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# Occurrence of *Panagrellus* (Rhabditida: Panagrolaimidae) Nematodes in a Morphologically Aberrant Adult Specimen of *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae)

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*Abstract:* An aberrant specimen of *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) also known as red palm weevil (RPW), the most economically important insect pest of palms in the world, was found among a batch of conspecifics reared for research purposes. A morphological analysis of this weevil revealed the presence of nematodes associated with a structured cuticle defect of the thorax. These nematodes were not able to be cultured, but were characterized by molecular analysis using 28S and 18S ribosomal DNA and shown to belong to the family Panagrolaimidae (Rhabditida), within a clade of *Panagrellus*. While most nematodes in the insect were juveniles, a single male adult was partially characterized by light microscopy. Morphometrics showed similarities to a species described from Germany. Excluding the entomopathogenic nematodes (EPN), only five other genera of entomophilic or saprophytic rhabditid nematodes are associated with this weevil. This is the first report of panagrolaimid nematodes associated with this invasive pest. Possible mechanisms of nematode-insect association are discussed.

*Key words:* insect thorax defect, invasive species, nematode phoresy, physiological ecology, saprophagous nematode, sour paste nematode.

*Rhynchophorus ferrugineus*, the RPW, is currently considered as the most damaging pest of palm species in the world (Giblin-Davis et al., 2013). A recent review highlighted the paucity of data concerning the natural enemies and the organisms associated with the RPW (Mazza et al., 2014). Although new knowledge has been acquired in recent years, much remains to be done (Mazza et al., 2014). Excluding the EPN belonging to *Steinernema* and *Heterorhabditis* genera, few other insect-associated nematodes with entomophilic, saprobiotic, phoretic, commensal, or parasitic associations (Sudhaus, 2008) are known to be associated with this weevil. Those genera already identified include *Acrostichus* Rahm, 1928; *Bursaphelenchus* Fuchs, 1937; *Caenorhabditis* (Osche, 1952) Dougherty, 1953; *Diplogasteritus* Paramonov, 1952; *Mononchoides* Rahm, 1928, and *Teratorhabditis* (Osche, 1952) Dougherty, 1953 (reviewed in Troccoli et al., 2015). Further studies are necessary to identify weevil nematodes, clarify their biology and type of association with RPW, and consider their possible effects as biocontrol agents (Mazza et al., 2014). With this aim, we report the first finding of nematodes belonging to Panagrolaimidae (Nematoda: Rhabditida) associated with the RPW and briefly discuss the possible modes of association.

## Materials and Methods

*Origin and rearing of the RPW:* Newly emerged RPW adults with a balanced sex ratio ($n = 384$) were provided by the UTAGRI-ENEA C.R. Casaccia, Laboratorio Gestione Sostenibile degli Agroecosistemi, Rome, Italy in 2013 to 2014. Larvae were individually reared, in a climatic room (29°C, about 100% relative humidity with a photoperiod of 12:12), in plastic containers with perforated lids, fed with apple slices, and incubated until adult emergence. Final larval instars made cocoons using coconut fibers or moist filter papers. In this batch of individuals, one adult with unusual behavior (less agility), and aberrant in the thorax region, was isolated and anaesthetized at −20°C for 5 min. The aberrant thorax region was dissected in phosphate buffer (PB) 0.1 M, pH 7.2. About 100 nematodes, including an adult male and many juveniles, along with biological tissue emerged. Both the RPW and the nematodes were processed for morphological and molecular purposes. Nematodes could not be put into bacterial plate culture, as they were not identified as potentially culturable until after they had been killed so the insect and nematodes could be preserved for diagnosis.

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*Light microscopy:* The aberrant thorax of the RPW was photographed using a Canon Powershot S50 mounted in a Nikon SMZ-10 stereomicroscope. For morphological observations of nematodes, specimens were mounted in glycerin on semipermanent microscope slides after room-temperature fixation in 2.5% glutaraldehyde in 0.1 M PB and examined with a Leitz ORTHOPLAN light microscope (Leica Microsystems, Wetzlar, Germany) at up to ×1,000 magnification.

*Scanning electron microscopy:* About 50 juvenile stages of the nematodes were washed in 0.1 M pH 7.2 PB, to which 3% sucrose was added. Samples were then fixed overnight with 2.5% glutaraldehyde in 0.1 M PB. Afterward, samples were rinsed with PB and postfixed with 1% osmium tetroxide in the same buffer for 3 hr at 4°C. Specimens were then dehydrated by washes with increasing ethanol concentrations (50%, 75%, 95%, and absolute) at room temperature. After dehydration, samples were freeze-dried in an Edwards Modulyo instrument. Finally, the material was mounted on aluminum stubs, coated with gold using a JEOL JFC-1300 sputter coater, and then examined with a JEOL Neoscope JCM-5000 SEM operating at 10 kV (JEOL Ltd., Tokyo, Japan).

*Nematode DNA extraction and polymerase chain reaction amplification:* Isolated nematodes were snap frozen in liquid nitrogen and placed at −80°C. For DNA extraction, single nematodes were homogenized with a pestle in a 1.5-ml tube containing 100 µl of 6% InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) and 10 µl of Proteinase K solution 20 mg/ml (5 PRIME GmbH, Hilden, Germany). The sample was incubated at 56°C for 30 min (block heater SBH130DC, Stuart; Bibby Scientific Ltd., Staffordshire, UK), then vortexed at high speed for 10 sec (Multi-Vortex V-32; Biosan, Riga, Latvia) and boiled at 100°C for 8 min in a block heater. Afterward, the sample was centrifuged at 12,000 rpm for 3 min at 22°C (rotor 11461, MPV-351R centrifuge; MPW Med. instruments, Warsaw, Poland). The resulting supernatant contained nematode DNA. All polymerase chain reactions (PCR) were carried out in 25 µl volume containing 0.2 µM of each dNTP, 0.3 µM of each primer, 1× PCR buffer; 1.5 mM $MgCl_2$, 1.5 U Taq DNA Polymerase (Invitrogen; Life Technologies, Grand Island, NY). Negative controls were included. PCR's were performed on an Applied Biosystem 2720 thermal cycler (Life Technologies).

For the 28S ribosomal DNA (rDNA) PCR reaction, 1 µl of supernatant was used for gene amplification using forward primer no. 391 (Nadler and Hudspeth, 1998) and reverse primer no. 536 (Stock and Nadler, 2006). The 28S PCR conditions were detailed by Stock and Nadler (2006). For 18S rDNA gene amplification, 1 µl

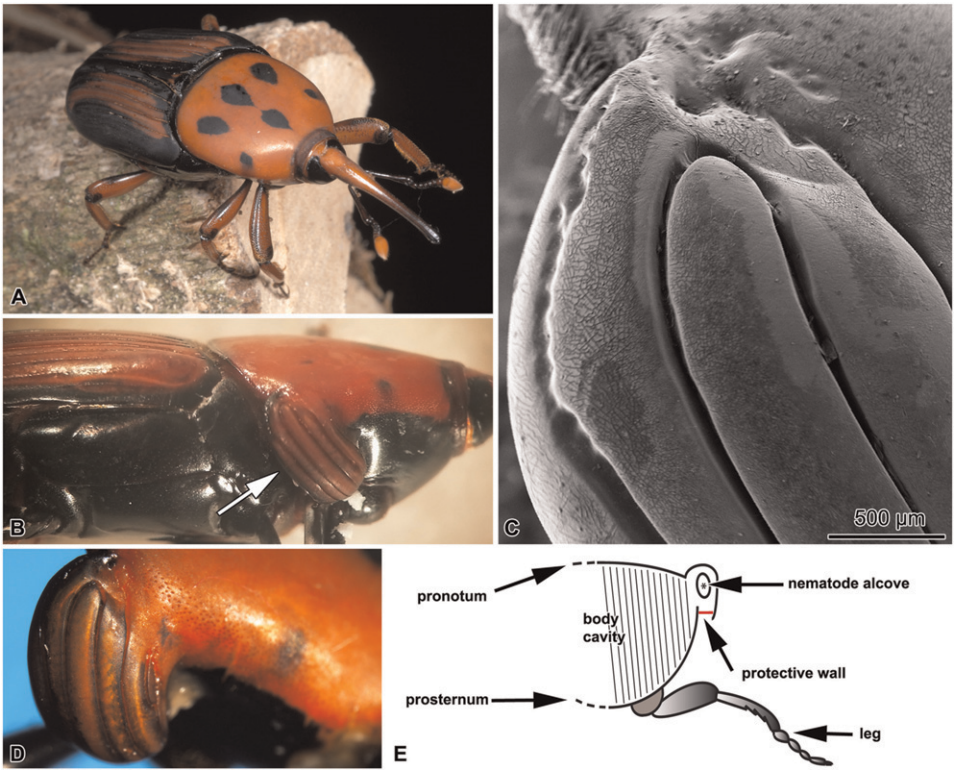


FIG. 1. A. A healthy specimen of *Rhynchophorus ferrugineus*. B. The aberrant specimen of *Rhynchophorus ferrugineus* showing the evident defect in the region of the pronotum. Note the structural similarity between this defect and the normal elytron. C. Scanning electron microscopy image showing the detail of the pronotum defect in which some cuticular furrows similar to those of the elytron are present. D. The pronotum defect seen from another point of view. E. Schematic representation of the thorax transverse section showing the abnormal pronotum and where nematodes were found.

of supernatant was used with forward primer nem1 and reverse primer nem 2 (Foucher and Wilson, 2002). The 18S PCR conditions were as follows: 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 54.1°C for 30 sec, and 72°C for 40 sec. PCR products (10 µl) for both 28S and 18S were loaded on 1% agarose gels with 1× SYBR Safe in DMSO (Invitrogen), then viewed and photographed on a Bio-Rad Gel Doc system (Bio-Rad Laboratories).

*DNA sequencing and phylogenetic analysis:* Both DNA strands of 28S and 18S PCR products were sequenced at the Centro di Servizi per le Biotecnologie di Interesse Agrario Chimico ed Industriale, Università degli Studi di Firenze, Italy. A multiple alignment between the 28S rDNA deposited sequence and other related 28S sequences from GenBank (National Centre for Biotechnology Information) was made with ClustalW (Larkin et al., 2007). *Rhabditophanes* sp. DQ145655 (Rhabditida: Alloionematidae), *Halicephalobus gingivalis* DQ145637, and *Panagrolaimus* sp. KC522702 were specified as outgroups, as informed by a previous study (van Megen et al., 2009), and ingroup sequences included *Panagrellus ceylonensis* DQ408251, *Panagrellus redivivus* DQ145647, *Panagrellus dubius* DQ145648, and the *Panagrellus* sp. discovered in the present study. A phylogenetic tree was inferred under the Bayesian criterion with the MrBayes plugin (Huelsenbeck and Ronquist, 2001) for Geneious v. 7.1.7 (Biomatters, Auckland, New Zealand), and employed the HKY85 model, a gamma distribution of rates, a chain length of 1,100,000 generations sampling trees every 100 generations, and a burn-in length of 110,000 generations.

The 18S sequences of *P. redivivus* PS1163 AF083007 and *Baujardia mirabilis* AF547385 were compared with the deposited 18S sequence for *Panagrellus* sp. using the Clustal W alignment plugin and trimmed as an extraction within Geneious ver. 7.

## Results and Discussion

In comparison with healthy specimens (Paoli et al., 2014; Inghilesi et al., 2015; Montagna et al., 2015), the body of the aberrant RPW male had an evident defect in the exoskeletal cuticle of the posterior right side of the pronotum (Fig. 1A–E). Such an exoskeletal defect showed an ovoid shape that extended laterally for a few millimeters and was characterized by several furrows (Fig. 1B–D). In this region the cuticle was red, except for the external edge that was black, and appeared to be curiously differentiated as an outlined elytron, obliquely oriented (Fig. 1B–D). This could represent a congenital aberration (Bownes, 1975) where

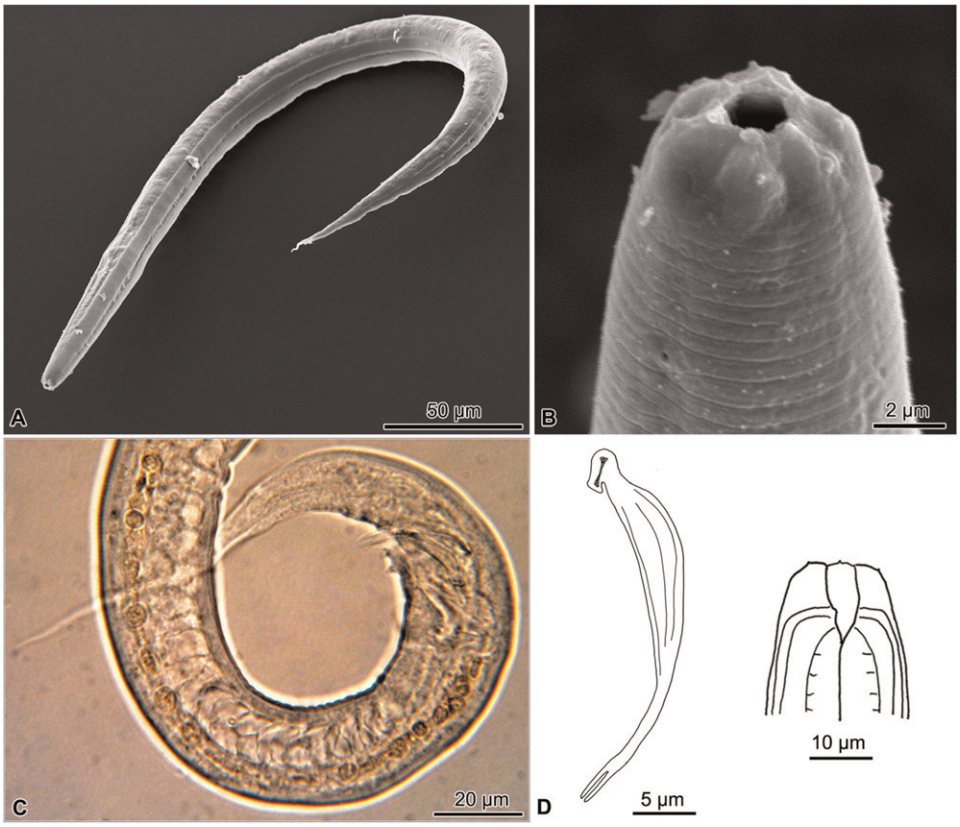


Fig. 2. *Panagrellus* sp. A. Scanning electron micrograph (SEM) of *Panagrellus* sp. nematode juvenile. B. SEM image of the buccal region of a *Panagrellus* sp. nematode juvenile. C. Male tail with distally bifurcate spicule outside body, lateral view. D. Male lateral view drawings of spicule (left), stoma (right). This nematode was fixed in 2.5% glutaraldehyde in 0.1 M in phosphate buffer.

nematodes in the neighborhood could have found suitable conditions to live and possibly reproduce in this cavity. It could also represent a cryptic infection, possibly of the larva or pupa stages, that arrived with the original weevil stock and was only recently noticed. Alternatively, it may be an accidental association that occurred in captivity. Since some *Panagrellus* and *Panagrolaimus* species are reported to be associated with fruits or material for rearing (Goodey, 1963; Félix and Duveau, 2012), it is possible that the origin of the infection could be sought in the apples used to feed the weevils or in the coconut or palm fibers used for the larvae pupation. Whether this was a chance infestation of a suboptimal host in the laboratory, or a repeatable phenomenon present in nature, it will be informed by further field sampling and laboratory testing.

In its distal region, the furrowed exoskeletal structure was not completely folded on itself, thus forming a cavity in which about 100 larvae and a few adult nematodes were found. However, the space in which nematodes were found was isolated from the outside by whitish, sac-like biological tissue that acted as a protective wall. This material had a wafer shape and was composed of several multilayered, elongated, noncuticular, biological elements arranged in parallel. Although it may have been produced by the RPW, it may also have been produced by the nematodes themselves. The entomophilic nematode *Ektaphelenchus obtusus* Massey, 1956 (Aphelenchida: Aphelenchoididae) produced cocoons under the elytra of spruce beetle *Dendroctonus rufipennis* Kirby (Curculionidae: Scolytinae) that contained adult nematodes (Massey, 1974). This phenomenon is quite different from that of the EPN *Steinernema feltiae* and *Heterorhabditis bacteriophora* (Rhabditida: Steinernematidae) that were individually melanized, encapsulated, and deformed the wings of Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) (Ebrahimi et al., 2011). Another related association with *R. ferrugineus* and *Steinernema carpocapsae* exhibited milder host defenses without wing deformation (Mastore et al., 2015), demonstrating great variation in nematode integrity with different insect associations.

The nematodes from aberrant *R. ferrugineus*, after being characterized by morphological and molecular analyses, belonged to *Panagrellus* sp. (Panagrolaimidae) (Fig. 2A–D). Measurements for a single adult male were as follows: L = 602 μm, pharynx = 146.8 μm, tail = 84.7 μm, corpus = 94.4 μm, isthmus = 26.6 μm, bulb = 25.4 μm, gubernaculum = 10 μm, spicules L = 29 μm, stoma L = 6.4 μm, stoma W = 4 μm, a = 21.3, b = 4.1, c = 7.1, and c' = 5.1 (Fig. 2). Of the 13 known *Panagrellus* species (Andrássy, 1984; Ferris, 2009), *P. ventrodentatus* (Heindl-Mengert, 1956) Baker, 1962 described from Germany is most similar to the species reported herein.

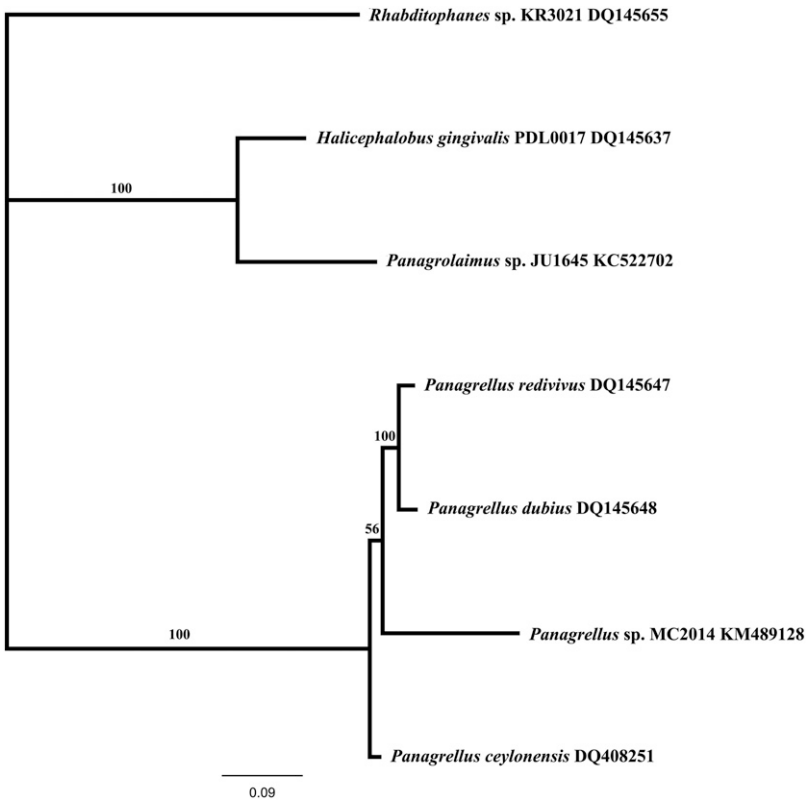


Fig. 3. Phylogenetic tree of Panagrolaimidae, inferred from Bayesian analysis of the 28S rDNA gene. The sequence from *Panagrellus* sp. (KM489128) from morphologically aberrant *Rhynchophorus ferrugineus* was included in the analysis. The tree has a mean log likelihood of −4,234.7. The numbers on each branch indicate support values, and the tree scale units represent nucleotide substitutions per site.

Differences between this individual and *P. ventrodentatus* are longer spicules (29 vs. 16–21 μm) and shorter stoma length (6.4 vs. 8 μm) (Heindl-Mengert, 1956). The long distal bifurcation of the male spicules uniquely characteristic of *Panagrellus* species is evident (Fig. 2C,D). However, male tail papillae (Fig. 2C) and stomatal dentition (Fig. 2D) were too obscure to be fully characterized.

The 28S rDNA gene amplification product of 992 bp obtained in this study was deposited in GenBank with the accession number KM489128. There was 87.3% sequence similarity in a 993 bp pairwise alignment between *Panagrellus* sp. and *P. ceylonensis*, and 86.8% similarity with *P. redivivus* (not shown). The Bayesian tree inferred from the larger alignment for selected Panagrolaimidae and rhabditid outgroup showed that sequence KM489128 belonged within a clade of *Panagrellus* species with 100% support (Fig. 3). The closest known genus to *Panagrellus* is *Baujardia*, a nematode genus with setae on its head (Bert et al., 2003) rather than the more typical rhabditid papillae seen here in Fig. 2B. The GenBank 18S sequences for *P. redivivus* AF083007 and *B. mirabilis* AF547385 are 97% similar when aligned pairwise (not shown). In a trimmed alignment of *P. redivivus, B. mirabilis*, and the deposited 18S *Panagrellus* sp. KP876562 (not shown), there are 46 nucleotide position differences out of 615 alignment positions between *Panagrellus* sp. and *P. redivivus*, or 92.5% similarity between them, but only 91.6% similarity between both *Panagrellus* species and *Baujardia*. Although there were three *Panagrellus* species with 28S sequences in GenBank to construct a relevant tree, there is no 28S rDNA sequence of *Baujardia* to compare. However, because the weevil nematode reported herein is typologically distinct from *Baujardia*, yet its 28S rDNA sequence is highly similar to three other *Panagrellus* species, it is identified here as *Panagrellus* sp. Further analysis of adult morphology in a range of other specimens will be necessary to clearly confirm the species identity of this nematode.

Many members of the Panagrolaimidae such as *Panagrolaimus*, *Panagrellus*, *Plectonchus*, and *Panagrobelus* (Massey, 1974) are saprophagous, bacterial-feeding nematodes often associated with insects that occupy a wide diversity of niches. Some species enter the body cavity of living insects, such as longhorn beetles or Chloropidae flies (reviewed in Poinar, 1972), thus behaving as facultative parasites. A number of *Panagrellus* species have been found in phoretic association with beetles such as *P. leperisini*, *P. dorsibidentatus*, and *P. dubius* (in frass) (Stock and Nadler, 2006), and many reports of association with fruit flies in nature and the laboratory (Goodey, 1963). Thus far, to the best of our knowledge, panagrolaimid nematodes were never reported in association with this weevil pest (Mazza et al., 2014), nor were they reported in the related weevil species *Rhynchophorus palmarum* that contained nematodes from Rhabditidae, Diplogastridae (Rhabditida), and Parasitaphelenchidae (Aphelenchida) (Esparza-Díaz et al., 2013).

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