Differentiation of *Diaprepes abbreviatus* and *Pachnaeus litus* (Coleoptera: Curculionidae) Egg Masses: PCR-Restriction Fragment-Length Polymorphism and Species-Specific PCR Amplification of 18S rDNA Products

A. A. WEATHERSBEE III, R. C. BULLOCK, T. D. PANCHAL, AND P. M. DANG

USDA–ARS, U.S. Horticultural Research Laboratory, 2001 South Rock Road, Fort Pierce, FL 34945

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ABSTRACT The root weevils *Diaprepes abbreviatus* (L.) and *Pachnaeus litus* (Germar) are both pests of Florida horticulture, but *D. abbreviatus* is a regulated exotic species that causes severe damage whereas *P. litus* is considered a minor pest. Egg masses of these two weevil species are indistinguishable when they are detected on host plants. Two approaches to differentiating the egg masses are described herein. Total genomic DNAs extracted from *D. abbreviatus* and *P. litus* were used for polymerase chain reaction (PCR) amplification and the PCR products were sequenced to obtain the 2014-bp sequence of the complete 18S rRNA gene for each species. A 446-bp region amplified from the 5′ end of the 18S rDNA of *P. litus* contained a restriction fragment-length polymorphism marker, an extra *Bst*U I recognition site that was not present in *D. abbreviatus*. Agarose gel electrophoresis of the restriction enzyme-digested PCR products produced a restriction pattern that enabled differentiation of the egg masses. Additionally, two species-specific reverse primers were designed to exploit a single nucleotide polymorphism (SNP) that occurred at the restriction fragment-length polymorphism marker site. These reverse primers differed only by a single nucleotide at the 3′ end. When used in concert with a standard 18S rDNA forward primer, each species-specific reverse primer distinctly amplified a 256-bp product only when the correct genomic DNA was present.

KEY WORDS *Diaprepes abbreviatus*, *Pachnaeus litus*, 18S rDNA, genotyping, molecular detection

THE ROOT WEEVILS *Diaprepes abbreviatus* (L.) and *Pachnaeus litus* (Germar) are pests of citrus and ornamental plants in Florida. Their ranges overlap in southern Florida where both species are found in commercial and dooryard plantings. *Diapepes abbreviatus*, an exotic species, is more destructive to Florida horticulture than the native *P. litus*. *Diaprepes abbreviatus* has become well established in the state since it was first discovered in Apopka, Florida, in 1964 (Woodruff 1964). It is known to be associated with at least 270 plant species and can complete its life cycle and cause economic damage in citrus, sugar cane, and many ornamental plants and vegetable crops (Simpson et al. 1996). Annual losses to the citrus industry alone are estimated at more than $75 million (Anonymous 1997). Conversely, *P. litus* is normally considered a minor pest although it can cause damage to young citrus plantings (Tarrant and McCoy 1989).

The life cycles of these root weevils are similar, particularly in commercial citrus. The adults feed on tender, flushing foliage of citrus to obtain the nutrients required for egg production. Eggs of both weevils are laid on mature leaves, often on the same trees from which the adults have been feeding. Upon hatching, the larvae drop to the ground and quickly burrow into the soil where they begin feeding on roots. Citrus is a preferred host for both weevils but damage caused by *D. abbreviatus* in particular reduces fruit production and tree fitness, and can lead to tree death (Schroeder and Beavers 1977).

Although both weevils are considered pests of commercial citrus, the potential for economic damage is much greater when *D. abbreviatus* is present (Duncan et al. 2001). Counts of egg masses have been used as indices of *D. abbreviatus* abundance and potential damage to infested groves (Doostdar et al. 2002, Schroeder and Sutton 1977). Also, monitoring programs for egg parasitoids, released as biological control agents against *D. abbreviatus*, rely on visual examination of weevil egg masses for signs of parasitism (Peña et al. 2000). Unfortunately, the egg masses deposited by *D. abbreviatus* and *P. litus* cannot be visually differentiated, while rearing-out and identifying the larvae are difficult and time-consuming.

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Because *D. abbreviatus* is an exotic species subject to quarantine regulations, species identification is absolutely required when detected egg masses can be confused with those of the unregulated pest. The need to differentiate eggs of root weevils was recognized by Jones et al. (1984), who developed an identification method based on enzyme electrophoresis to differentiate the eggs of root weevils. Alternative methods are warranted because of currently available technologies. Two simple and quick molecular approaches were developed and compared here to distinguish the egg masses of *D. abbreviatus* and *P. litus* with greater efficiency. The ability to differentiate root weevil egg masses will improve predictions of prospective damage to citrus, facilitate in monitoring activities of egg parasitoids and their effectiveness in targeting *D. abbreviatus*, and also improve species identification for regulatory purposes.

Materials and Methods

Insects and Plant Material. Adults of *D. abbreviatus* and *P. litus* were collected from citrus groves in Saint Lucie County, FL. Insects were maintained in small cylindrical cages (25 cm tall × 23 cm diameter) with screened lids in the laboratory at 25°C and a photoperiod of 10:14 (L:D) h and fed flushing foliage of *Citrus macrophylla* Wester. Insects deposited their eggs on mature leaves of *C. macrophylla* and on artificial substrates fashioned from waxed paper strips (Wolcott 1933). Egg masses were excised from citrus leaves and waxed paper strips and stored at −80°C until genomic DNA was extracted. Some egg masses were incubated inside plastic bags in the laboratory for 10 d until first-instar larvae emerged. Immediately after hatching, the larvae were stored singly at −80°C until genomic DNA was extracted.

Isolation of Genomic DNA. Total genomic DNA was extracted from single first-instar larvae and single egg masses of *D. abbreviatus* and *P. litus* using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI). Genomic DNA was isolated following the manufacturer's protocol for animal tissue with the following modifications to account for the small amounts of insect tissue that were used. Insect tissues were ground in 300 μl of chilled ethylenediaminetetraacetic acid (EDTA)/nuclei lysis solution buffer (60 μl of 0.5 M EDTA, 250 μl of nuclei lysis solution); then, 3 μl of 20 mg/ml proteinase K was added and the suspension was incubated overnight, or for 3 h with gentle shaking, at 55°C. The remaining procedures were the same as described by the manufacturer except that the purified DNA was resuspended in 30 μl of rehydration solution.

PCR and Sequencing of 18S rDNA. Genomic DNA isolated from the egg masses and first-instar larvae of *D. abbreviatus* and *P. litus* was used as template to amplify 18S rDNA products in a PCR. The 18S rDNA was PCR amplified as three fragments using the primer sets 18SFrontF/18SFrontR and 18SBackF/18SBackR reported by Campbell et al. (1994) and 18SFrontI2 F/18SBackI1R designed herein, and as the full-length gene with the primer set 18SFrontF/18SBackR (Table 1). The reaction mixtures were prepared using a Qiagen PCR Core Kit (Qiagen, Valencia, CA) in 50-μl volumes containing 1× PCR buffer solution, 1× Q solution, 0.2 mM of each dNTP, 0.2 *u*M of each primer, and 1.25 U of *Taq*DNA polymerase. Amplifications were performed using a PTC-100 thermocycler (MJ Research, Watertown, MA) with PCR conditions as follows: 2 min at 98°C; then 35 cycles of 1 min at 96°C, 1 min at 56°C, and 3 min at 74°C; and a final extension step of 7 min at 74°C.

Table 1. Primers used to PCR amplify and sequence, whole and partial, 18S rDNA products from genomic DNA extracted from *D. abbreviatus* and *P. litus* egg masses and larvae

Primer name[a]	Primer sequence	Tm[b]
18SFrontF*	CTGGTTGATCCTGCCAGTAGT	57.65
18SFrontR*	GGTTAGAACTAGGGCGGTATC	56.37
18SBackF*	GATACCGCCCTAGTTCTAACY	55.11
18SBackR*	TCCTTCCGCAGGTTCACC	59.58
18SFrontI1F	GTCTGCCTTATCAACTGTCGA	56.22
18SFrontI1R	TCGACAGTTGATAAGGCAGAC	56.22
18SFrontI2F	TTACTTTGAACAAATTAGAGTGCT	54.66
18SFrontI2R	AGCACTCTAATTTGTTCAAAGTAA	54.66
18SBackI1F	AGCTCTTTCTTGATTCGGTGG	59.70
18SBackI1R	CCACCGAATCAAGAAAGAGCT	59.70
18SBackI2F	TTAGATGTTCTGGGCCGC	57.51
18SBackI2R	GCGGCCCAGAACATCTAA	57.51
18SFrontR-DA	TGTACGATGACGAGCGCA	57.89
18SFrontR-PL	TGTACGATGACGAGCGCG	60.34
18SFrontR-DAPL	CTGCCTTCCTTGGATGTGG	59.36

[a] Primers followed by an asterisk were reported by Campbell et al. (1994). Abbreviations in primer names: F, forward; R, reverse; I, internal; DA, *D. abbreviatus;* and PL, *P. litus.* 'Front' and 'Back' refer to 5′ and 3′ portions of the 18S rRNA gene.

[b] Melting temperatures of primers.

The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. The products were direct sequenced in both directions using Big Dye Version three chemistry, and sequences were generated using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) located at the U.S.Horticultural Research Laboratory, Ft. Pierce, FL. Multiple overlapping sequences were generated using combinations of the primers reported by Campbell et al. (1994) and the eight internal primers designed here (Table 1). The PCR products obtained from whole egg masses and at least three individuals of each species were sequenced to generate consensus data and to confirm gene conservation at the species level. Nucleotide base calls were analyzed for data quality with TraceTuner (Paracel, Pasadena, CA) software. Contiguous sequences were generated using Sequencher (Gene Codes, Ann Arbor, MI) software and consensus data were formed from replicates. Automated alignment of complete 18S rDNA sequences for both species was achieved using the default parameters for CLUSTAL W (Thompson et al. 1994). The sequences were deposited in the GenBank database under the accession numbers AY157729 and AY157730 for *D. abbreviatus* and *P. litus*, respectively.

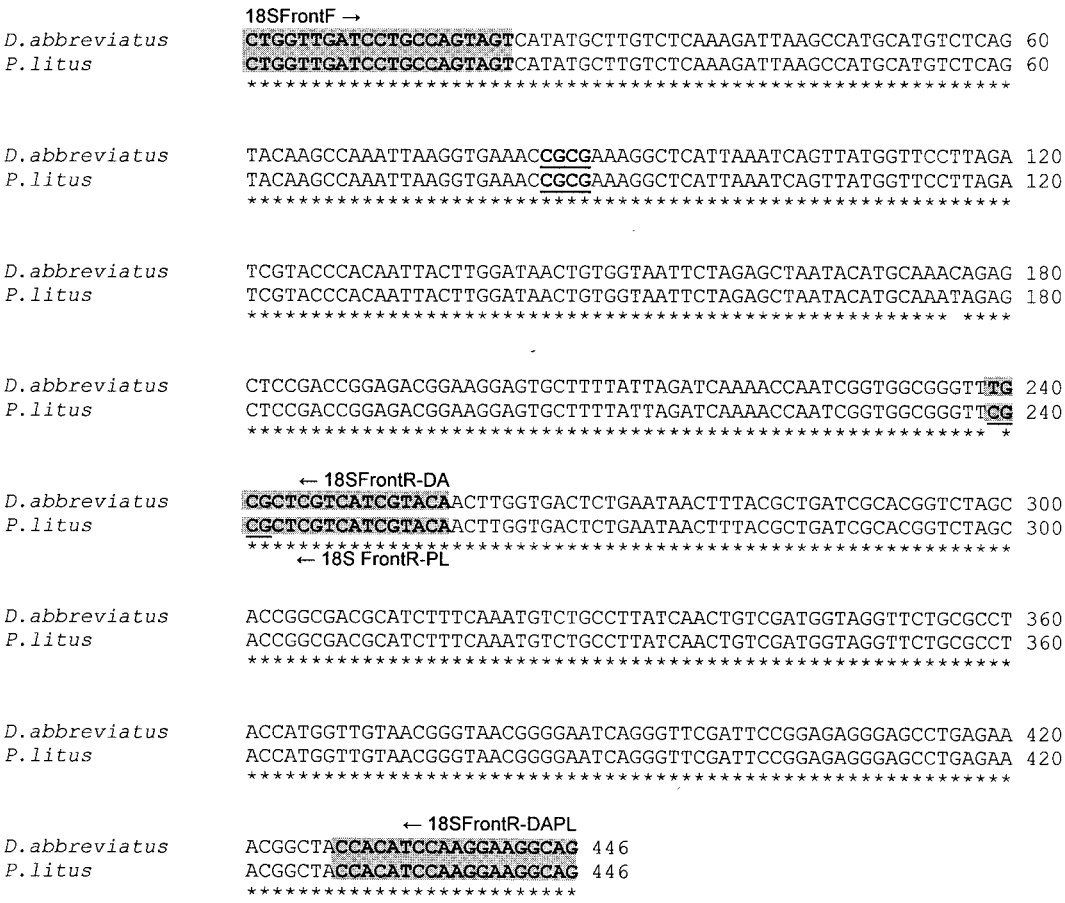

Fig. 1. Clustal W alignment of *D. abbreviatus* and *P. litus* 18S rDNA partial sequences. The locations of selected priming sites are in bold case and shaded boxes. Names of primers are given adjacent to the boxes. Restriction enzyme (*Bst*U I) recognition sites are in bold and underlined case. Note the SNP at position 239 was used to genotype the two species.

PCR-restriction Fragment-Length Polymorphism Analyses. Genomic DNA derived from single egg masses of each weevil species was used in the PCR-restriction fragment-length polymorphism and species-specific primer analyses. Egg masses removed from waxed paper strips and egg masses adhered to excised sections of citrus leaf tissue were tested to ascertain whether the plant tissues would interfere with DNA extraction or the PCR.

Sequencher software was used to analyze the 18S rDNA sequences for restriction enzyme recognition sites that could be used to differentiate *D. abbreviatus* and *P. litus* egg masses. An extra *Bst*U I (New England Biolabs, Beverly, MA) recognition site found in the *P. litus* sequence was used as a restriction fragment-length polymorphism marker to distinguish the two species.

The common reverse primer, 18SFrontR-DAPL, was designed and used in concert with the forward primer, 18SFrontF (Table 1), to amplify a 446-bp region from the 5′ end of each 18S rDNA. The PCR conditions were as previously described. The amplified products encompassed the region containing the restriction fragment-length polymorphism marker, and their small size simplified the PCR-restriction fragment-length polymorphism procedure. Restriction analyses were performed using the restriction enzyme *Bst*U I according to the manufacturer's protocol. The DNA fragments were separated on a 0.8% agarose gel, stained with ethidium bromide, and visualized and photographed under ultraviolet light.

Species-Specific Primer Analyses. Two species-specific reverse primers were developed to eliminate the need for restriction analyses and increase the economy of the differentiation procedure. The reverse primers 18SFrontR-DA and 18SFrontR-PL were designed to exploit the SNP at position 239 in the aligned sequences (Fig. 1) whose location was synonymous with the restriction fragment-length polymorphism marker. These primers differed only by the terminal nucleotide at the 3′ extension end (Table 1), and both amplified 256-bp PCR products. Species-specific amplification of 18S rDNA products was first attempted using the same PCR conditions described above. Subsequently, the PCR conditions were optimized for annealing temperature to increase primer specificity.

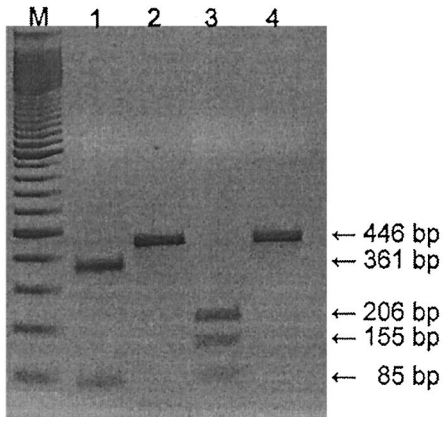

Fig. 2. Restriction fragment length polymorphism analysis of 18S rDNA PCR products amplified with primers 18SFrontF and 18SFrontR-DAPL from genomic DNA of weevil eggs. Lane M: wide range DNA marker, 50–10,000 bp (Sigma). Lanes 1 and 2: *D. abbreviatus* 18S rDNA fragment cut with *Bst*U I and uncut, respectively. Lanes 3 and 4: *P. litus* 18S rDNA fragment cut with *Bst*U I and uncut, respectively.

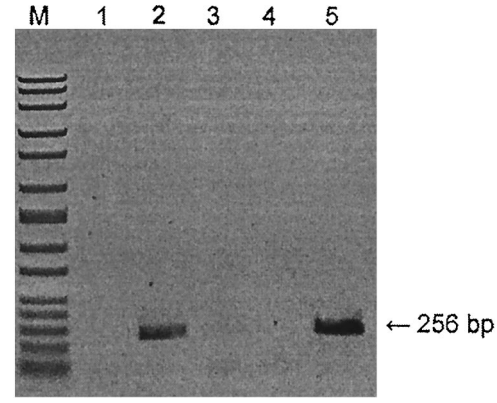

Fig. 3. 18S rDNA PCR products amplified using species-specific reverse primers to differentiate weevil genomic DNA extracted from eggs on citrus leaf tissue. Lane M: wide range DNA marker, 50–10,000 bp (Sigma). Lane 1: negative control. Lanes 2 and 3: *D. abbreviatus* and *P. litus* genomic DNAs, respectively, amplified with 18SFrontF and 18S-FrontR-DA primers. Lanes 4 and 5: *D. abbreviatus* and *P. litus* genomic DNAs, respectively, amplified with 18SFrontF and 18SFrontR-PL primers.

The annealing temperature was raised to 63°C; other conditions remained the same. The DNA fragments were stained, visualized, and photographed as previously described.

Results

The 18S rRNA gene was PCR amplified as a full-length gene product and in partial fragments from the genomic DNA of both weevils (data not shown). Sequencing reactions and processing of the data were simplified by using the full-length gene product as template for DNA sequencing. Consensus sequences generated from replicates confirmed that the gene is conserved at the species level in both *D. abbreviatus* and *P. litus*.

A CLUSTAL W (Thompson et al. 1994) alignment of the consensus sequences for the complete 18S rDNA sequences of *D. abbreviatus* and *P. litus* indicated that they are >99% homologous (Fig. 1). There were only six SNPs observed between the two sequences along the entire length of the gene (2014 bp). Percentages of the nucleotides adenine (A), thymine (T), guanine (G), and cytosine (C) were 24, 24, 23, and 28, respectively, for both species. The total GC content was 51.45%, whereas that for AT was 48.55% averaged over both species.

The restriction enzyme, *Bst*U I, recognizes the nucleotide sequence CGCG. The 446-bp fragments amplified by the primers 18SFrontF and 18SFrontR-DAPL contained only one *Bst*U I recognition site when derived from *D. abbreviatus* genomic DNA and two sites when derived from *P. litus* (Fig. 1). The *Bst*U I digestion of the PCR product obtained from *D. abbreviatus* resulted in two fragments of 361 and 85 bp in length, whereas that from *P. litus* resulted in three fragments of 206, 155, and 85 bp (Fig. 2).

The novel recognition site for *Bst*U I, present in the 18S rDNA of *P. litus*, was caused by a single nucleotide polymorphism (SNP) occurring at position 239 in the 446-bp PCR fragment. The species-specific reverse primers, 18SFrontR-DA and 18SFrontR-PL, that targeted the SNP were able to discriminate which weevil genomic DNA was present in the PCR. Initially, small amounts of nonspecific product were PCR amplified for both species when the annealing temperature was 56°C; however, differentiation was possible based on band intensity of the PCR products under ultraviolet light (data not shown). When the annealing temperature was increased to 63°C, the PCR was highly specific and 256-bp products were distinctly amplified only in the presence of the correct genomic DNA (Fig. 3). The reaction was consistent whether or not the DNA was extracted from egg masses adhered to leaf tissue or egg masses excised from waxed paper strips. The results presented in Fig. 3 were developed using genomic DNAs extracted from samples containing leaf tissue. The PCR products were formed only when the 3′ terminal nucleotide of the reverse primer complimented the template nucleotide present at the position of the SNP.

Discussion

The 18S rRNA genes have been used extensively to infer evolutionary relationships among arthropod taxa because they consist of both conserved regions and rapidly evolving, highly variable expansion regions—V2, V4, V7, and V9 (Chong et al. 1999). These variable expansion regions can be used to distinguish recently diverged taxa for interspecific differences (Campbell et al. 1994). Although the differences between *D. abbreviatus* and *P. litus* sequences were minimal in these variable expansion regions, successful 18S rDNA

genotyping of their egg masses was demonstrated because of the presence of a SNP that occurred in the V2 region of the gene. The 18S rRNA gene was chosen because it is recognized as one of the most conserved at the species level. Although a less conserved gene could be used, this could lead to false-negative results. Scheffer et al. (2001) raised the concern that recent divergence at the subspecies level among unsampled populations could lead to misidentification when detection methods based on variability are used in place of sequence data. Using the highly conserved 18S rRNA gene abridged this concern. It is important to note that another root weevil species, *Pachnaeus opalus* Olivier, occurs in northern Florida. Although only *P. litus* occurs in southern Florida, the two species may be coexistent where their ranges intersect (Futch et al. 2002). The adults of *P. litus* and *P. opalus* are distinguishable but the eggs are similar. This genotyping approach is not recommended where *P. opalus* is present until that species has been evaluated.

The 18S rDNAs of some insects are difficult to amplify by PCR as full-length products (Campbell et al. 1994), so those of *D. abbreviatus* and *P. litus* initially were amplified as fragments. Subsequently, full-length products were amplified from both species under the same PCR conditions and these were used as templates for sequencing. The length of the 18S rRNA gene in insects often exceeds 2000 bp, and unusually long sequences of 2469 bp and 3316 bp have been reported for the aphid, *Acryrthosiphon pisum* (Kwon et al. 1991), and the strepsipteran, *Xenos vesparum* (Chalwatzis et al. 1995), respectively. The 2014-bp lengths found here for this gene in *D. abbreviatus* and *P. litus* are typical of those reported for other weevil species (Farrell 1998). The GC contents of 51.4% observed for both species are slightly less than the average of GC contents for insects in general (54.4%), but are well within the reported ranges of 50–60% (Chong et al. 1999). Notably, the level of homology for the 18S rDNA sequences of *D. abbreviatus* and *P. litus* is substantially greater than among representatives of other genera in the same tribe, Entimini (Farrell 1998). Although these insects represent different genera, the level of sequence divergence indicated (<1%) is consistent with that at or below the species level in other related taxa (Farrell et al. 2001). These two weevil species are probably recently diverged.

Although the 18S rDNA sequences of *D. abbreviatus* and *P. litus* are nearly identical, the PCR-restriction fragment-length polymorphism approach consistently distinguished the egg masses of these two species in a simple four-step process: genomic DNA extraction, PCR amplification of the 18S rDNA product, restriction enzyme digestion, and agarose gel electrophoresis. The procedure can be completed in 1 d with the 3 h proteinase K incubation step during the DNA extraction protocol. Scheffer et al. (2001) reported that species identification by PCR restriction fragment-length polymorphism should not be based only on negative results. This is particularly true when a restriction enzyme cuts the DNA fragment of one species but not the other, because a negative reaction can not be differentiated from a reaction failure. This was not a concern in the procedure described here because the enzyme, *Bst*U I, cuts the DNA fragments from both weevils but with different frequency.

The species-specific primer approach is more efficient and less costly than the PCR-restriction fragment-length polymorphism approach in differentiating *D. abbreviatus* and *P. litus* eggs. The process was reduced to three steps by removing the need for a restriction digestion. Differentiation is based on the specificity of the 3′ terminal nucleotides of each species-specific primer for the complementary template nucleotide at the position of the targeted SNP, and is dependent on PCR conditions. The utilities and potential uncertainties of this approach to SNP detection have been discussed previously (Ahmadian and Lundberg 2002). For example, some 3′ end nucleotide mismatches between primer and template may be extended as well as perfectly matched primer and template (Newton et al. 1989). Nevertheless, optimizing the PCR conditions (e.g., temperature) for the targeted DNA templates can avoid false amplification (Kwok et al. 1990). The method demonstrated here with species-specific primers requires attention to detail, specifically regarding the optimization of the primer annealing temperature for *D. abbreviatus* and *P. litus* templates. The PCR was specific and consistent at an annealing temperature of 63°C. Recently, apyrase-mediated allele-specific extension (AMASE) was shown to improve the reliability of genotyping based on SNPs by taking advantage of the slower reaction kinetics that occur under conditions of primer and template mismatch (Ahmadian et al. 2001). Combinations of AMASE, fluorescently-labeled nucleotides, and real-time PCR will further improve the utility of SNPs in genotyping both at and below the species level.

References Cited

Anonymous. 1997. Diaprepes Task Force Minutes, July 17, 1997. University of Florida. Lake Alfred, FL.

Ahmadian, A., B. Gharizadeh, D. O'Meara, J. Odeberg, and J. Lundberg. 2001. Genotyping by apyrase mediated allele-specific extension. Nucleic Acids Res. 29: E121.

Ahmadian, A., and J. Lundberg. 2002. A brief history of genetic variation analysis. Biotechniques 32: 1122–1137.

Campbell, B. C., J. D. Steffen-Campbell, and R. J. Gill. 1994. Evolutionary origin of whiteflies (Hemiptera: Sternorrhyncha: Aleyrodidae) inferred from 18S rDNA sequences. Ins. Mol. Biol. 3: 73–88.

Chalwatzis, N., A. Baur, E. Stetzer, R. Kinzelbach, and F. K. Zimmerman. 1995. Strongly expanded 18S rRNA genes correlated with a peculiar morphology in the insect order Strepsiptera. Zoology 98: 115–126.

Chong, P. C., J. M. Hancock, U. W. Hwang, and W. Kim. 1999. Analysis of the primary sequence and secondary structure of the unusually long SSU rRNA of the soil bug, *Armadillidium valgare.* J. Mol. Evol. 49: 798–805.

Doostdar, H., R. C. Bullock, R. A. Adair, and R. T. Mayer. 2002. When to Spray? Population Dynamics of *Diaprepes abbreviatus* in Indian River County Citrus Groves. Diaprepes Task Force Meeting, February 8, 2002, University of Florida, Lake Alfred, FL.

Duncan, L. W., C. W. McCoy, P. A. Stansly, J. H. Graham, and R. F. Mizell. 2001. Estimating the relative abundance of adult citrus root weevils (Coleoptera: Curculionidae) with modified Tedders traps. Environ. Entomol. 30: 939–946.

Farrell, B. D. 1998. "Inordinate Fondness" explained: why are there So many beetles? Science 281(5376): 555–559.

Farrell, B. D., A. S. Sequeira, B. C. O'Meara, B. B. Normark, J. H. Chung, and B. H. Jordal. 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). Evolution 55: 2011–2027.

Futch, S. H., C. W. McCoy, and H. N. Nigg. 2002. A guide to soil insect pests identification. Document HS-868. Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL.

Jones, I. F., J. B. Beavers, and W. J. Schroeder. 1984. Identification of weevil egg masses (Coleoptera: Curculionidae) by enzyme electrophoresis. Fla. Entomol. 67: 573–575.

Kwok, S., D. E. Kellog, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky. 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type I model studies. Nucleic Acids Res. 18: 999–1005.

Kwon, O-Y., K. Ogino, and H. Ishikawa. 1991. The longest 18S ribosomal RNA ever known. Eur. J. Biochem. 202: 827–833.

Newton, C. R., A. Graham, L. E. Heptinstall, S. J. Powell, C. Summers, N. Kalsheker, J. C. Smith, and A. F. Markham. 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. 17: 2503–2516.

Peña, J. E., D. G. Hall, R. Nguyen, R. Duncan, D. Amalin, P. Stansly, C. McCoy, R. Adair, S. L. Lapointe, and H. Browning. 2000. Efforts toward establishment of biological control agents of Diaprepes Weevil. Citrus Industry 81: 30–38.

Scheffer, S. J., A. Wijesekara, D. Visser, and R. H. Hallett. 2001. Polymerase chain reaction-restriction fragment-length polymorphism method to distinguish *Liromyza huidobrensis* from *L. langei* (Diptera: Agromyzidae) applied to three recent leafminer invasions. J. Econ. Entomol. 94: 1177–1182.

Schroeder, W. J., and J. B. Beavers. 1977. Citrus root weevils in Florida: Identification, biology, and control. Proc. Int. Soc. Citriculture 2: 498–500.

Schroeder, W. J., and R. A. Sutton. 1977. Citrus root damage and the spatial distribution of eggs of *Diaprepes abbreviatus.* Fla. Entomol 60: 114.

Simpson, S. E., H. N. Nigg, N. C. Coile, and R. A. Adair. 1996. *Diaprepes abbreviatus* (Coleoptera: Curculionidae): Host plant associations. Eviron. Entomol. 25: 333–349.

Tarrant, C. A., and C. W. McCoy. 1989. Effect of temperature and relative humidity on the egg and larval stages of some citrus weevils. Fla. Entomol. 72: 117–123.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

Wolcott, G. N. 1933. Otiorhynchids oviposit between paper. J. Econ. Entomol. 26: 1172–1173.

Woodruff, R. E. 1964. A Puerto Rican weevil new to the United States (Coleoptera: Curculionidae). Fla. Dep. Agr., Div. Plant Ind., Entomol. Circ. 30: 1–2.

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