatively small number of scattered studies that provide essential biological information about a few species, but an incomplete picture of the life cycles of these insects (3, 4, 5).



Several hundred billbug adult and larval specimens were collected from Indiana and Idaho using linear pitfall traps (shown above).

DOUGLAS S. RICHMOND, Ph.D., Associate Professor; BRANDON J. SCHEMERHORN, Ph.D., Adjunct Assistant Professor; and MOHAMED G. ABDELWAHAB, Ph.D., Post-doctoral Scientist; Department of Entomology, Purdue University, West Lafayette, IN.

USGA Turfgrass and Environmental Research Online 10(21):1-7.
TGIF Record Number: 194009

With few exceptions (1), information about larval population dynamics is almost completely lacking. Because the damaging larval stages of these insects cannot be identified to species, the seasonal population dynamics of our most common billbug pests remains largely unresolved. As a result, researchers, extension professionals, and turfgrass managers have little scientific foundation on which to establish or refine management programs.

In recognizing these facts, a long-term project to better understand the seasonal ecology of the billbug species complex associated with turfgrass in North America has been initiated. In working toward this goal, we set out to develop a DNA-based billbug larval identification tool that could be employed in this endeavor. The development of such a tool could greatly facilitate the collection of baseline information on billbug seasonal ecology that is needed in order to formulate and improve management strategies and clarify new management opportunities— especially in areas where multiple species are present.

Current Knowledge of Billbug Ecology

More than 60 native billbug species exist in the United States (8), with 25 species occurring in Florida (7) and at least 9 species attacking turfgrass in the contiguous United States (12). In New Jersey, four billbug species (*Sphenophorus inaequalis, S. minimus, S. parvulus*, and *S. venatus*) may occur in roughly equal numbers (5) in turfgrass environments, whereas at least four species (*S. parvulus* Gyllenhal, *S. minimus* Hart, *S. venatus* (Say), and *S. inaequalis* (Say)) are known to co-exist in the Midwest.

Unfortunately, the seasonal population dynamics of most billbug species associated with turfgrass are poorly understood and regional climatic differences can lead to a high degree of variability in the seasonal activity patterns of even the most widely studied species. Furthermore, most of what is known about billbug seasonal ecology is generalized from reported observations of only two species (*S. parvulus and S. venatus*) (3, 4, 10).

According to the reported literature, the number of generations per year may range from one to as many as six with overwintering populations being comprised primarily of adults or adults and larvae depending on latitude and location (3, 4, 5). Both adults and larvae may feed on turfgrass plants and although damage from larval feeding has been well documented, recent evidence indicates that *S. venatus* adults may also be capable of causing significant damage in warm-season grasses (1). Novel interactions among billbug species occurring in mixed populations could have unknown effects on billbug seasonal ecology. Because proper timing and targeting of insecticide applications depends heavily on a sound understanding of the seasonal ecology of the target insect(s), turfgrass professionals have increasingly struggled to manage this growing pest complex.

Current Status of Billbug Management

Although cultural tools, including the use of resistant plant varieties, provide alternatives to chemical management of billbugs in some situations, agronomic, ecological, and site-specific use requirements that exclude the use of these grasses (e.g., endophyte-enhanced perennial ryegrass and tall fescue) may dictate the use of chemical intervention as the only viable billbug management option. In this regard, three general management strategies can be found in the extension literature: 1) adult preventive, 2) larval preventive, and 3) larval curative.

The adult preventive strategy relies on the contact or oral activity of insecticides to reduce adult populations and break the reproductive cycle. This strategy relies on accurately timing insecticide applications to kill adults before significant numbers of eggs are deposited. When systemic insecticides such as neonicotinyls are used to target adults, accurate timing may be somewhat less critical because these insecticides are thought to possess some degree of activity against larvae feeding on or inside plant tissue.

When neonicotinyl or diamide insecticides are used as part of an adult preventive approach, this approach overlaps and becomes more or less indistinguishable from the larval preventive

approach. The larval preventive approach relies on the use of such extended residual, systemic insecticides to reduce larval populations before visible damage occurs. As such, proper application timing depends on the time required for the plant to acquire biologically active levels of the insecticide after it is applied, and how long the material maintains appropriate levels of systemic activity. Naturally, this time period may vary with environmental conditions and the chemical properties of the specific compound used.

The last and least common chemical management strategy employed against billbugs is the larval curative strategy. This strategy typically requires the use of soil insecticides such as carbaryl, trichlorfon, or faster acting neonicotnyls (thiamethoxam or chlothianidin) to reduce larval populations once damage becomes visible. Effective management can be difficult to achieve using this approach because the application window, the time between diagnosis of damage and pupation (pupae are not susceptible to most insecticides), is significantly reduced. Furthermore, damage may be difficult to reverse at this time.

Although these three strategies can be effective against billbug populations composed primarily of one dominant species, their adaptability for situations where mixed species coexist or multiple and overlapping generations of one or a few key species occur is not clear. Difficulties experienced by professional turfgrass managers in reducing mixed populations to acceptable levels implies that strategies based on our current understanding of billbug seasonal ecology are not adequate. A better understanding of the seasonal ecology of these mixed populations could provide the insight necessary to significantly improve management strategies, increase efficiency, and reduce insecticide inputs.

A New Approach for Studying Billbug Population Dynamics

Clearly, the problems associated with billbug management require an innovative approach to enhance our understanding of the population dynamics of these insects and improve management programs. Although trapping and monitoring of adult populations can be used to determine the species composition of a billbug complex, a much better understanding of larval population dynamics will be required to identify how different co-occuring species may contribute to damage over the course of a season. In doing so, new and more efficient management opportunities may be forthcoming.

The tandemly arrayed nuclear ribosomal RNA multigene family (nrDNA) is a common target of efforts to differentiate closely related species because of useful features of its sequence organization and evolution. Portions of the nrDNA coding sequences are highly conserved even between distantly related species, allowing the application of 'universal' primers for amplification from any species. The non-coding nrDNA spacer sequences, however, can be highly variable in length and sequence even among closely related species. Concerted evolution acting on nrDNA arrays maintains sequence homogeneity within species as it drives differentiation between species, a pattern that explains the utility of nrDNA for species-diagnostic assays.

Ribsomoal DNA-based PCR assays have been developed for many species complexes, including the *Anopheles gambiae* complex, the *Anopheles funestus* complex, the *Rhodnius* species complex and numerous other species complexes both within and outside of the Class Insecta (2, 6, 9). It may also be possible to exploit the characteristics of certain nrDNA arrays in order to develop a genetic fingerprinting technique for differentiating billbug species, making accurate identification of billbug larvae a realistic possibility for researchers and managers alike.

The long-term goal of this research is to understand the seasonal ecology of the billbug species complex associated with managed turfgrass in North America. In working toward this goal, this project aimed to exploit the properties of the nrDNA multigene family to identify species-specific markers that could be used to develop diagnostic assays for a major portion of the billbug species complex associated with turfgrass environments. Once identified, these unique DNA

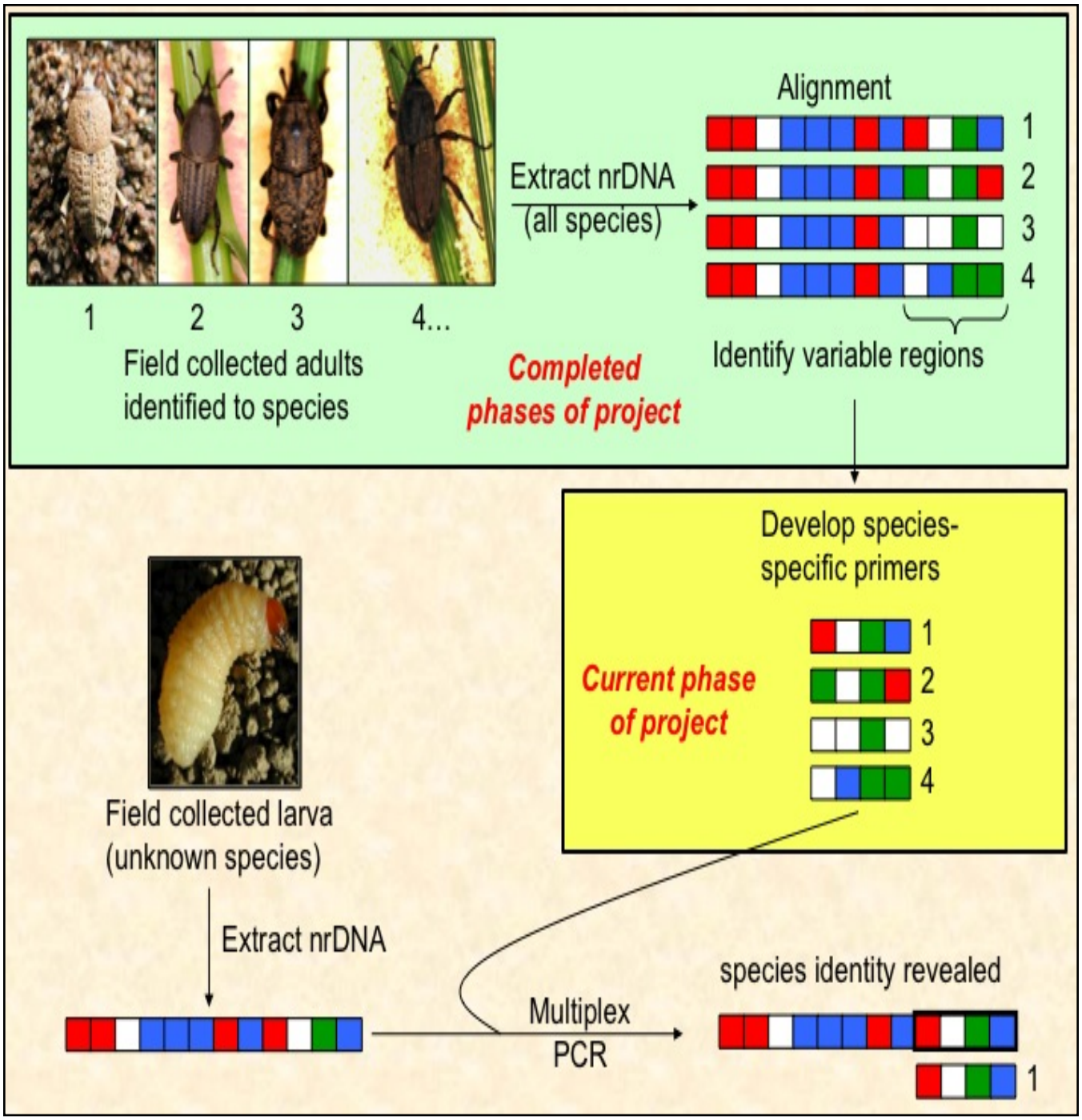


Figure 1. Schematic diagram describing the multiplex PCR procedure being developed to facilitate identification of billbug larvae.

markers could provide the basis for development of a simple and dependable diagnostic tool that could be compatible with a multiplex PCR reaction using species-specific primers (Figure 1). This technology could be readily accessed by superintendents, consultants, and researchers who require proper diagnosis of billbug infestations in order to prescribe, and improve management programs. Development of such a diagnostic tool could vastly improve our ability to answer basic questions about billbug seasonal ecology and population dynamics.

Experimental Procedures

Several hundred billbug adult and larval specimens were collected from Indiana and Idaho

using linear pitfall traps, and preserved in 90% ethanol between 2006 and 2010. All adult specimens were identified to species using morphological characters (5, 8, 12). The specific locations from which specimens were collected are shown in Table 1.

Billbug genomic DNA was extracted from the previously identified adult specimens using a modified version of the procedure described in the DNeasy kit (QIAGEN, Valencia, CA). PCR was performed using the primer of regions ITS1 and ITS2 using a 50 ul reaction mixture containing 2.5 units of Taq polymerase, 100 ng of template DNA, 0.5 ul of each primer, 200 uM of four dNTP and a reaction buffer. Thirty amplification cycles were performed in a thermocycler with each cycle.

The amplified DNA fragments were analyzed in 1.5% agarose gels in TAE buffer containing 0.5 ug/ml ethidium bromide, using short wave ultraviolet to visualize the DNA. The amplicons from each species were extracted from the gel using a gel extraction kit. The purified DNA fragments were ligated into TOPO vector (Invitrogen) and the ligated mixture was introduced into TOP 10 chemically competent *E.coli* . The transformants were selected on Luria-Bertani plates containing 100 ug of ampicillin per milliliter. The inserted DNA fragments were amplified using T3 of the vector and reverse primer of the inserted gene. The PCR products of ~ 800 -1000 bp for ITS1 and ~570-670 bp for ITS2 were confirmed using 1% agarose/TAE gel electrophoresis.

Plasmid DNA including the fragment were also purified from overnight-cultured bacteria using a DNA purification system (Wizard plus SV Minipreps, Promega). Three clones of Purified plasmids were sequenced with a DNA sequence system (Beckman 5000). The DNA sequences were aligned using Clustal X (11).

Results and Discussion

In order to discriminate between billbug species using nrDNA sequences, the length and/or order of nucleotides composing the target sequences must be measurably different among species. When one region provides resolution between some, but not all species, additional regions may be included in combination with the first in order to further resolve the remaining species.

Two regions were selected as the focus of our analysis (ITS1 and ITS2) to differentiate 6 common billbug species (*Sphenophorus venatus*, *S. parvulus*, *S. minimus*, *S. inaequalis*, *S .cicatristriatus*, and *S. phoenesiensis*) associated with cultured turfgrass. While ITS1 yielded amplicons with predicted sizes ranging across species from 800 to 1,000 bp, ITS2 yielded amplicons with predicted sizes ranging from 600 to 700 bp. For diag-

Billbug Species	Collection Location(s)	ITS1 (bp)	ITS2 (bp)
S. cicatristriatus	Boise, ID	884	616
S. inaequalis	West Lafayette, IN		
	Hamlet, IN	1,012	679
S. minimus	West Lafayette, IN	895	579
S. parvulus	West Lafayette, IN	882	614
S. phoeniciensis	Boise, ID	904	629
S. venatus	Evansville, IN	855	612

Table 1. Size (in base pairs, bp) of the nrDNA regions ITS1 and ITS2 and collection locations for six species of billbugs (*Sphenophorus* spp.) associated with managed turfgrass in the United States.

nostic purposes, both ITS1 and ITS2 each provided enough variation in size and nucleotide sequence among species to allow for complete differentiation of the species examined based on either nrDNA egion. Table 1 lists the size (in base pairs, bp) of each respective region (ITS1 and ITS2) for each billbug species examined. The specific order of nucleotides in each sequence remains to be published.

Findings of this research indicate that it should be possible to design specific primers that will facilitate identification of the otherwise indistinguishable larval stage of these insects based on only a single nrDNA region (ITS1 or ITS2). However, due to the relatively low mobility of adult billbugs, it is likely that geographically separated populations are also reproductively isolated, resulting in the potential for geographically distinct populations of most if not all species examined.

For this reason, some level of genetic variation between populations of any single billbug species may be expected. Whether or not such variation is expressed in ITS1 or ITS2 remains a critical question to be examined, but a large, multi-regional collaborative effort will be required to achieve this goal. In further exploring genetic variation among geographically isolated billbug populations, the development of more geographically specific primers may prove useful for species identification and tracking of novel infestations. Nonetheless, results herein represent a crucial step that will facilitate the development of species-specific primers for studies aimed at understanding the population dynamics of the billbug complex.

Acknowledgements

The authors would like to thank USGA Turfgrass and Environmental Research Program and the Midwestern Regional Turf Foundation for providing financial support for this project. The assistance of Tom Salaiz (University of Idaho) and Joe Aholt (Hillcrest Country Club) is gratefully acknowledged.

References Cited

1. Doskocil, J. P. 2010. The biology and ecology of hunting billbug in North Carolina turfgrass. Ph.D. Dissertation. North Carolina State University, Raleigh, NC. (TGIF Record 177538)

2. 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.

3. Hansen, J. D. 1987. Seasonal history of bluegrass billbug, *Sphenophorus parvulus* (Coleoptera: Curculionidae) in a range grass nursery. *Environ. Entomol.* 16:752-756. (TGIF Record 192827)

4. Huang, Ta-I, and E. A. Buss. 2009. Billbug (Coleoptera: Curculionidae) species composition, abundance, seasonal activity, and developmental time in Florida. *J. Econ. Entomol.* 102:309-314. (TGIF Record 147052)

5. Johnson-Cicalese, J. M., G. W. Wolfe, and C. R. Funk. 1990. Biology, distribution and taxonomy of billbug turf pests (Coleoptera:Curculionidae). *Environ. Entomol.* 19:1037-1046. (TGIF Record 29148)

6. 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.

7. Peck, S. B., and M. C. Thomas. 1998. A distributional checklist of the beetles (Coleoptera) of Florida. Arthropods of Florida and neighboring land areas. Vol. 16. Florida Department of Agriculture and Consumer Services, Gainesville, FL.

8. Niemczyk, H. D., and D. J. Shetlar. 2000.

Destructive turf insects, 2nd edition. H.D.N. Books, Wooster, OH. (TGIF Record 71482)

9. 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.

10. Tashiro, H., and K. E. Personius. 1970. Current status of the bluegrass billbug in its control in western New York home lawns. *J. Econ. Entomol.* 63:23-29. (TGIF Record 1380)

11. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The Clustal X windows interface flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* 24:4876-4882.

12. Vittum, P. J., M. G. Villani, and H. Tashiro. 1999. Turfgrass insects of the United States and Canada, 2nd edition. Cornell University Press, Ithaca, NY. (TGIF Record 64756)