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CHEMICAL SCIENCES

Orthoporus fuscipes (PORAT, 1888) (Juliformia; Spirostreptidae): population structure and defensive secretion chemical analysis

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Abstract: Diplopods are terrestrial arthropods important for the dynamics of terrestrial ecosystems. One of the reasons for that can be their low predation rate due to their defensive secretion. Thus, *Orthoporus fuscipes*, a species belonging to this group and endemic to northeastern Brazil, was investigated as to its population structure and chemical constituents of defensive secretion. The population structure showed that females are larger and have greater mass than do males, along with negative allometric growth between males and females. The defensive secretion hexane extract was submitted to fractionation using SiO_2 open-column chromatography and the gas chromatographic coupled to mass spectrometric analysis was applied in the fraction possibilities to identify major fatty acid methyl esthers, along with minor alkanes, alkenes and fatty acids derivatives and the known quinoids 2-methoxy-3-methylhydroquinone, 2-methoxy-3-methyl-1,4-benzoquinone, 2,3-dimethoxy-1,4-benzoquinone and 2,3-dimethoxyhydroquinone. In addition, the cytochrome oxidase I sequence for the species was deposited for the first time.

Key words: benzoquinones, gas Chromatography, cytochrome c oxidase, methyl esthers, millipedes.

INTRODUCTION

Diplopoda (millipedes) comprises the third largest group of terrestrial arthropods after Hexapoda and Arachnida (Golovatch et al. 1995, Hoffman et al. 1996). This class plays a vital role in the cycling of matter, energy and nutrients, and comprises 15 orders and numerous species, accounting for the most abundant and diverse group within Myriapoda (Geoffroy 2015). About 80,000 species are estimated to exist on the planet, but only 10 to 11% have been already described (Golovatch et al. 1995, Hoffman et al. 1996). Diplopods have different defense mechanisms against threats from the external environment (Hopkin & Read 2002). In addition to the exoskeleton, the production of defensive secretions (repellents or poisonous) provides protection to these animals (Arab et al. 2003, Taira & Arakaki 2002, Taira et al. 2003), which is the main reason why they have few natural enemies (Hopkin & Read 2002). Many species of millipedes have defense glands in the form of integumentary sacs arranged in each segment along the body, from which they release fluids with unpleasant odors in response to the disturbance. More than 30 compounds have been already identified as defense substances (Abraham et al. 2011), and several of these compounds may be useful for medicine and, possibly, industry (Geoffroy 2015).

The family Spirostreptidae Brant, 1833 (Hoffman, 1979), is included in the order Spirostreptida, and is the largest family in the order, with serious nomenclatural and taxonomic problems. It has a wide geographical distribution spanning Africa, South America, Central America and the southern United States (Hoffman et al. 2002). At present, it consists of 280 species distributed in 61 genera (Enghoff et al. 2015). The order Spirostreptida consists of about 1,300 species. This order is of medical importance because of the recorded incidents in humans caused by the release of quinones (Eisner et al. 1978, Enghoff et al. 2014, 2015). Compounds of this class are usually the dominant components found in the defensive secretions from millipedes of the order Spirostreptida (Deml & Huth 2000, Eisner et al. 1978, 1965, Shear 2015). Benzoquinones are reported to be responsible for the antibacterial (Williams et al. 1997) and antifungal (Stanković et al. 2016, Williams et al. 1997) properties observed in the defensive secretions from millipedes. The aim of this study was to conduct the first biological and chemical studies of *Orthoporus fuscipes*. In addition, the DNA Barcode was sequenced, due to the fact that there are no reports in databases of this gene in this group of millipedes.

MATERIALS AND METHODS

Collection and morphological identification of diplopodes

The specimens of *Orthoporus fuscipes* (Porat, 1888) (Diplopoda, Spirostreptidae) (Figure 1) were manually collected during two days, in the early hours of the morning, in the month of December of the years 2009, 2012 and 2013 in a preserved fragment of the Seasonal Forest (13º41'S; 40º05'W - SisGen AD9837D) located in the municipality of Jequié, Bahia, Brazil. The choice of this month/period for the collection was due to the fact that the diplopods are in the reproductive phase and foraging on the soil, besides being the period of high temperatures and higher humidity, factors that influence the greater activity of those animals. The municipality of Jequié is located in the southwest of the state of Bahia in Brazil, extending through a transition zone between the Seasonal Forest and the Caatinga, with a predominance of dry semi-arid climate (BAHIA/SEI 2021). The rainy season is more intense in the months of November to January (summer rainfall), and the average temperature in the region is around 24.3ºC (data for the period 2000 - 2010, provided by the Instituto de Meio Ambiente e Recursos Hidricos (Institute of Environment and Water Resources) - INEMA / BA).

After collection, the specimens were separated into groups. Each group was accommodated in organizer boxes. Inside the boxes, a soil rich in organic matter was provided, with dried leaves to create a covering layer, mimicking the natural environment. To ensure their hydration, a hydration source was provided *ad libitum*. This separation was made for each year of collection.

Dr. Carmem Silvia Fontanetti Christofoletti (Universidade Estadual Paulista - UNESP/Rio Claro) performed the morphological identification of the collected specimens. In the laboratory, the animals were maintained in terraria containing moist soil and organic material from the places where they were collected, under constant surveillance, with an average room temperature of 25ºC. After recording population data, the animals were returned to the collection site. (13º41'S; 40º05'W - SisGen AD9837D)

Population structure of *O. fuscipes*

The animals collected and kept alive at the Laboratório de Zoologia dos Invertebrados

Figure 1. a: Dorsal photo of *Orthoporus fuscipes*, showing its coloration ranging from yellow to brown. b: *O. fuscipes* feeding in captivity covered with dry leaves. c: Defensive behavior (curling up). d: *O. fuscipes* hiding in a clay burrow made in captivity.

(UESB) were separated into males and females (n = 915). The results were analyzed qualitatively in terms of abundance (%), without and with sexual distinction each year of collection. Sexual proportions were calculated using the Chi-square test - X^2 ($p < 0.05$) using the BioEstat version 5.0 software. The body mass of each diplopod was measured using a semi-analytical balance with an accuracy of 0.01 gram. The total length was measured with animals stretched and immobilized between a bulkhead and a metal ruler (for technical drawing) with an accuracy of 0.1 millimeter.

For each individual collected, the following morphological aspects were recorded: total length (centimeter) and body mass (grams). Subsequently, the length and mass means and respective standard deviations were calculated and compared using the t-test, with a 5% significance level. The mass-length ratio given by the equation $M = aL^b$ was also analyzed, where M is the mass in grams, L is the length in centimeters, **a** and **b** are constants. These constants were estimated by the linear regression of the transformed equation: W = log a + b x log. The level of significance of r was estimated and the value of b tested using the t test to find out if b = 3. The estimated values of the regression constant (b) can vary from 2.00 to 3.50 (Le Cren 1951). This wide variation in b

occurs due to biotic and abiotic factors (Gomiero & Braga 2003). When growth is isometric (b = 3.00), it suggests an increase in weight and length in the same proportion. To perform these calculations, the Statistica version 7.0 software was used.

Molecular Identification: DNA extraction and sequencing

For the extraction of the DNA of *O. fuscipes*, ten individuals were used: five males (M) and five females (F). The testimony material was deposited in the Arthropod Collection of the Zoology Museum of the State University of Feira de Santana (MZFS), identified by a registration number. The Canadian Center for DNA Barcoding (CCDB) protocol for arthropods, with some modifications, was used to perform DNA extraction. The sequencing reactions were performed by the direct method, in both directions - forward and reverse - containing: 50 ng of purified PCR product, 1.0 µL of sequencing buffer, 0.5 µL of BigDye v3.1 (Applied Biosystems®), 0.25 µL of each primer and an amount of ultrapure water that completes 10 µL. Forward and reverse sequences for each sample were edited using the Geneious software (Kearse et al. 2012); the consensus sequences were generated and submitted to the BLAST tool for comparison and identification by similarity with the nucleotide sequence database GenBank™ from the National Center for Biotechnology Information – NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). More details of the amplification and sequencing are in Supplementary Material - Figure S1.

Chemical study of the defensive secretion of *O. fuscipes*

Extraction and fractioning

To obtain the extract of the secretions, 80 individuals of *O. fuscipes* were immersed in hexane (1000 mL), at room temperature, until the release of the yellow material (approximately for 2 min). Next, the solvent was removed using a rotaevaporator at 70°C to furnish the respective hexanic extract (2.2 g). All extracts were fractionated by open-column chromatography containing silica gel (70-230 mesh F254 Fluka® Analytical) as the stationary phase. The eluent system was composed by different mixtures of solvents in crescent polarity order (Petroleum ether, *n*-hexane, ethyl acetate, ethanol, methanol) resulting in eleven fractions. Each fraction was evaluated using thin-layer chromatography (TLC) (Merck), using n-hexane:ethyl acetate (7:3) as eluent. The plates were revealed using UV light (365 nm) and methanol/sulfuric acid solution (10 %, V/V), with heating (220ºC). After TLC analysis, the fractions with similar chromatographic profile were grouped resulting in 4 fractions (A-D).

GC-MS analysis

The gas chromatography analyses coupled to mass spectrometry (GC-MS) were performed on a Shimadzu chromatograph model CG 17A, coupled to the GCMS QP5050A mass spectrometer, operated by electron impact (EI) with 70 eV ionization energy, in the range m/z 40-400. Helium was used as the carrier gas at a constant flow of 0.92 mL, and the general analysis conditions were as follows: capillary column of fused silica DB-5 (30 m x 0.25 mm x 0.25 µm with 5% diphenyldimethylsiloxane) (Agilent Technologies); injector and detector temperature were maintained at 280ºC and the split injection mode (20/1) was used. The

injection volume was 2µL. The oven temperature was programmed from 150 °C with an increase of 30 °C/min to 270 °C (for 10 minutes), then increasing at a rate of 10 °C/min to 290 °C, ending with a 14-minute isotherm at 300 °C (at 50 minutes).

RESULTS

Population structure of *O. fuscipes* and DNA Barcode

Table I shows the sex ratio, the distribution of males and females, as well as the proportions of each year when the *O. fuscipes* were collected in northeastern Brazil. The Table II shows body weight and length for these animals. In general, without sexual distinction, the body mass of the diplopods ranged from 0.16 to 8.74 grams, while the length varied from 3.9 to 14.7 cm. When the results of body mass and length were analyzed with sexual distinction, differences between males and females were verified. In general, females have higher body masses and longer lengths than do males. The means were compared using the t test for independent series and showed a significant difference ($p < 0.05$). When the mass-length ratio of *O. fuscipes* was evaluated, a predominance of negative allometric growth (values of b <3.00) was observed between males and females, except for males collected in 2009, which showed an isometric growth (b = 3.02) (Supplementary Material - Table SI). That indicates an increase in body mass and growth in the same proportion, which theoretically is ideal.

Table I. Chi-square test to analyze the sexual proportion of individuals of *O. fuscipes*.

Years	Males	Females	Total	Proportions	X^2	GL	p
2009	78	267	345	1 : 3.4	103.5	1	< 0.0001
2012	125	169	294	1 : 1.4	6.6	1	0.0103
2013	70	206	276	1 : 2.9	67.0	1	< 0.0001
Total	273	642	915	1 : 2.4	148.8	1	< 0.0001

Table II. Body weight (g) and length (cm) of the *O. fuscipes* collected in Jequié (Bahia, Brazil).

Body weight (g)						
	2009		2012		2013	
Parameters	M	F	M	F	M	F
Mean	1.82	2.40	3.47	4.80	3.94	5.70
± SD	± 0.87	± 1.15	± 0.55	±1.18	± 0.69	± 1.40
min	0.21	0.16	2.07	1.18	2.64	1.86
max	4.20	5.87	4.91	7.54	6.20	8.74
Length (cm)						
Mean	7.72	8.70	9.99	10.63	10.58	11.80
± SD	± 1.38	± 1.75	± 0.68	± 1.12	± 0.83	± 1.14
min	4.2	3.9	8.20	6.20	9.0	7.4
max	10.0	13.00	12.00	12.8	12.8	14.7

M – male; F – Female; min – minimal value measured; max – maximal value measured.

About the DNA Barcode, eight sequences with about 622 bp were obtained, the low number of insertions and deletions (indels) and the undetected stop codons provided an easy alignment of the sequences, as expected for protein coding genes. The rarity of indels in the animal mitochondrial genes is already known (Hebert et al. 2003). During the alignment of the consensus sequences of all individuals, the non-overlapping ends were eliminated for uniformity and total overlapping of the sequences was maintained. The similarity between the sequences of the COI fragment for the eight individuals of *O. fuscipes* analyzed was 99.9%. Information on the obtained sequences is listed in Table SII. The composition of the mean sequences for *O. fuscipes* in our study was G = 15.9%, C = 22.0%, A = 28.5% and T = 33.6%.

Chemical Characterization of the defensive secretion of *O. fuscipes*

The hexane extract in open SiO_2-column chromatography fractionation resulted in four fractions (A-D). The chemical composition of these fractions was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) and the chemical structure of the compounds was identified by mass fragmentation patterns and comparison with literature data (Urbanová et al. 2012, Van Der Horst & Oudejans 1973). Fraction A showed a waxy appearance with color of opaque white indicating that it is a mixture of lipophilic compounds. The Figure 2 and Table SIII show the total ion chromatogram and the mass spectra of the compounds of this fraction, respectively. This fraction showed the presence of eighteen peaks being the fatty acid methyl esters the major class (peaks **4, 7-10** and **12-18**) together with the minor alkanes (Peak **2**), alkenes (Peaks **1, 3** and **6**) and fatty acids (Peaks **5** and **11**). The Figure 3 and Table III show the total ion chromatogram and the mass spectra of the compounds present in the fraction B, C and D, possibilities to characterize four quinonic derivatives, known as; quinone 2-methoxy-3-methylhydroquinone (peak **19**), 2-methoxy-3-methyl-1,4-benzoquinone (peak **20**), 2,3-dimethoxy-1,4-benzoquinone (peak **21**) and 2,3-dimethoxyhydroquinone (peak **22**).

DISCUSSION

The factors such as ancestry, age, sex, available food, latitude, altitude, habitat and coexistence can affect the size of millipedes (Cooper 2018, Enghoff 1990). Furthermore, females of millipedes tend to have a higher diameter than males (Crawford et al. 1987, Gerlach et al. 2005),

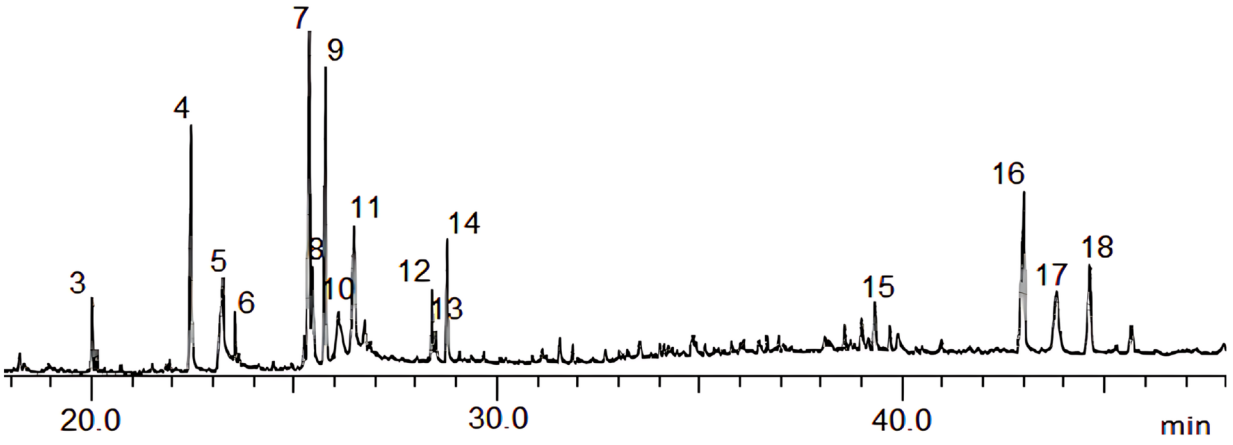


Figure 2. Total ion chromatogram obtained by GC-MS of fraction A.

which may help explain the higher body weight observed for the females of *O. fuscipes*. One can observe that the population of *O. fuscipes* declined over the years of study, and this can be a consequence of climatic stress, parasitism (Gerlach et al. 2005) or food availability (Enghoff 1990). Differences in the population structure between males and females *O. fuscipes* may be related to reproduction and/or different energy allocation strategies between different sexes, a finding that deserves further study. It is also noteworthy, as pointed out in the introduction section, that the scarcity of taxonomic studies and population dynamics of this taxonomic group have made it difficult to perform comparative studies with Diplopoda, mainly in the neotropical region.

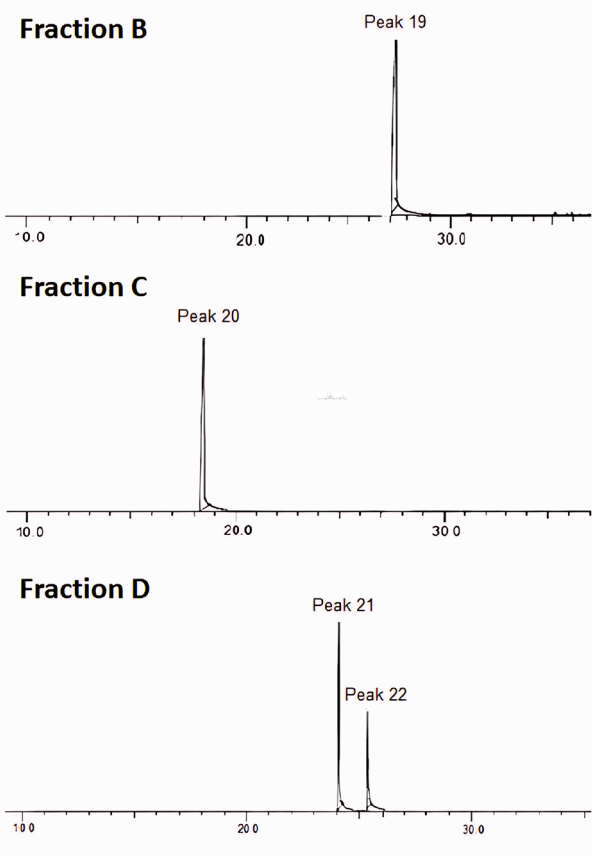
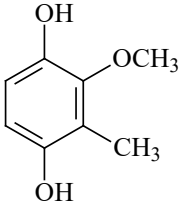
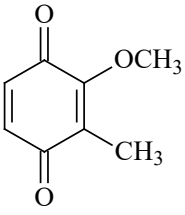
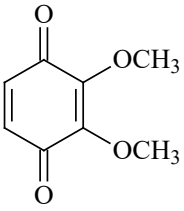
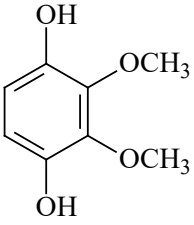


Figure 3. Total ion chromatograms obtained by GC-MS of the fractions B, C and D of the hexanic extract from *Orthoporus fuscipes*.

To identify these species, the genitalia are considered as the safest character complex for morphological taxonomy, as it is species-specific for the vast majority of animal groups. Nonetheless, in some groups, this character is very similar, and its use in species identification is not recommended (Greilhuber 1984). These obstacles have led researchers to seek a way to accelerate and facilitate the process of identifying species. Among these strategies is the use of standardized molecular tools in taxonomic research, such as the DNA barcoding, which have shown to be promising (Leite 2012). In this technique, a short (648-bp) and standardized region of the cytochrome c oxidase I (COI) gene forms the primary barcode sequence for members of the animal kingdom, aiding in the identification and discovery of species (Ratnasingham & Hebert 2007). DNA Barcode shows pronounced bias towards A and T, found in COI gene of *O. fuscipes*, characteristic of arthropods, as demonstrated by other authors (Spelda et al. 2011). The use of this technique for molecular identification of millipedes was already reported (Hassan & Hassan 2021). After searching the NCBI (GenBank®, database), no similar sequences were found, thus there is no genetic 'barcode' (DNA Barcode) for *O. fuscipes*. Therefore, the sequences of the COI fragments obtained in this study were deposited for the first time in GenBank® as a reference sequence (DNA Barcode) for the species *O. fuscipes* and can be used for future identification.

Arthropods are rich in chemical defenses used offensively for the incapacitation of the prey, and defensively against predators (Eisner et al. 1978). In millipedes, except for the orders Polyxenida, Glomeridesmida, Sphaerotheriida and Chordeumatida, which lack glands (Shear 2015), the release of these repellent and noxious fluids is made from serial exocrine glands serially arranged as segmental pairs along the length

Table III. GC-MS analysis of the fractions B, C and D (quinonic compounds) obtained of the defensive secretion from *O. fuscipes*.

Peak	[M$^+$]	RT (min)	Principal fragmentations (rel. Intensity)				Others Fragmentation (rel. Intensity)	Chemical Structure
			CH_3	CH_3-CH_3	CO + CH_3	CO + H_2 + CH_3		
FRACTION B								
19	154	27.0	139 (33)	124 (2)	111 (100)	-----	65 (15), 111 (100), 214 (3), 139 (33)	
FRACTION C								
20	152	18.5	137 (5)	122 (28)	109 (15)	-----	53 (50), 54 (30), 66 (44), 82 (29), 83 (28), 109 (15), 122 (6), 137 (5)	
FRACTION D								
21	168	24.0	153 (36)	138 (3)	125 (11)	123 (82)	54 (58), 82 (57), 94 (12), 95 (18), 153 (36), 168 (74)	
22	170	25.4	155 (61)	-----	127 (5)	125 (3)	109 (32), 112 (23), 155 (61), 170 (75)	

of the body (Eisner et al. 1978). This secretion released by millipedes can be composed of different classes of molecules, depending on the taxonomic groups (Shear 2015). Nonetheless, in millipedes from the order Spirostreptida, quinone derivatives (hydroquinones and benzoquinones) are usually the dominant components (Deml & Huth 2000, Eisner et al. 1965, Shear 2015).

The n-alkanes, saturated methyl-branched components and alkenes are the major components of the cuticular lipids on the surface of arthropods, and this wax layer is essential to prevent water loss and desiccation of these animals (Blomquist et al. 2018). Some

esters, including fatty acid methyl esters, were reported in the defensive secretions from different millipedes. These esters may act as informative molecule for intra or interspecific communication, but may be also important for the effectiveness of other compounds present in the defensive secretion (Sekulic et al. 2014, Shear 2015, Shimizu et al. 2012, Stanković et al. 2016, Vujisić et al. 2014, 2011). The alkanes and alkenes are also already found in the defensive secretions of arthropods, which can suggest that these hydrocarbons may serve as solvents for quinones, and also may act as surfactant, facilitating the spread of the secretion over the body of the arthropod (Shimizu et al. 2012, Vujisić et al. 2014, Eisner et al. 2000). On the other hand, using a methodology similar to ours, the authors did not find any compounds in hexane whole body extract of the millipede *Anaulaciulus* sp. (Julida: Julidae). In this way, we can not rule out that the presence of hydrocarbons in the hexane whole body extract of *O. fuscipes* may be from the defensive secretions, since the presence of alkanes and alkenes was reported in the defensive secretions of arthropods.

The presence of hydroquinone derivatives in the defensive secretion of the *O. fuscipes* is not surprising since hydroquinones may be expected to be the chemical precursors of the quinones in these secretions (Eisner et al. 1978). Interspecific comparison within the genus *Orthoporus* is limited. Studying the composition of the defensive secretions of millipedes from the genus *Orthoporus*, Eisner et al. (1965) and Williams et al. (1997), the authors described the characterization of the 2-methoxy-3-methyl-1,4-benzoquinone (19) for *O. antillanus* and 2,3-dimethoxy-1,4-benzoquinone (21) for *O. favor*, *O. punctilliger* and *O. conifer*, respectively. Williams et al. (1997) also reported that 2-methoxy-3-methyl-1,4-benzoquinone (20) was among the major components in the defensive secretions of *O. antillanus*.

Studies with the genera *Orthoporus* have been previously reported, indicating a high similarity between the secretion compositions in different species. Two benzoquinones were identified in three different species: 2-Methyl-3-methoxy-1 ,4-benzoquinone in *O. conifer*, *O. flavior* and *O. ornatus;* 2-Methyl-1,4-benzoquinone in *O. flavior* and *O. ornatus* (Eisner et al.1965). Six benzoquinones were detected in *O. antillanus*: two were the major compounds and were identified as 2-methyl-1,4-benzoquinone and 2-methoxy-3-methyl-1,4-benzoquinone, representing 96% of the total secretion composition. This secretion displayed antifungal, bactericidal and antinematode activities (Williams et al. 1997). Additionally, two benzoquinones, 2-methyl-1,4-benzoquinone and 2-methyl-3-methoxy-1,4 benzoquinone were identified in *O. dorsovittatus* (Valderrama et al. 2000).

CONCLUSION

Our study described, for the first time, the COI sequence, the population structure and the chemical composition of the defensive secretion of *O. fuscipes*. The sequences of the COI genes (DNA barcode) obtained in this study were deposited in database (GenBank®) and will be useful for future studies with *O. fuscipes*, helping in the taxonomic identification of this diplopod, and minimizing the problems related to the scarcity of classic taxonomists (morphological identification). The population of millipedes chosen (from Jequié, Bahia, Brazil) showed that, generally, females of *O. fuscipes* are predominant in number, are larger and have greater body mass than do males. Our results also confirm that quinonic derivatives are the major components found in the defensive secretion of millipedes

from the order Spirostreptida. In addition to quinonic compounds, several components were found in the hexane whole body extract of *O. fuscipes*, demonstrating that this millipede may be an interesting source of compounds with possible biotechnological applications.

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SUPPLEMENTARY MATERIAL

Figure S1.
Tables SI,SII, SIII.

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Julia A. Romão contributed to the procedures of the extract and isolation of the compound. Luiz Marcelo R. Tomé and Aristóteles Góes Neto performed the DNA analysis. Júlia A. Romão, Lilian Boccardo, Êuder Reis Dias and Matheus Nolasco performed the analysis of results. Ivo José C. Vieira and Raimundo Braz-Filho contributed to the performance of spectroscopic analyses and identification of the quinone. Êuder R. Dias and Matheus Nolasco contributed to writing the manuscript. Alexsandro Branco obtained the funding, designed and coordinated the work and the review of the manuscript. All authors read and approved the final manuscript.