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DETECTION OF *RHYNCHOPHORUS PALMARUM* (COLEOPTERA: CURCULIONIDAE) AND IDENTIFICATION OF ASSOCIATED NEMATODES IN SOUTH TEXAS

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ABSTRACT

This study reports a survey conducted to find the South American palm weevil *Rhynchophorus palmarum* (L.) and the red palm weevil *R. ferrugineus* (Olivier) (Coleoptera: Curculionidae), 2 invasive species of palm trees. The study was performed in the Rio Grande Valley of south Texas and near the border with Tamaulipas state, Mexico. A total of 40 traps were inspected biweekly from 26 Sep 2011 to 20 Sep 2012 and 4 traps were inspected from 20 Sep 2012 to 4 Sep 2013. To attract *R. palmarum* and *R. ferrugineus*, the lures 2-methylhept-5-en-4-ol and 4-methyl-5-nonanol were used, respectively. We used these lures in combination with sugar and the ethyl acetate aggregation kairomone for both species, plus an ethylene glycol kill solution. Two specimens of *R. palmarum* were found and identified next to a commercial palm plantation on 11 Mar and 5 May 2012 near the city of Alamo, Texas, but no *R. ferrugineus* was detected during the entire study. Nematodes found in the 2 *R. palmarum* specimens were dauer juveniles of the order Rhabditida, and in one of these weevils only 1 nematode of an undetermined species within the family Aphelenchoididae was found. It is of great importance that *Bursaphelenchus cocophilus* Cobb Baujard (Nematoda: Parasitaphelenchinae), the nematode causal agent of coconut red ring disease, was not found within these insects. This is the first detection of *R. palmarum* in Texas, and the second in the United States.

Key Words: *Bursaphelenchus,* invasive pest, South American palm weevil, sugarcane

RESUMEN

Este estudio reporta una inspección para la detección del gorgojo sudamericano, *Rhynchophorus palmarum* (L.) y el picudo rojo, *R. ferrugineus* (Olivier) (Coleoptera: Curculionidae), ambos son plagas invasivas de palmas. El estudio se realizó en el Rio Grande Valley en el sur de Texas y en las cercanías de la frontera con el estado de Tamaulipas, México. Un total de 40 trampas fueron inspeccionadas cada dos semanas desde el 26 de septiembre de 2011 al 20 septiembre del 2012 y adicionalmente 4 trampas se inspeccionaron desde el 20 de septiembre 2012 hasta el 4 de septiembre 2013. Para atrapar a *R. palmarum* y *R. ferrugineus* fueron utilizados los atrayentes 2-metilhept-5-en-4-ol y 4-metil-5-nonanol, respectivamente. Conjuntamente se utilizó azúcar como atrayente, una kairomona de agregación para ambas especies y una solución de etilenglicol para aniquilar al picudo. Dos especímenes de *R. palmarum* fueron encontrados en la proximidad de una plantación comercial de palmas el 11 de Marzo y 5 de Mayo 2012 cercas de la ciudad de Alamo, Texas; ningún *R. ferrugineus* fue detectado durante el estudio. Los nematodos encontrados en los dos ejemplares de *R. palmarum* fueron juveniles dauer rabdítidos además en un gorgojo, un ejemplar de una especie no determinada de la Familia Aphelenchoididae. Es de gran importancia que el agente nematodo *Bursaphelenchus cocophilus* Cobb Baujard (Nematoda: Parasitaphelenchinae) causal de la enfermedad anillo rojo de la palma, no se haya encontrado dentro de estos insectos. Esta es la primera detección de *R. palmarum* en Texas, y la segunda en los Estados Unidos de América.

Palabras Clave: *Bursaphelenchus,* plagas invasivas, gorgojo sudamericano de las palmas, caña de azúcar

Invasive palm weevils (*Rhynchophorus* spp.; Coleoptera: Curculionidae) are a threat to many palm species in subtropical areas and to the ornamental industry of the U.S. In the Rio Grande

Valley, the southern-most region of Texas, many species of palm are used as ornamentals in backyards and in landscape design on highways, commercial centers, city parks, and on palm plantations. Two species of weevils have been reported in California: the red palm weevil (CDFA 2010), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), and the South American palm weevil (USDA-APHIS 2011), *R. palmarum* (L.) (Coleoptera: Curculionidae). The introduction of these two exotic invasive species and their associated nematodes could be devastating to the palm and landscape industry of many areas of the southern U.S.

Rhynchophorus ferrugineus is a native of southern Asia and Melanesia, whereas *R. palmarum* is a native of South America. The former is a devastating pest of palms, and has spread over the last few decades to the Middle East (Faleiro 2006), North Africa (Cox 1993), and south Europe (Barranco & Cabello 1996). Since 1996, *R. ferrugineus* has invaded Japan (Abe et al. 2009), the Mediterranean Sea rim, Egypt, and spread in Spain. *Rhynchophorus ferrugineus* was recently found in the Caribbean, where it threatens palms of that region (Roda et al. 2011), and spread in Murcia, Islas Baleares, and Islas Canarias (CABI/EPPO 2003). Ferry (2010) and the North American Plant Protection Organization (NAPPO 2010) confirmed that *R. ferrugineus* was found infesting *Phoenix canariensis* Chabaud (Arecales: Arecaceae) in Laguna Beach, California. Although *R. ferrugineus* was found in California in 2010, Fiaboe et al. (2012) hypothesized that this species can be confused with *R. vulneratus* and it is necessary to do both morphological and molecular studies for a proper identification. However there are discrepancies on this identification because both species have color morphs and may be combined under *R. ferrugineus*.

The primary hosts of *R. ferrugineus* include 40 species of palms (Naturjardins 2013), which include *Areca catechu, Arenga pinnata, Borassus flabellifer, Caryota maxima, C. cumingii, Cocos nucifera, Corypha gebanga, C. umbraculifera, C. elata, Elaeis guineensis, Metroxylon sagu, Oreodoxa regia, Phoenix canariensis, P. dactylifera, P. sylvestris, Sabal umbraculifera* and *Washingtonia* sp.

Rhynchophorus palmarum is a primary pest of palms in Central and South America (Alpizar et al. 2002), and it is a main pest of coconut palm (Oehlschlager et al. 1995), oil palm (Oehlschlager 2002), and sugarcane. Wattanapongsiri (1966) indicated that *R. palmarum* was distributed from California to Texas based on identification of specimens borrowed from collections in the U.S. or from overseas; however this report did not provide the locations where the specimens had originated. A report from CABI/EPPO (2006) confirmed that in 1996 *R. palmarum* was found in Central America (Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, and Panama) and the West Indies; and in South America, *R. palmarum* was detected in Argentina, Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Paraguay, Peru, Surinam, Uruguay, and Venezuela. Esser & Meredith (1987) indicated that *R. palmarum* was in Barbados, Dominican Republic, Grenada, Saint Lucia, Saint Vincent and Grenadines, and the Commonwealth of Dominica (OEPP/EPPO 2005). Wattanapongsiri (1966) indicated that *R. palmarum* was established in Cuba, and CABI/EPPO (2006) reported this species in Guadeloupe, Martinique, and Trinidad and Tobago. In North America, *R. palmarum* was present only in Mexico (CABI/EPPO 1969). However, the Animal and Plant Health Inspection Service (APHIS) confirmed the first US detection of *R. palmarum*, in the San Ysidro area of San Diego, California on 9 May 2011 (USDA-APHIS 2011). The National Agricultural Pest Information System indicates that *R. palmarum* has not yet been detected in Florida and Hawaii. *Rhynchophorus palmarum* larvae damage the apical growth of the palm tree while feeding inside the trunk. In addition, *R. palmarum* is a vector of red ring nematode, *Bursaphelenchus cocophilus* (Cobb) Baujard (Nematoda: Rhabditida, Tylenchina).

Bursaphelenchus cocophilus causes red ring disease with major economic impacts on coconut and commercial oil palms (Griffith 1968). Murphy & Briscoe (1999) established that other nematodes found in *R. palmarum* included members of the Rhabditidae (*Teratorhabditis* sp.) and Diplogasteridae (*Diplogasteritus* sp., and *Mononchoides* sp.). This putative *Diplogasteritus* sp. was later fully described as *Acrostichus rhynchophori* by Kanzaki et al. (2009a).

While *R. palmarum* causes physical damage, if a particular nematode parasite causing a plant disease is present, the potential for damage is much greater than that of the weevil itself. In this study we describe a 2-yr survey to detect 2 invasive weevil species and their phoretic nematodes in the Lower Rio Grande Valley of southern Texas, an area bordering the state of Tamaulipas, Mexico.

Materials and Methods

The survey was conducted in 3 counties of the Lower Rio Grande Valley of south Texas (Hidalgo, Cameron and Willacy) from 26 Sep 2011 to 4 Sep 2013. *Rhynchophorus palmarum* and *R. ferrugineus* were targets of a survey near the international bridges connecting Mexico and Texas (see Fig. 1), the highways with much commercial trade between Mexico and the US, and areas within cities and near palm plantations in the Rio Grande Valley. The survey was conducted using bucket traps attached to palm trees located in areas of

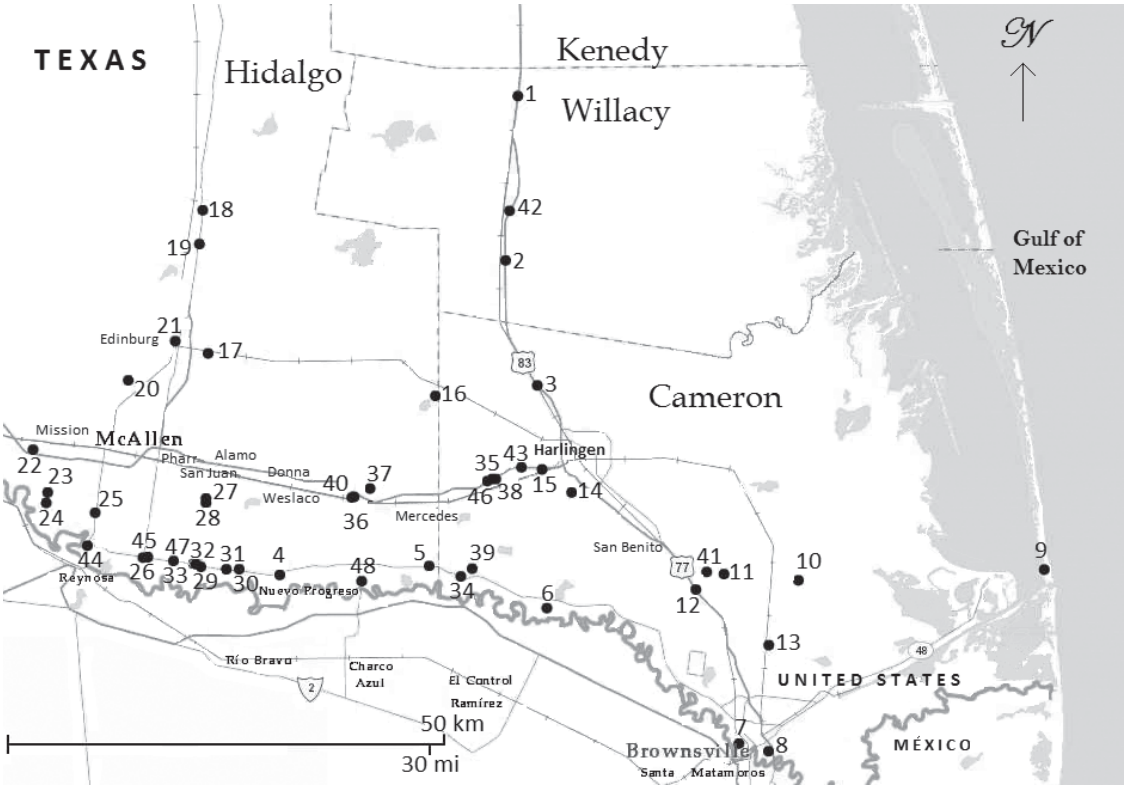

Fig. 1. Locations of the 48 traps to survey for *Rhynchophorus ferrugineus* and *R. palmarum* in the lower Rio Grande Valley, south Texas *Rhynchophorus palmarum* was found in locations 27 and 28.

easy access. Sites were registered with a global positioning system by a GARMIN® GTM35 unit based on the World Geodetic System (WGS) 1984 using spheroid and datum standards. On all places we used one or more of the following conditions to set traps on palms: palm trees needed to be located within 100 ft (30.5 m) of a palm plantation, a sugarcane plantation, an international border, a highway, or a wildlife area.

In year 1, usually 40 traps were inspected biweekly throughout 1 year; however, we used 47 locations that were moved or relocated when traps became inaccessible, or when palms were removed, or when the location was inundated, or if traps were vandalized (trap numbers 1 to 47 of Fig. 1). The above inspections were conducted from 26 Sep 2011 to 20 Sep 2012. Eleven traps were placed near international bridges between Texas and Tamaulipas, Mexico, and near the towns of Mission, Hidalgo, Pharr, Donna, Progreso, Los Indios, and Brownsville. Some traps were placed at several points along 3 major highways (Hwy 83, which runs parallel to the border between Mexico and United States, and highways 77 and 281, which are routes of trade with San Antonio and Houston, respectively). Sixteen traps were located on palm trees next to sugarcane or palm plantations, and 1 trap was placed in the Santa Ana National Wildlife Refuge. The remaining traps were placed at various towns in south Texas, i.e., Mission, Hidalgo, McAllen, Edinburg, Alamo, Donna, Progreso, Weslaco, Harlingen, Los Fresnos, Olmito, Brownsville, Rangerville, Lyford, Blue Town, Pharr, South Padre Island, and La Feria. In year 2, the survey continued until 4 Sep 2013 but only with 4 traps (trap numbers 27, 28, 39 and 48) shown in Fig. 1.

Each bucket trap consisted of a 25-liter plastic container. Traps were strapped to palm trees (Oehlschlager 2007) with fencing wire. To increase the number of *R. palmarum* attracted to traps, we used a solution of 50% sugar with the specific pheromone (2-methylhept-5-en-4-ol), with an aggregation kairomone (pouch with 99% ethyl acetate; release rate 200-400 mg per day) (Oehlschlager 2007), and a kill solution of 50% ethylene glycol. In the same trap, a lure was installed for *R. ferrugineus* (4-methyl-5-nonanol). All the pheromones and the kairomone (ChemTica USA, Durant, Oklahoma) were replaced every 6 wk, on 26 Sep and 22 Nov 2011, 3 Jan, 14 Feb, 27 Mar, 22 May, 3 Jul, 14 Aug, 26 Sep, 7 Nov, 19 Dec in 2012, and 30 Jan, 13 Mar, 24 Apr, 5 Jun, 17 Jul in 2013. The ethylene glycol solution (an-

tifreeze) was refilled as required to maintain at least a 2-cm depth level in the container.

Weevil identification was initially conducted by Raul Villanueva and Gabriela Esparza-Díaz, and confirmation of weevil specimens were made by USDA scientists. The nematodes found within the weevils were identified by microscopy and molecular sequencing at the Nematology Laboratory, USDA-ARS, Beltsville, Maryland. Thus a *R. palmarum* specimen was removed from the sample vial, rinsed with water, and this rinse and the alcohol from the specimen bottle were inspected under an Olympus SZX16 (Olympus America Inc., Center Valley, Pennsylvania) dissecting microscope. After imaging ventrally and laterally with a Nikon CoolPix 990 camera (Nikon Inc., Melville, New York), the aedagus was removed with a pointed forceps, water was pipetted into the abdomen to flush out any internal nematodes, and the insect tilted and nearly immersed in water within a Stender dish over the next 12 h. Nematodes were removed with pipettes and eyelash picks from these liquids both initially and after flushing and soaking. The weevil and the aedagus were replaced in an alcohol solution and deposited with the USDA-ARS Systematic Entomology Laboratory at the Smithsonian Institution insect collection, Washington, D.C.

Nematodes were imaged on a Zeiss Ultraphot II with differential interference contrast (DIC) optics, (Zeiss, Inc., Thornwood, New York), and on an Olympus BX51 (Olympus America Inc., Center Valley, Pennsylvania) with polarization optics, and photographed with a Q-Imaging Micropublisher 5 digital camera (Q-Imaging, Vancouver, British Columbia, Canada).

Nematodes for molecular analysis were suspended in buffer containing proteinase K (Williams et al. 1992) and DNA extracts prepared as described previously (Skantar et al. 2007). The large ribosomal subunit (LSU) D2-D3 expansion segment and the mitochondrial cytochrome oxidase gene (mtCOI) were amplified using the primers and PCR conditions described in Ye et al. (2007) and Platinum Taq (Invitrogen, Carlsbad, California). The 18S small ribosomal subunit (SSU) was amplified in 3 fragments, as described previously. The 5′ end of 18S was amplified with a mixture of forward primers [G18S4, 18S-82F, EukF(10), and SSU_F_03] paired with reverse primer 18SR530 (http://nematol.unh.edu/Method-Protocol/18sprotocal/18s550.html); the middle fragment was amplified with 550F and 1108R (Carta et al. 2011); and the 3′ end with primers 18S1.2 and 18Sr2b (Powers et al. 2005). PCR products were analyzed by electrophoresis on agarose gels in 1X SB (sodium borate-EDTA), excised, and purified using Qiaquick Gel Extraction Kit (Qiagen, Valencia, California). Sequencing was performed at the University of Maryland Center for Biosystems Research. DNA sequences were assembled using Sequencher 5.0 (Genecodes, Ann Arbor, Michigan) and analyzed using blastN to search for highly similar sequences from the GenBank nr database. Putative translation of mtCOI was also analyzed using tblastN and blastP (http://www.ncbi.nlm.nih.gov/blast).

Results and Discussion

In year 1, 89% of traps were active between 300 and 350 days, 5% between 250 and 299 days, and 7% for less than 200 days. The number of checked traps was 977 of which 51% were on *Livistona chinensis*, 25% *Washingtonia robusta*, 21% *Washingtonia* sp. and 3% *Phoenix sylvestris*. Across all traps, *Phoenix sylvestris* was examined during 338 days, *L. chinensis* for a mean of 332 ± 1.8 days, *W. robusta* for a mean of 311± 4.6 days, *Washingtonia* sp. for a mean of 301 ± 4.6 days. In 0.2% of inspections *Rynchophorus* weevils were found. In year 2, the 4 traps were checked for 344 days.

Rhynchophorus ferrugineus was not detected in this survey (Fig. 1). However, 2 male specimens of *R. palmarum* were detected in this study. The first weevil was found on 11 Mar 2012 next to a commercial palm plantation in Alamo, Texas in trap No. 28 (Table 1 and Fig. 1). The species was confirmed as *R. palmarum* (Fig. 2) by Kira Metz (USDA APHISPPQ in College Station, Texas) and Jenz Prena (USDA ARS Systematic Entomology Laboratory in Washington DC). The trap was attached to a *Washingtonia robusta* H.Wendl. (Arecales: Arecaceae; Mexican Fan Palm or Mexican washingtonia). Nematodes of 2 distinct types were detected in this specimen.

Identification of an adult nematode in Beltsville, Maryland was inconclusive as to genus and

Table 1. Trap numbers and geolocations where *Rhynchophorus palmarum* were found indicating the initial and end inspection period in the lower Rio Grange Valley, Texas. For the other trap number locations see Fig. 1.

		Geo location		Quantification	Inspection period	
Trap No.	Point of interest	Latitude	Longitude	*R. palmarum*	Initial	End
27	Palm plantation	26.149598°	-98.129027°	1	9/26/2011	8/29/2013
28	Palm plantation	26.154235°	-98.128855°	1	9/26/2011	8/29/2013

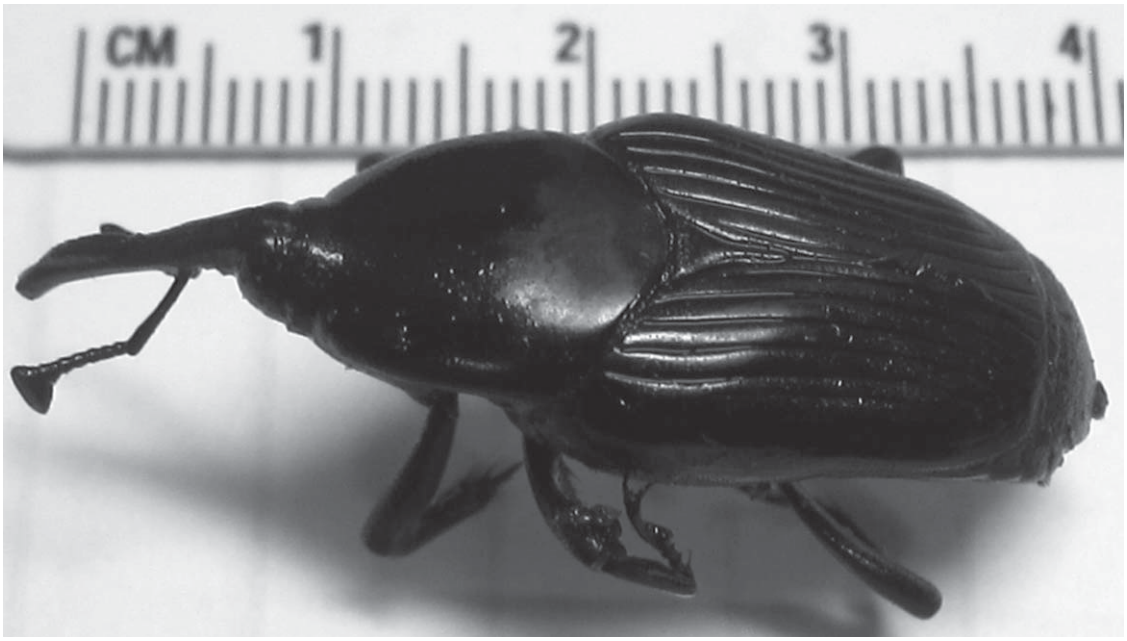

Fig. 2. *Rhynchophorus palmarum* male from Alamo, Texas.

species (Fig. 3); but by morphology it was clearly in the Family Aphelenchoididae. The specimen was contaminated with fungi, which complicated molecular analysis due to cross-reaction of some primers with fungal DNA. Using DNA extracted from this adult, amplification of mtCOI was the only marker that gave a clear match to *Bursaphelenchus*, although the similarity was not high enough to definitively identify the exact species. The result of blastN, limited to Aphelenchoidea, showed a 91% identity with mtCOI sequence from *B. abruptus* (AY508036 and AB0677062) and some other insect-associated nematodes. The result of blastP showed 95% identity to *B. borealis* (AAS90522), *B. rufipennis* (BAG80582), and *B. luxuriosae* (BAD04986). While the red ring nematode, *B. cocophilus,* was not found in this specimen, Magalhães et al. (2007) suggested that nematodes associated with *B. cocophilus* could probably be co-participants in the etiology of red ring disease. *Rhynchophorus* spp. is also known to be associated with *B. gerberae* (Giblin-Davis et al. 2006). The sequence determined in the present study is different from both other associated species *B. gerberae* and *B. cocophilus*, so it appears to be a third *Bursaphelenchus* species from *Rhynchophorus* weevils. The inconclusive identification of this *Bursaphelenchus* nematode to species level might be due to the nature of this survey, the *R. palmarum* specimens could have remained submerged in the ethylene glycol from 1 to 14 days causing degeneration, or denaturation of the nematode's DNA that provided inconclusive molecular data in addition to contamination by fungi.

Amplification of 28S rDNA from the adult nematode yielded a single fragment whose sequence was identical to the two reported from *Rhabditis rainai* (EU195966 and JN572919), a nematode previously found in association with termites (Carta & Osbrink 2005; Kanzaki et al. 2009b). The 18S rDNA gene was amplified in 3 parts; however, only the 3′ end fragment yielded a nematode sequence that was identical to that from *Rhabditis rainai* (JQ237848, AF083008). The other fragments turned out to be from amplification of fungal contaminants.

PCR of 28S rDNA using DNA from dauer juveniles found in this weevil resulted in products of ~600 bp and ~800 bp. The larger fragment sequence was 99% similar to *Cephalobus* sp. JB-67 (DQ145628). The 28S sequence from a second dauer was 80% similar to *Neodiplogaster* sp. (AB478641).

Others studies of *R. palmarum* had reported *Teratorhabditis palmarum* (Gerber & Giblin-Davis 1990a; Magalhães et al. 2007), *Mononchoides* sp., *Bursaphelenchus* sp. (Gerber & Giblin-Davis 1990b), *Diplogasteritus* sp. (Magalhães et al. 2007) and *Acrostichus rhynchophori* (Kanzaki et al. 2009a). *Bursaphelenchus cocophilus* is sometimes associated with several different termite species such as *Coptotermes niver, Leucotermes tenius* (Esser & Meredith 1987; Griffith 1987). This seemed to be an accidental association when nematodes in dead palms were picked up by ter-

Fig. 3. Adult female nematode (unidentified member of Aphelenchoididae) found inside *Rhynchophorus palmarum*, polarization optics.

mite workers foraging there. The association of *R. rainai* and *R. palmarum* could be an inverse association from that of the *B. cocophilus*-termite association from a habitat that supports diverse invertebrates.

The second specimen of *R. palmarum* was found in trap 27 (Table 1 and Fig. 1;) on 5 May 2012 across a road from the same commercial palm plantation and 0.3 mile distant from the first finding in Alamo, Texas. Its host was *Livistona chinensis* (Jacquin) R. Brown ex Martius (Arecales: Arecaceae; Chinese Fan Palm). Only rhabditid nematodes and no alphelenchid nematodes were found in this second specimen. Features of these rhabditid dauer juvenile nematodes were not sufficient to identify to nematode genus, although one diplogastrid image (Fig. 4) was similar to *Acrostichus rhynchophori* from Caribbean *R. palmarum* specimens (Kanzaki et al. 2009b). Sequences of 28S rDNA were of insufficient quality to allow clear comparisons with GenBank accessions to make a species identification based on DNA. The only non-fungal ITS sequence obtained from one of these dauers was also of poor quality with many ambiguous base calls; the closest match was to *Ditylenchus* sp. This contamination issue was not too surprising because Miguens (2011) had reported that *R. palmarum* was highly infected by a fungus.

The nearby plantation where these 2 specimens were found was growing *W. robusta* (Mexican *Washingtonia*) in addition to other species of ornamental palm trees, including *P. canariensis* (Canary palms), *P. dactylifera* (date palms), and *Sabal* spp. (sabal palms). It might be possible that *R. palmarum* has become established there, or these insects could have been hitchhikers on palm leaves from Mexico. Weevils might have been introduced on juvenile palm fronds that are exported from Mexico to the USA for floral arrangements. This plantation was using these fronds to fabricate floral arrangements for the local market or for distribution elsewhere in the USA.

Several factors are important pertaining to the detection of *R. palmarum*. Firstly, *R. pal-*

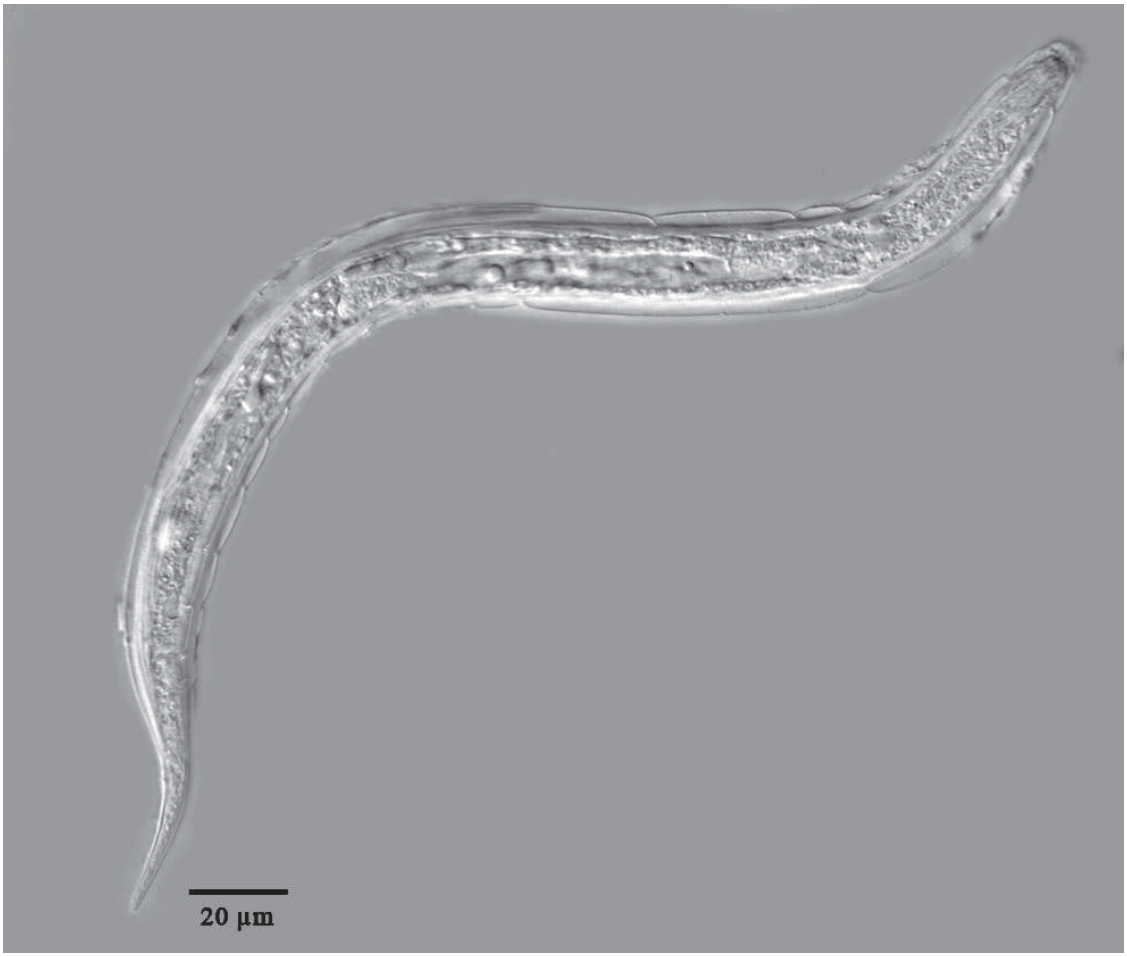

Fig. 4. Dauer juvenile diplogastrid nematode (Unidentified Rhabditida) from *Rhynchophorus palmarum* showing bunched, sticky outer cuticle, differential interference contrast optics.

marum can be easily established in subtropical environmental conditions, especially with the presence of the wide variety of host plants found in the Lower Rio Grande Valley including several ornamental palms, coconut and oil palms, and sugarcane. Secondly, if *R. palmarum* does become successfully established, it could be moved to the rest of the southern U.S., affect the ornamental palm industry, and infest and negatively impact sugarcane plantations in South Texas, Louisiana, and Florida. Finally, this weevil is a vector of red ring nematode *B. cocophilus,* which causes red ring disease that is lethal to many palm tree species, and that further increases economic damage to commercial palm plantations and urban landscapes.

Although in this study we did not confirm the presence of *B. cocophilus,* the nematode species found in the first *R. palmarum* might be this species or a closely related species based on all morphological characteristics. Aside from *B. cocophilus*, there is also a potential environmental risk of other damaging nematodes and mites being carried by *R. palmarum* in a kind of cryptogenic invasion.

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