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Palm Weevil Diversity in Indonesia: Description of Phenotypic Variability in Asiatic Palm Weevil, *Rhynchophorus vulneratus* (Coleoptera: Curculionidae)

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ABSTRACT

Palm weevils (Coleoptera: Curculionidae) are the most destructive pest of many palm species worldwide, including Indonesia. Accurate species identification and knowledge of their diversity is crucial for implementing management strategies. Some samples of palm weevils from six main islands of Indonesia were found exhibiting intermediate color and markings between those of Asiatic palm weevil (APW), *Rhynchophorus vulneratus* Panzer and red palm weevil (RPW), *R. ferrugineus* (Olivier). To test the hypothesis that intermediate occurring phenotypes in Indonesia are only phenotypic color variations of APW, morphometric analyses, mating trials, and the analysis of cytochrome b (CyB) and random amplified polymorphism DNA (RAPD-PCR) were carried out. Additionally, RPW from the Kingdom of Saudi Arabia (KSA) and Pakistan were compared to Indonesian samples. Morphology-based identification of Indonesian palm weevils recognized three putative taxa: *R. ferrugineus*, *R. vulneratus*, and the black palm weevil, *R. bilineatus* (Montrouzier). It was suspected that intermediate color phenotypes of APW from Indonesia had been ambiguously considered RPW. Discriminant function analysis of morphometric measurements indicated that an intermediate weevil, which was previously determined to be RPW, are highly similar to APW. The mating trials of intermediate weevils and APW produced fertile progenies for three successive generations. The CyB and RAPD-PCR analyses showed that the intermediate phenotypes previously identified as RPW are distinguishable from RPW from the KSA. Therefore, our findings indicate that these are color phenotypes of APW. Thus, based on the palm weevil samples in this study, only two species exist, namely, *R. vulneratus* and *R. bilineatus*.

Key words: Morphometric, palm weevils, Indonesia, phenotype, polymorphic.

INTRODUCTION

Palm weevils are large curculionid beetles belonging to the subfamily Rhynchophorinae. This subfamily consists of seven species that infest a wide range of palms (Booth *et al.*, 1990). Five species, including red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier); African palm weevil, *R. phoenicis* (Fabricius); black palm weevil, *R. bilineatus* (Montrouzier); palmetto weevil, *R. cruentatus* (Fabricius), and South American palm weevil, *R. palmarum* (L.) are reported to be the most damaging pests. Among these species, *R. ferrugineus* is considered to be the most invasive pest of palms worldwide (Soroker *et al.*, 2005; Malumphy and Moran, 2007; Bozbuga and Hazir, 2008; EPPO, 2008; Pelikh, 2009; USDA-CPHST, 2010; CABI, 2015). Based on the last five years studies in South East Asia, the RPW distribution has been evaluated and was confirmed that it exists in Thailand, Philippines, Vietnam, Cambodia, and several states in Malaysia (Azmi *et al.*, 2013; Rugman-Jones *et al.*, 2013).

In Indonesia, palm weevils have been relatively poorly studied. This is most likely because the Indonesian Ministry of Agriculture (IMA) has declared it, based on surveys in East Java during 2007 and 2008, as secondary pests only (DITJENBUN, 2010). Recently, the consequences of palm weevil infestations have become evident, and a high degree of damage to coconut palms has been reported by several districts of East Java, i.e., Ponorogo, Kediri, Jombang, and Probolinggo (Ernawati and Yuniarti, 2013; Wibowo and Ernawati, 2013; Trisnadi, 2014). Beginning 2016, the weevil has been declared the second most damaging pest of coconut in East Java region (Nadiah, 2016; Santosa, 2016).

Identifications of palm weevils from Indonesia have been open to question. IMA has reported three palm weevils: red palm weevil, *R. ferrugineus*, which has black spots on the pronotum; Asiatic palm weevil (APW), *R. vulneratus* or *R. schach*, which is black with a red pronotal stripe; and black palm weevil (scientific name not provided by source), which lacks pronotal spots (DITJENBUN, 2010). Kalshoven (1981) and Pracaya (1991) recognized these weevils as *R. ferrugineus*, *R. vulneratus*, and *R. bilineatus*, respectively.

A previous study on the taxonomy of palm weevils confirmed that these three species were present in Indonesia (Wattanapongsiri, 1966). However, there has been confusion about the identification of *R. vulneratus* in Indonesia (Santosa, 2016) and neighboring countries. For example, in Malaysia, this taxon has sometimes been considered *R. ferrugineus*, or the South American palm weevil, *R. palmarum* (L.), or *R. schach* (Wattanapongsiri, 1966).

The wide range of colors and markings exhibited by palm weevil adults has been a challenge for researchers and taxonomists in making conclusive identifications of these beetles, including those from Indonesia (Hallett *et al.*, 2004; Rugman-Jones *et al.*, 2013). Some specimens from Indonesia have been identified as *R. ferrugineus* based on morphology (Wattanapongsiri, 1966). After studies of aggregation pheromones and analysis by random amplified polymorphic DNA polymerase chain reaction (RAPD PCR) on these palm weevils from West Java, Indonesia, it was proposed that *R. vulneratus* was a synonym of *R. ferrugineus* because both were capable of mating and producing fertile progenies (Hallett *et al.*, 2004; Hallett *et al.*, 1993). However, a study

by Rugman-Jones *et al.* (2013) using the mitochondrial cytochrome c oxidase subunit I gene (COI) and pronotal shape to analyze a larger number of samples of *R. vulneratus* from Indonesia (Java, Sumatra, and Bali) and *R. ferrugineus* samples from 18 countries, determined that *R. vulneratus* is a distinguishable species from *R. ferrugineus*.

The identifying characteristics of palm weevils having a rusty red body with a high variability of black pronotal markings, morphologically very similar to that of RPW, are inconclusive, like in the case of *R. palmarum* in Colombia (Lohr *et al.*, 2015). Although the previous studies of Rugman-Jones *et al.* (2013) have found a wide range of phenotypic chromatic variations in APW and RPW, they have not aided species descriptions or identification of their specimens. By analyzing APW samples, they concluded that genetically there was no evidence of RPW presence in Indonesia. Nevertheless, until now there was no proof that APW exhibits atypical phenotypic variation, that is, a rusty red body with black pronotal markings instead of the typical black body with a red pronotal stripe.

Unambiguous taxonomic identification of palm weevils, as well as knowledge of their diversity, is critical, as it is the foundation for appropriate pest management programs. Although efforts have been made to identify palm weevils, including those in Indonesia, there are unanswered questions. In the present study, sampling of palm weevil populations was carried out on the six main islands of Indonesia to provide a better understanding of palm weevil diversity and to evaluate the extent of color variation in *R. vulneratus* and the other closely related species, *R. bilineatus*. Samples were subjected to morphological measurements to identify the characteristics useful for weevil species discrimination. Additionally, genetic compatibility, CyB and RAPD-PCR analyses of these putative species were evaluated for morphometric confirmation. The approaches were designed to test if there are color phenotypes of APW from Indonesian samples that were perhaps ambiguously considered RPW.

MATERIALS AND METHODS

Palm weevil collection

Larval, pupal, and adult stages of palm weevils collected from seven provinces covering six main islands of Indonesia from June to September of 2012 and 2014 were used in this study (Table 1). From these, two hundred and thirty-seven individuals were collected from 25 localities (Fig. 1). The sampling was conducted by hand picking adults, pupa, and larva from infested coconut palms (*Cocos nucifera* L.), toddy (*Borassus flabellifer* L.), and/or sago (*Metroxylon sago* Rottb.). Palm weevil infestation was recognized by the typical symptoms of oozing sap and frass along the palm trunk, apical leaf dieback, and a collapsed crown (Abraham *et al.*, 1998). Adults were kept in 96% ethanol in 50 ml conical tubes. Collected larvae were maintained on sugarcane, while pupae were kept in plastic boxes until emergence. The emerged adults were then preserved in 96% ethanol. Adults of RPW collected by using pheromone traps from date palm farms in Al 'Ammariyah, Riyadh Region, the Kingdom of Saudi Arabia (KSA) and Khudai, Punjab Province of Pakistan, were used for species comparisons.

Morphometric studies

We suspected that RRPW was a color phenotype of APW. RPW collected from the KSA and BPW collected from Indonesia were used as controls to determine the effectiveness of the morphometric species separation. Forty-nine morphometric characters were measured using a Dino-Lite Edge Digital Microscope, AM4815ZT (AnMo Electronics Corp., USA). The proportion of black spots existing on the pronotum was measured using Digimizer image analysis software, version 4.3.1 (MedCalc Software, Belgium). The morphological species identification was conducted using a determination key (Wattanapongsiri, 1966).

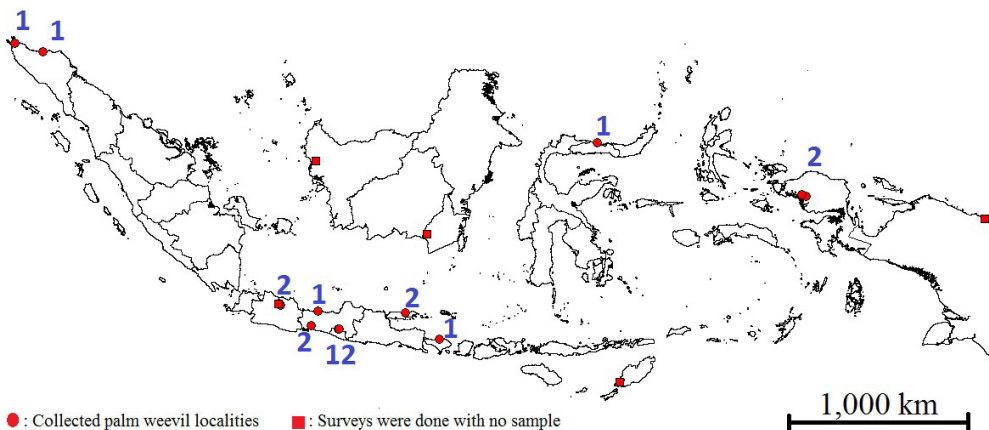


Fig. 1. Survey and collection sites of palm weevils from Indonesia, showing the number of localities at each site (● : localities where palm weevils were found/collected, ■ : localities where surveys were made but weevils were not found).

RRPW and APW mating experiment

Twenty-six palm weevil pupae were collected from coconut palms in Kebonalas, Klaten, Central Java, and brought to the laboratory. The pupae were kept individually in a plastic jar (d: 80 mm, h: 80 mm) covered with a perforated lid, and incubated at room temperature. The mating experiments were performed as heterospecific pairings

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(RRPW x APW, RRPW x RRPW, and APW x APW) using unmated males and females. These mating experiments were repeated three times, whereas conspecific mating was performed twice.

Table 1. Localities (latitude and longitude) of palm weevil collections covering seven provinces in Indonesia, and several locations in Saudi Arabia and Pakistan.

No	Locality/Province*)	Code	Latitude	Longitude	Species
1.	Bireun, Aceh IN	BIR A	5.195158	96.71058	<i>R. vulneratus</i>
2.	Lambrita, Aceh IN	LAM A	5.576197	95.38698	<i>R. vulneratus</i>
3.	Katiasa, Bali IN	BAL A	-8.16072	115.1387	<i>R. bilineatus</i>
4.	Bumi Ayu, Central Java IN	BAY A	-7.53806	109.1794	<i>R. vulneratus</i>
5.	Jetis A, Central Java IN	JTS A	-7.66841	110.4987	<i>R. vulneratus</i>
6.	Jetis B, Central Java IN	JTS B	-7.66782	110.4967	<i>R. vulneratus</i>
7.	Jetis C, Central Java IN	JTS C	-7.66781	110.4965	<i>R. vulneratus</i>
8.	Jetis D, Central Java IN	JTS D	-7.66857	110.4988	<i>R. vulneratus</i>
9.	Jetis E, Central Java IN	JTS E	-7.66852	110.4973	<i>R. vulneratus</i>
10.	Kebon Alas A, Central Java IN Java	KBN A	-7.68646	110.4885	<i>R. vulneratus</i>
11.	Kebon Alas B, Central Java IN	KBN B	-7.68688	110.4885	<i>R. vulneratus</i>
12.	Kebon Alas C, Central Java IN	KBN C	-7.68708	110.4884	<i>R. vulneratus</i>
13.	Gondang, Central Java IN	GDG A	-7.67528	110.4967	<i>R. vulneratus</i>
14.	Pemalang, Central Java IN	PEM A	-6.85977	109.5075	<i>R. vulneratus</i>
15.	Madura A, East Java IN	MAD A	-6.94456	113.5491	<i>R. vulneratus</i>
16.	Madura B, East Java IN	MAD B	-6.93524	113.5553	<i>R. vulneratus</i>
17.	Sumalata, Gorontalo, Sulawesi IN	SUM A	0.974586	122.5041	<i>R. vulneratus</i>
18.	Aitinyo, West Papua IN	AIT A	-1.46081	132.0229	<i>R. bilineatus</i>
19.	Moswaren, West Papua IN	MOS A	-1.50742	132.233	<i>R. bilineatus</i>
20.	Teminabuan, West Papua IN	PAP A	-1.44905	132.0236	<i>R. bilineatus</i>
21.	Pendowoharjo, Jogjakarta IN	PND A	-7.68413	110.4341	<i>R. vulneratus</i>
22.	Kuwung A, Jogjakarta IN	KUW A	-7.67367	110.4581	<i>R. vulneratus</i>
23.	Kuwung B, Jogjakarta IN	KUW B	-7.67388	110.4581	<i>R. vulneratus</i>
24.	Alamariyah A, Riyadh SA	AMR A	24.80447	46.50868	<i>R. ferrugineus</i>
25.	Alamariyah B, Riyadh SA	AMR B	24.81748	46.51401	<i>R. ferrugineus</i>
26.	Khudai A, Punjab PK	KUD PK1	30.059377	71.179379	<i>R. ferrugineus</i>
27.	Khudai B, Punjab PK	KUD PK2	30.059377	71.179379	<i>R. ferrugineus</i>
28.	Khudai C, Punjab PK	KUD PK3	30.059377	71.179379	<i>R. ferrugineus</i>
29.	Muzafargarh, Punjab PK	MZF PK1	30.059377	71.179379	<i>R. ferrugineus</i>

Note: *) IN: Samples from Indonesia, PK: Samples from Pakistan, SA: Samples from Saudi Arabia.

RRPW and APW mating experiment

Twenty-six palm weevil pupae were collected from coconut palms in Kebonallas, Klaten, Central Java, and brought to the laboratory. The pupae were kept individually in a plastic jar (d: 80 mm, h: 80 mm) covered with a perforated lid, and incubated at room temperature. The mating experiments were performed as heterospecific pairings (RRPW x APW, RRPW x RRPW, and APW x APW) using unmated males and females. These mating experiments were repeated three times, whereas conspecific mating was performed twice.

Each pair was kept separately in a one liter plastic box (l: 160 mm, w: 100 mm, and h: 70 mm) covered with a perforated cover. Cotton saturated in a 10% sugar solution was provided as a food source. This cotton also served as a substrate for egg laying and allowed the weevils to remain upright. The observation and collection of eggs laid were carried out daily. The collected eggs from each pairing were kept in a plastic cup (d: 50 mm, h: 70 mm) and provided with water-saturated filter paper. The eggs were incubated at room temperature and their hatchability was observed daily. The hatched larvae were collected daily, then transferred to sugarcane for feeding and rearing (Kaakeh *et al.*, 2001; Shahina *et al.*, 2009). The interbreeding follow-up was conducted for one month, beginning on the first day of egg-laying by each pair. The experiment was repeated with three successive generations using these interbred progeny. Information on larval duration, pupal duration, full-grown larval weight, and adult weight of *R. vulneratus* on sugarcane was noted.

Identification using CyB and RAPD-PCR markers

Thirty-one specimens for the representatives of *R. vulneratus* (RRPW, APW, and IPW), *R. ferrugineus* (RPW) from Saudi Arabia and Pakistan, and *R. bilineatus* (BPW) were used to confirm the morphological identifications using CyB and RAPD markers. The proteinase-K lysis method (Collard *et al.*, 2007) with several modifications was used for extracting the DNA. Fifty-five microliters of proteinase-K lysis buffer [20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 400 mM NaCl, 0.3% SDS and 100 μ M proteinase-K] were added to each dried sample in a thin-wall 200 μ L PCR tube, vortexed vigorously for 10 sec, and spun in a microcentrifuge (IKA® mini G IKA® – Werke GmbH and Co. KG, Germany). The sample was then incubated in an aluminum block bath (Cool Thermo Unit CTU-Neo, Taitec Corp., Japan) at 55°C for 3 h. After incubation, the sample was kept at ambient temperature for 10 min, briefly vortexed for 10 sec, and finally centrifuged at 10000 rpm for 1 min (Hermle Z216 MK, Germany) at 25°C to precipitate the debris. The crude DNA samples were used directly for the subsequent DNA amplifications. Each of 1 μ L the supernatant was used as a PCR template to amplify the CyB gene and the random polymorphic DNA.

The CyB gene amplification was carried out in a 30 μ L volume of KOD FX Neo polymerase kit solution (Toyobo Co., LTD., Japan) containing 0.2 μ M each of MCB 398 and MCB 869 primers (Verma and Singh, 2002) synthesized by IDT DNA technologies (IDT DNA, Belgium), and 1 μ L of crude DNA template. The CyB was amplified in a thermocycler (GeneAmp® PCR System 9700 Applied Biosystem, USA) with a heated

lid. The amplification conditions were as follows: initial denaturation at 95°C for 2 min; then 45 amplification cycles of 98°C for 10 sec, 54°C for 30 sec, and 68°C for 40 sec; and a final extension at 68°C for 5 min, followed by 4°C for an indefinite time.

The polymorphic DNA amplification was carried out in a 30 µL volume of KOD FX Neo polymerase kit (Toyobo Co., LTD., Japan) containing each of 0.2 µM of ten-mers random primers (Gadelhak and Enan, 2005) synthesized by IDT DNA technologies (IDT DNA, Belgium), and 1 µL of crude template. The DNA was amplified in a thermocycler (GeneAmp® PCR System 9700 Applied Biosystem, USA) with a heated lid. The amplification conditions were as follows: initial denaturation at 95°C for 2 min; then 45 amplification cycles of 98°C for 10 s, 36°C for 1 min, and 68°C for 2 min; and a final extension at 68°C for 5 min, followed by 4°C for an indefinite time.

For the analysis of amplicon, one microliter each of unpurified CyB amplicon was checked using 1% gel agarose electrophoresis at 100 V for 25 min in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) in an electrophoresis system (Mupid® - 2 Plus Submarine electrophoresis system Takara, Japan). The 1 kb Plus DNA Ladder (Invitrogen Life Technologies, USA) was used as a marker. The gels were then placed in an ethidium bromide solution (4 µL per 200 mL 1× TAE). Staining was performed by gentle shaking for 30 min at 45 rpm on an orbital shaker (Orbital incubator SI 500 Stuart®, Bibby scientific Ltd., UK). The stained gels were visualized under UV light in a gel documentation system (BioDocAnalyze, Biometra, Denmark). The amplified PCR products of CyB (~500 bp) were sent to Beijing Genomics Institute (BGI China) for sequencing in both directions using the Sanger method.

The RAPD-PCR amplicons were checked using 1.8% agarose gel, with the same electrophoresis, staining, and observation procedures as described above for the CyB analysis. The 1 kb Plus DNA Ladder (Invitrogen life technologies, USA) was used to estimate the molecular size of the amplicons. The presence of bright and reproducible bands was scored as one (1) while their absence was scored as zero (0). The score was only given to amplicons that had clear bands, and were subsequently converted to binary data by using the Jaccard index for the further construction of a phylogenetic tree (Sneath and Sokal, 1973). Finally, hierarchic clustering analysis using SPSS 13.0. was performed (SPSS Inc., 2005).

Both directions of the CyB sequences were aligned separately in BioEdit ver. 7.2.2 (Hall, 1999). The primers were trimmed to obtain 421 bp nucleotides. Basic local alignment search tool (BLAST) of the nucleotides was carried out using NCBI GenBank databases to confirm the obtained CyB sequences. The validated sequences were then aligned using ClustalW multiple alignments in the BioEdit program (Hall, 1999). The Kimura 2-parameter model available in MEGA 6.06 was used to estimate the pattern and rate of substitution, transition/transversion bias, and genetic diversity. This model was also used for constructing the neighbor-joining (NJ) phylogeny (Tamura *et al.*, 2013) with 1000 bootstrap values. The transition and transversion substitutions were included in the analysis. Gaps or missing data were treated as complete deletions. A comparison of the NJ analysis based on the CyB and RAPD-PCR phylogenetic relationships from the same specimens was used for species identification.

Statistical analysis

A multivariate analysis of variance (MANOVA) at $\alpha = 0.05$ was conducted to determine whether there were significant differences between the three main phenotypes and RPW species based on the entire morphometric dataset. Subsequently, a one-way ANOVA was performed to evaluate the contribution of each characteristic in the color morph/species separations. Means were separated by least significant difference (LSD). Eleven morphometric characters contributing to strong color phenotypic differences were used for species separation. The morphometric and statistical analyses for species separation were based on (Sánchez-Ruiz and Sanmartín, 2000). All analysis procedures were performed using SPSS 13 (SPSSInc, 2005).

RESULTS

Palm weevil diversity in Indonesia

Based on morphological identification using a palm weevil identification key, three weevil species could be identified in Indonesia: RPW, *R. ferrugineus* (Fig. 2) APW, *R. vulneratus* (Fig. 3), and BPW, *R. bilineatus* (Fig. 4). RRPW and APW populations were sympatric in the Aceh Special Province, West Java, Jogjakarta Special Province, Central Java, East Java, North Sulawesi, and Madura. They were sympatric on the same host plant, from either coconut or toddy palms. BPW was found only in Bali and Sorong of West Papua. It was found on coconut palms and sago in Bali and West Papua, respectively. No palm weevil were found infesting oil palm plantations in the South and West Kalimantan provinces. Additionally, coconut plantations in East Nusa Tenggara were also surveyed, but no palm weevil or infested palm was found.

Phenotypic color variation of palm weevils in Indonesia was high. For RRPW, 20 distinguishable color phenotypes could be separated (Fig. 2). The average percentage contribution of black spot pronotal markings to the total pronotal area for the above three main weevil color phenotypes was 29%, 68%, and 99%, respectively (Table 2). Twelve RRPW color phenotypes were commonly found in both sexes (M_{RR1} – M_{RR12}) (Fig. 2), whereas eight color phenotypes were identified as male-specific (M_{RR13} – M_{RR20}). This suggests a higher tendency for color variation in males (20 color phenotypes) than in females (12 color phenotypes). In addition, M_{RR7} , with an average spot area of 19%, had the highest incidence (30%) in all localities, while the color phenotypes M_{RR11} , M_{RR12} , M_{RR13} , M_{RR14} , M_{RR15} , and M_{RR19} represented only 4%.

In the case of APW, eight color phenotypes were recognized from Indonesia (Fig. 3). Five pronotal markings were common in both sexes (M_{RS1} – M_{RS5}) while the other three color phenotypes were found to be male-specific (M_{RS6} – M_{RS8}). Moreover, color morph 4 (M_{RS4}) presented the highest incidence (30%) in the 25 localities, while the color phenotypes M_{RS3} , M_{RS6} , and M_{RS8} had very low incidence values (only 4%). In addition, M_{RS4} was the most abundant color morph (25%) of the total collected individuals. Four individuals (3 females and 1 male) from North Sulawesi exhibited

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a hybrid color pattern, suggesting the possibility of RRPW and APW hybridization. These weevils exhibited a rusty red color but with a red stripe on the pronotum ($M_{RS}3$).

BPW were collected from two localities (Bali and Sorong of West Papua) and four color phenotypes were observed (Fig. 4). Among these, two were commonly found in both sexes (M_B1 - M_B2), whereas one was male-specific (M_B3) and one was female-specific (M_B4). We observed that the M_B1 color morph was found to be the most abundant in the collected samples (contributed 41%) while M_B4 (with two reddish longitudinal lines) was the least abundant (8.33%).

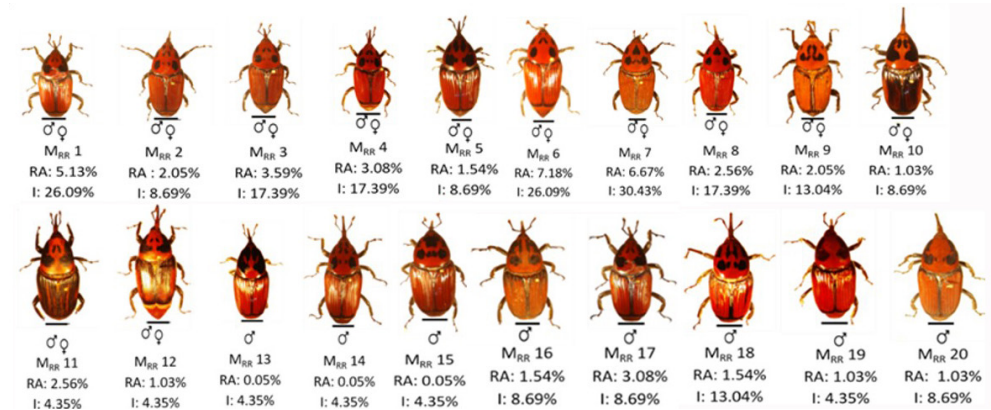


Fig. 2. Rusty red palm weevil (RRPW) color polymorphisms collected from Indonesia. Twenty rusty red color phenotypes ($M_{RR}1$ - $M_{RR}20$) were collected/identified from Indonesia. $M_{RR}1$ - $M_{RR}12$ are common in both males and females, while $M_{RR}13$ - $M_{RR}20$ are male specific. RA and I indicate the percentage (%) of relative abundance and incidence, respectively (— bar: 5mm).

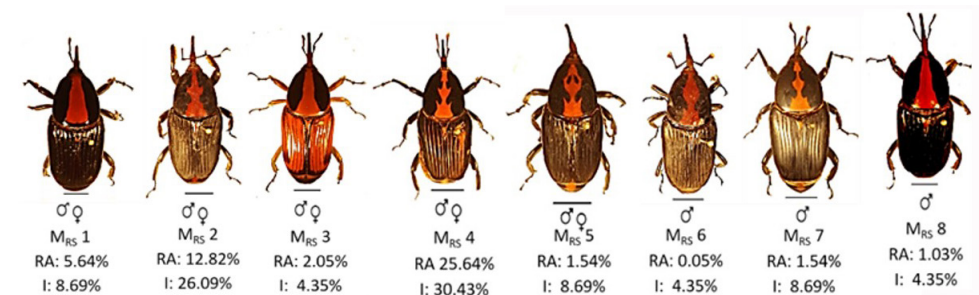


Fig. 3. Asiatic palm weevil (APW) polymorphisms collected from Indonesia. Eight red stripe color phenotypes ($M_{RS}1$ - $M_{RS}8$) were collected/identified from Indonesia. $M_{RS}1$ - $M_{RS}5$ are common in both males and females, while $M_{RS}6$ - $M_{RS}8$ are male specific. $M_{RS}3$ are weevils representing a cocktail of characteristics, with a rusty red color and a red stripe. RA and I indicate the percentage (%) of relative abundance and incidence, respectively (— bar: 5mm).

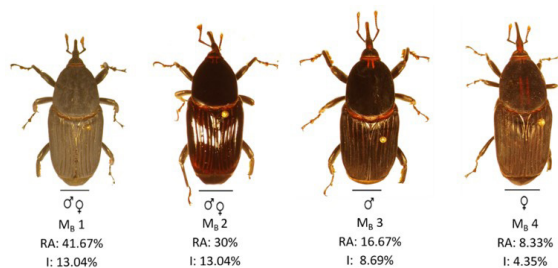


Fig. 4. Black palm weevil (BPW) polymorphisms collected from Indonesia. Four black color phenotypes (M_B1-M_B4) were collected/identified from Indonesia. M_B1 and M_B2 are common in both males and females, while M_B3 and M_B4 are male and female specific, respectively. RA and I indicate the percentage (%) of relative abundance and incidence, respectively (— bar: 5mm).

Morphometric analysis

The three palm weevils from Indonesia identified in the present study as RRPW, APW, and BPW were compared with RPW from the KSA, and were further analyzed based on their morphometric characters (Table 2). The table indicates the 49 morphometric characteristics with means and standard errors. All characteristics showed significant differences, except for onychium width, which was not significant ($F=1.02$, $df=73$, $P=0.427$). Our morphometric analyses indicated that 11 characteristics could be potentially useful for identification of these four palm weevil species: scutellum length ($F=8.99$, $df=71$, $P<0.001$), pronotum width at apex ($F=14.43$, $df=79$, $P<0.001$), profemur length ($F=13.92$, $df=79$, $P<0.001$), protibial width ($F=19.33$, $df=79$, $P<0.001$), 3rd protarsomere length ($F=19.40$, $df=77$, $P<0.001$), 3rd midtarsomere length ($F=14.28$, $df=76$, $P<0.001$), 1st protarsomere width ($F=14.46$, $df=77$, $P<0.001$), 2nd protarsomere width ($F=17.25$, $df=76$, $P<0.001$), 3rd protarsomere width ($F=10.3$, $df=74$, $P<0.001$), pygidium length ($F=18.87$, $df=77$, $P<0.001$), and the size of the black spots ($F=133.7$, $df=33$, $P<0.001$).

Although 48 morphometric measurements indicated significant differences among RRPW, APW, and BPW as compared to RPW from the KSA, the discriminant function analysis based on the 11 main characteristics (Fig. 5) placed RRPW and APW as overlapping clusters, whereas both of BPW and RPW were clearly separate clusters. Figure 5 showed that RRPW has higher morphometric similarities to APW than to RPW, and they are even further separated from BPW.

RRPW and APW color phenotypes interbreeding

The interbreeding data for three successive generations of the RRPW and APW color phenotypes is provided in Table 3. RRPW and APW were capable of mating and producing fertile progenies. The average period of newly hatched to pre-pupa, and pupae to adult was 55.2 days ($n=22$) and 34.8 days ($n=22$), respectively. The average weight of full-grown larvae, adult males, and adult females was found to be 6.5 g ($n=27$), 1.3 g ($n=11$), and 1.5 g ($n=12$), respectively.

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Table 2. The means and standard errors of the morphometric measurements (in mm) of red stripe palm weevil (RRPW), Asiatic palm weevil (APW), and black palm weevil (BPW) collected from Indonesia and red palm weevil (*R. ferrugineus*) from KSA.

No.	Characters	RSPW ^a	N	RRPW ^a	N	BPW ^a	N	RPW ^a	N
1	Rostrum width on pterygia (WRP)	1.58 (0.02)c	28	1.25 (0.03)b	44	1.16 (0.04)b	10	0.97 (0.04)a	40
2	Rostrum width between scrobes (WRS)	1.02 (0.02)a	28	1.68 (0.05)b	44	1.81 (0.04)b	10	1.61 (0.04)b	40
3	Rostrum length (LR)	9.18 (0.24)a	28	11.28 (0.28)bc	44	11.98(0.48)c	10	1.19 0.22)ab	40
4	Frons width between eyes (WF)	0.86 (0.02)a	28	0.96 (0.02)b	43	0.96 (0.03)b	10	1.13 (0.02)c	40
5	Scape length ^{*)} (LSC)	3.63 (0.07)a	28	4.09 (0.12)b	36	4.49 (0.19)b	10	3.61 (0.09)a	40
6	Scape width at maximum (WSC)	0.59 (0.01)a	28	0.70 (0.02)a	36	0.73 (0.02)a	10	0.59 (0.02)a	40
7	1 st funicular joint length (LF1)	2.54 (0.12)a	26	2.99 (0.09)b	36	3.14 (0.16)b	10	2.60 (0.07)a	40
8	2 nd funicular joint length (LF2)	0.72 (0.01)a	26	0.89 (0.03)b	36	0.86 (0.03)b	10	0.72 (0.03)a	40
9	Antennal club length (LC)	1.26 (0.03)a	25	1.42 (0.04)b	36	1.55 (0.07)b	10	1.19 (0.05)a	40
10	Antennal club width (WC)	1.84 (0.06)a	25	2.10 (0.06)b	36	2.10 (0.08)b	10	1.71 (0.05)a	40
11	Pronotum length at midline (LP)	12.23 (0.20)a	28	13.85 (0.35)b	44	14.26 (0.60)b	10	12.08 (0.19)a	40
12	Pronotum width at maximum (WP)	10.05 (0.25)a	28	11.75 (0.29)b	44	12.08 (0.53)b	10	9.98 (0.18)a	40
13	Pronotum width at base (WPB)	9.85 (0.23)a	28	11.49 (0.29)b	44	11.82 (0.51)b	10	9.28 (0.19)a	40
14	Pronotum width at apex ^{*)} (WPA)	3.99 (0.11)a	28	4.63 (0.10)b	44	5.16 (0.19)c	10	4.28 (0.07)ab	40
15	Scutellum length at midline (LS)	3.72 (0.10)a	28	4.63 (0.14)b	44	4.58 (0.19)b	10	3.88 (0.09)a	40
16	Scutellum width at base (WS)	2.07 (0.07)a	28	2.70 (0.09)b	44	2.85 (0.15)b	10	2.14 (0.04)a	40
17	Elytra width at maximum (WE)	6.51 (0.14)a	28	7.28 (0.16)b	44	7.19 (0.29)b	10	5.99 (0.15)a	40
18	Elytra length at maximum (LE)	14.58 (0.27)a	28	17.59 (0.42)b	43	17.95 (0.68)b	10	15.28 (0.20)a	40
19	Profemur length ^{*)} (LPF)	6.20 (0.12b)a	28	7.11 (0.19)b	44	8.28 (0.42)c	10	5.74 (0.12)a	40
20	Profemur width at maximum (WPF)	2.35 (0.06)a	28	2.65 (0.07)b	44	2.64 (0.11)b	10	2.31 (0.05)a	40
21	Protibia length (LPT)	6.11 (0.15)a	28	7.07 (0.19)b	44	7.70 (0.36)b	10	6.01 (0.15)a	40
22	Mid tibia length (LMT)	4.72 (0.12)a	28	5.82 (0.16)b	44	6.37 (0.30)b	10	4.52 (0.10)a	40
23	Hind tibia length (LHT)	5.70 (0.13)a	27	7.05 (0.19)b	44	7.69 (0.32)b	9	5.32 (0.13)a	40
24	Protibial width at maximum ^{*)} (WPT)	1.04 (0.03)a	28	1.29 (0.03)b	44	1.44 (0.07)c	10	1.27 (0.05)b	40
25	Mid tibia width at maximum (WMT)	1.07 (0.03)a	28	1.22 (0.03)b	44	1.24 (0.03)b	10	1.16 (0.04)ab	40
26	Hind tibia width at maximum (HMT)	1.09 (0.05)a	28	1.38 (0.05)b	44	1.36 (0.05)b	9	1.18 (0.02)ab	40
27	Protarsus length (PLT)	4.94 (0.12)a	28	5.79 (0.18)b	41	6.26 (0.24)b	10	4.89 (0.07)a	40
28	Mid tarsus length (MLT)	4.60 (0.11)a	23	5.51 (0.15)b	42	5.92 (0.26)b	10	4.37 (0.31)a	38
29	Hind tarsus length (HLT)	4.80 (0.11)a	24	5.53 (0.14)b	39	5.82 (0.26)b	9	4.74 (0.06)a	39
30	1 st protarsomere length (PLT1)	1.06 (0.03)a	28	1.26 (0.06)ab	42	1.42 (0.07)b	10	1.07 (0.04)a	40

Table 2. Continued.

No.	Characters	RSPW ^a	N	RRPW ^a	N	BPW ^a	N	RPW ^a	N
31	2 nd protarsomere length (PLT2)	0.65 (0.02)a	28	0.79 (0.02)b	42	0.81 (0.04)b	10	0.69 (0.02)a	40
32	3 rd protarsomere length ^{*)} (PLT3)	0.96 (0.02)a	28	1.18 (0.04)b	42	1.37 (0.06)c	10	1.04 (0.04)a	40
33	1 st mid tarsomere length (MLT1)	0.97 (0.03)a	26	1.19 (0.04)b	44	1.23 (0.07)b	10	1.03 (0.05)a	39
34	2 nd mid tarsomere length (MLT2)	0.61 (0.01)a	25	0.75 (0.02)b	44	0.80 (0.04)b	10	0.62 (0.03)a	39
35	3 rd mid tarsomere length ^{*)} (MLT3)	0.93 (0.02)a	25	1.12 (0.03)b	44	1.25 (0.06)c	10	0.98 (0.03)a	39
36	1 st hind tarsomere length (HP1)	0.94 (0.03)a	27	1.11 (0.03)b	44	1.12 (0.06)b	9	1.04 (0.04)ab	40
37	2 nd hind tarsomere length (HP2)	0.59 (0.01)a	26	0.70 (0.02)b	42	0.71 (0.03)b	9	0.62 (0.01)a	40
38	3 rd hind tarsomere length (HP3)	0.89 (0.02)a	26	1.14 (0.07)b	42	1.24 (0.06)b	9	1.03 (0.05)ab	40
39	Pro pretarsus length (LT1)	2.46 (0.05)a	28	2.94 (0.10)b	41	3.23 (0.15)b	10	2.38 (0.07)a	40
40	Mid pretarsus length (LT2)	2.24 (0.11)a	24	2.91 (0.09)b	42	3.21 (0.13)b	10	2.46 (0.05)a	40
41	Hind pretarsus length (LT3)	2.56 (0.06)a	24	3.05 (0.10)b	38	3.36 (0.13)b	9	2.46 (0.06)a	40
42	Onychium length at mid line (LON)	0.91 (0.02)a	28	1.02 (0.03)a	44	1.16 (0.04)b	10	0.90 (0.05)a	40
43	1 st pro tarsomere width ^{*)} (WT1)	0.89 (0.02)a	28	1.05 (0.03)b	42	1.21 (0.05)c	10	0.99 (0.05)ab	40
44	2 nd pro tarsomere width ^{*)} (WT2)	0.86 (0.02)a	25	1.01 (0.03)b	44	1.16 (0.05)c	10	0.98 (0.03)ab	40
45	3 rd pro tarsomere width ^{*)} (WT3)	0.84 (0.01)a	26	0.94 (0.02)b	42	1.04 (0.04)c	9	0.91 (0.03)ab	40
46	Onychium width (WON)	0.22 (0.02)a	28	0.23 (0.01)a	44	0.27 (0.02)a	10	0.23 (0.02)a	40
47	Body width at maximum ^{*)} (WL)	5.34 (0.24)a	27	6.93 (0.21)b	43	7.81 (0.29)c	10	6.85 (0.26)b	40
48	Pygidium length (TL)	12.48 (0.23)a	28	14.49 (0.34)b	44	14.54 (0.60)b	10	12.52 (0.28)a	40
49	Spots size ^{*)} (SPOTS)	68.64 (2.81)c	12	29.26 (2.15)b	20	99.74 (0.09)d	4	8.95 (3.55)a	20

*Numbers in the same row followed by the same letter showing no significant differences at α 0.05. The character no. 5, 14, 19, 24, 32, 35, 43, 44, 45, 47, and 49 indicate a strong separation for the three species. All the unit are in mm, except SPOTS that is in %. N indicates the number of measured individuals. a The numbers in parantheziz indicate the standard error of mean.

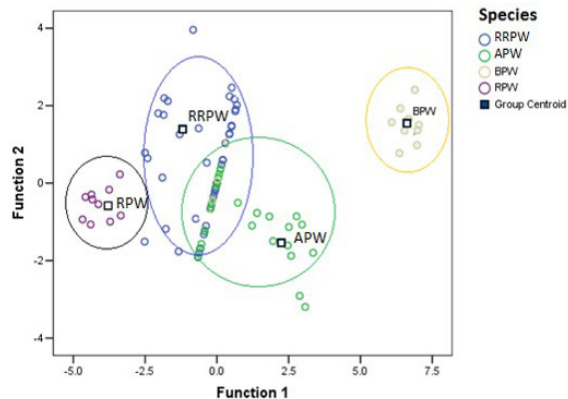


Fig. 5. The plots of first and second discriminant functions based on a set of 11 morphological measurements of rusty red palm weevil (RRPW), Asiatic palm weevil (APW), and black palm weevil (BPW) from Indonesia, and red palm weevil (*R. ferrugineus*) from Saudi Arabia.

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Table 3. The means and standard errors of eggs produced and percentage of eggs hatching within one month of *R. vulneratus* color phenotypes mating for three successive generations.

Pairings	First Generation		Second Generation		Third Generation	
	Eggs laid ^a	Hatched (%) ^a	Eggs laid ^a	Hatched (%) ^a	Eggs laid ^a	Hatched (%) ^a
RRPW-APW (n= 3)	78 (10.1)	85 (8.2)	97.3 (10.6)	86.7 (7.2)	181 (26.9)	61.8 (7.85)
RRPW-RRPW (n= 2)	88.5 (56.6)	71 (16.3)	129 (65.7)	82.2 (26.3)	109 (20.5)	51.7 (8.3)
APW-APW (n= 2)	182 (28.3)	57.5 (7.9)	87.7 (28.9)	65.4 (11.9)	77.7 (10.6)	81.9 (4.9)

Note: RRPW-APW= cross breeding of rusty red palm weevil with red stripe palm weevil, RRPW-RRPW= in breeding of rusty red palm weevil, and APW-APW= inbreeding of red stripe palm weevil with red stripe palm weevil. n indicates the number of replicate. a The numbers in parantheziz indicate the standard error of mean.

Identification based on CyB

The primers amplified all the samples with a single unique band of ~450 bp. The primers were trimmed and clean sequences with a length of 421 bp were obtained. All of the CyB sequences have been deposited into the BOLD with the accession numbers PWINA133-16 to PWINA163-16 (accessible at <http://boldsystems.org/>). The genetic diversity analysis based on CyB showed that most of the weevil color morphs or species had identical sequences ($d = 0$), except for *R. ferrugineus* from Saudi Arabia, which had 0.001 genetic diversity (Table 4).

Table 4. Several species of *Rhynchophorus* collected from Indonesia, Saudi Arabia, and Pakistan used in this study, and their genetic diversity within the populations based on cytochrome b (CyB) gene analysis.

No.	Species (N)		N	Sample Code	Locality*)	CyB ^o
1.	<i>R. bilineatus</i>	BPW	3	BAL-A1-3	Katiasa, Bali IN	0.000
2.	<i>R. vulneratus</i>	RRPW	2	KUW – C2; KUW– D1	Kuwung, Jogjakarta IN	0.000
3.	<i>R. vulneratus</i>	RRPW	6	MAD –A2-4; MAD –B1,3-4	Pamekasan, Madura IN	0.000
4.	<i>R. vulneratus</i>	IPW	2	SUM –A5-6	Sumalata, Gorontalo IN	0.000
5.	<i>R. vulneratus</i>	APW	5	KUW – E1-2,4-6	Kuwung, Jogjakarta IN	0.000
6.	<i>R. vulneratus</i>	APW	4	SUM –A1-4	Sumalata, Gorontalo IN	0.000
7.	<i>R. vulneratus</i>	APW	1	LAM –A1	Lambrita, Aceh IN	na
8.	<i>R. ferrugineus</i>	RPW	3	MZF – PK1-3	Muzafargarh, Pakistan PK	0.000
9.	<i>R. ferrugineus</i>	RPW	5	AMR –A3,4; MAD –B1-3	Alamariyah, Saudi Arabia SA	0.001

Note: N indicates the number of individuals used in the analysis. *) IN: Samples from Indonesia, PK: Samples from Pakistan, SA: Samples from Saudi Arabia. Numbers in column CyBA) indicate the genetic distance within the population, na: no available data because only had 1 sequence.

The study showed that based on the CyB marker, the interspecific variation was greater than intraspecific variation (Table 5). The genetic diversity of all *R. vulneratus* color morphs (RRPW, APW, and IPW) was zero. When compared to BPW (*R. bilineatus*) and *R. ferrugineus* from Saudi Arabia and Pakistan, the genetic distance was 0.241, 0.383, and 0.382, respectively. The genetic distance between

R. ferrugineus from Saudi Arabia and Pakistan was <0.001 , while the distance was 0.130 when compared with BPW.

Table 5. Genetic distances between rusty red palm weevil (RRPW), Asiatic palm weevil (APW), intermediate palm weevil (IPW), and black palm weevil (BPW) phenotypes in Indonesia, and *Rhynchophorus ferrugineus* in Saudi Arabia and Pakistan, based on cytochrome b (CyB) gene.

Samples-Color Morphs	N	Locality*)	1	2	3	4	5	6	7	8
1.SUM-IPW	3	Sumalata, Gorontalo IN								
2. MAD-RRPW	2	Pamekasan, Madura IN	0.000							
3. KUW-RRPW	6	Kuwung, Jogjakarta IN	0.000	0.000						
4. KUW-APW	2	Kuwung, Jogjakarta IN	0.000	0.000	0.000					
5. SUM-APW	5	Sumalata, Gorontalo IN	0.000	0.000	0.000	0.000				
6. LAM-APW	4	Lambrita, Aceh IN	0.000	0.000	0.000	0.000	0.000			
7. BAL-BPW	1	Katiasa, Bali IN	0.241	0.241	0.241	0.241	0.240	0.240		
8. MZF-RPW	3	Muzafargarh, Pakistan PK	0.382	0.382	0.382	0.382	0.382	0.382	0.130	
9. AMR-RPW	5	Alamariyah, Saudi Arabia SA	0.383	0.383	0.383	0.383	0.383	0.383	0.130	0.000

Note: *) IN: Samples from Indonesia, PK: Samples from Pakistan, SA: Samples from Saudi Arabia. *R. vulneratus* color polymorphism representatives: 1 - intermediate palm weevil color morph; 2, 3 - rusty red palm weevil color morph; 4, 5, 6 - Asiatic palm weevil color morph. *Rhynchophorus bilineatus* only exhibited a completely black color morph (no. 7). *Rhynchophorus ferrugineus* color morph representatives: 10 - two-spotted RPW; 11 - three-spotted RPW; 3 - three-spotted RPW; 4 - seven-spotted RPW. N indicates the number of individuals used in the analysis. The genetic distances were calculated only using non-identical sequences from each population.

A neighbor joining analysis of CyB showed that the specimens strongly separated into species clusters. In this analysis, the specimens were separated into three clusters, designated as C1, C2, and C3, representing the three different species (Fig. 6). Cluster C1 contained all the *R. vulneratus* color morphs: RRPW, APW, and IPW. Meanwhile, clusters C2 and C3 comprised of *R. bilineatus* and *R. ferrugineus*, respectively.

Identification based on RAPD-PCR markers

The analysis of RAPD-PCR showed bands of 200-1900 bp. All amplified bands were polymorphic (Table 6). The genetic variation between the different color morphs and species was investigated using the Jaccard index distance (dJ) (Table 7). The highest genetic distance was found between RRPW from Madura, Indonesia, and two-spotted RPW from Khudai, Pakistan (dJ = 0.96). The lowest dJ index (dJ = 0.30) was found between two- and three-spotted RPWs from Khudai, Pakistan. The range of genetic distance between IPW and RRPW, RRPW and APW, RRPW and BPW, and APW and BPW was 0.5-0.75, 0.36-0.87, 0.60-0.84, and 0.66-0.75, respectively.

The UPGMA dendrogram showed four main clusters, which were designated as Ca, Cb, Cc, and Cd (Fig. 7). Most of the clusters correspond to the species (*R. ferrugineus*, *R. vulneratus*, and *R. bilineatus*). The cluster Ca was composed of *R. ferrugineus* samples from Pakistan and Saudi Arabia. The spotted RRPW, maple leaf RRPW, and APW color morphs were grouped into the cluster Cb. The RRPW

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color morphs exhibiting bilaterally symmetrical shading and BPW (*R. bilineatus*) were separated into clusters Cc and Cd, respectively.

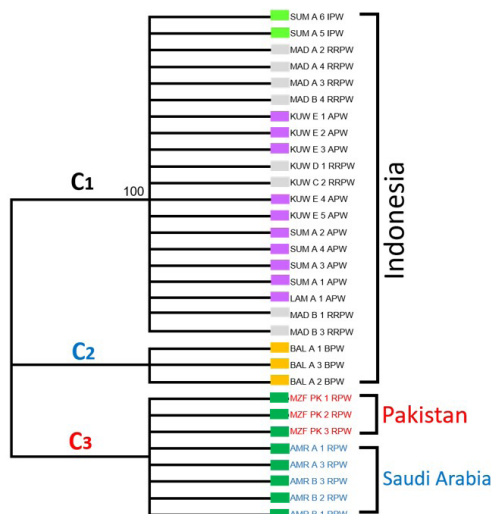


Fig. 6. The genealogical relationships of 31 sequences of the rusty red palm weevil (RRPW), Asiatic palm weevil (APW), intermediate palm weevil (IPW), and black palm weevil (BPW) from Indonesia and *Rhynchophorus ferrugineus* from Saudi Arabia and Pakistan, based on the 421 bp sequence of cytochrome b (CyB) gene using the neighbor-joining method with 1000 bootstraps. The numbers at the branching points indicate the bootstrap support values. The colors indicate the palm weevil phenotypes or species: ■ - rusty red palm weevil (RRPW), ■ - Asiatic or red stripe palm weevil (APW), ■ - intermediate palm weevil (IPW), ■ - black palm weevil (BPW), and ■ - red palm weevil (RPW). The bootstrap values below 50% were cut off.

Table 6. Total number of bands calculated from palm weevils representing *Rhynchophorus vulneratus* and *R. bilineatus* collected from Indonesia, and *R. ferrugineus* from Saudi Arabia and Pakistan.

Primers	Palm weevil species															
	<i>R. vulneratus</i> ^{a)}									<i>R. ferrugineus</i> ^{b)}					<i>R. bilineatus</i>	Total
	1	2	3	4	5	6	7	8		1	2	3	4			
Primer 1	7	7	4	2	7	7	3	6		1	2	3	3		4	62
Primer 2	1	1	4	4	0	4	2	3		2	2	2	2		4	33
Primer 3	2	2	1	2	0	1	1	1		4	4	4	4		1	27
Primer 4	0	0	4	4	4	4	1	5		3	4	2	2		3	36
Primer 5	0	1	0	0	1	1	1	1		1	4	0	5		0	15
Primer 6	1	0	3	1	5	1	4	2		3	3	0	3		4	30
Total	11	11	16	13	17	18	12	18		14	19	11	19		16	203

Note: The numbers in column a) indicate the *R. vulneratus* color morphs: 1, 2 - intermediate palm weevil morphs; 3, 4, 8 - rusty red palm weevil morphs; 5, 6, 7 - Asiatic palm weevil morphs. The numbers in column b) describe the *R. ferrugineus* morphs: 1 - two-spotted RPW; 2 - three-spotted RPW; 3 - three-spotted RPW; 4 - seven-spotted RPW. The *R. bilineatus* only used completely black-colored morph.

Table 7. Approximate genetic distances between rusty red palm weevil (RRPW), Asiatic palm weevil (APW), intermediate palm weevil (IPW), and black palm weevil (BPW) collected from Indonesia and *Rhynchophorus ferrugineus* collected from Saudi Arabia and Pakistan, based on the Jaccard index distance.

Specimen Code	1	2	3	4	5	6	7	8	9	10	11	12
1. SUMA5-IPW												
2. SUMA6-IPW	0.44											
3. MADA2-RRPW	0.50	0.50										
4. MADA4-RRPW	0.78	0.75	0.79									
5. SUMA1-APW	0.58	0.65	0.70	0.61								
6. SUMA4-APW	0.81	0.83	0.87	0.36	0.68							
7. KUW E1-APW	0.64	0.71	0.75	0.44	0.62	0.63						
8. MAD B4-RRPW	0.54	0.61	0.71	0.46	0.59	0.48	0.42					
9. BALA3-BPW	0.84	0.79	0.82	0.60	0.84	0.75	0.66	0.71				
10. RPW MZF PK1	0.84	0.87	0.96	0.58	0.70	0.80	0.70	0.76	0.81			
11. RPW MZF PK2	0.86	0.85	0.93	0.59	0.74	0.80	0.70	0.71	0.77	0.30		
12. RPW AMRA3	0.81	0.71	0.83	0.64	0.76	0.73	0.81	0.67	0.79	0.56	0.57	
13. RPW AMRA4	0.86	0.80	0.88	0.59	0.78	0.80	0.70	0.71	0.73	0.52	0.35	0.42

Note: *Rhynchophorus vulneratus* morph representatives: 1, 2 - intermediate palm weevil (IPW) morphs; 3, 4, 8 - rusty red palm weevil (RRPW) morphs; 5, 6, 7 - red stripe palm weevil morphs. The *R. bilineatus* specimens only exhibited a completely black-colored morph (no. 9). *Rhynchophorus ferrugineus* morph representatives: 10 - two-spotted RPW; 11 - three-spotted RPW; 3 - three-spotted RPW; 4 - seven-spotted RPW.

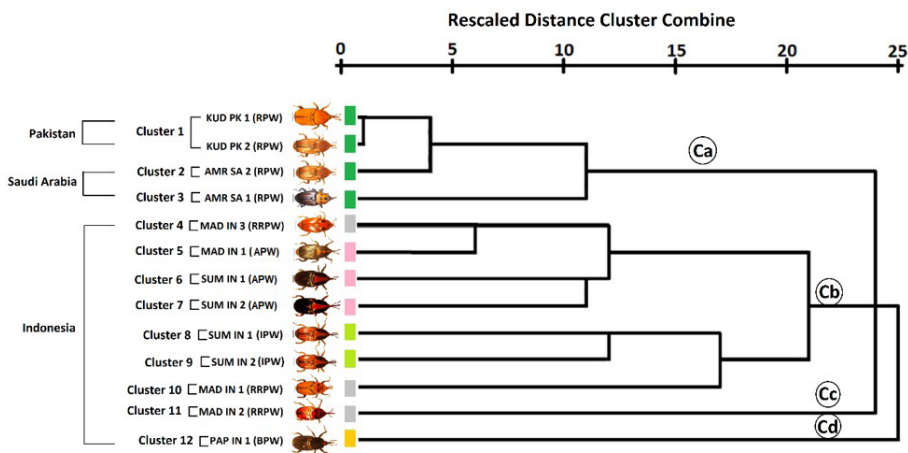


Fig. 7. Dendrogram showing the species separation of palm weevils collected from Indonesia and red palm weevils from Saudi Arabia and Pakistan, constructed based on the random amplified polymorphism DNA binary Jaccard index using UPGMA method. The colors indicate the following morphs: grey - rusty red palm weevil (RRPW), pink - Asiatic palm weevil (APW), green - intermediate palm weevil (IPW), yellow - black palm weevil (BPW), blue - red palm weevil (RPW).

DISCUSSION AND CONCLUSIONS

Recently, severe infestations of palm weevils in coconut palms were reported in Indonesia (Ernawati and Yuniarti, 2013; Trisnadi, 2014; Ratmawati, 2015; Yuliyanto and Ernawati, 2015). It was believed that three species of palm weevils were present in Indonesia: *R. ferrugineus*, *R. schach* (a synonym of *R. vulneratus*), and *R. bilineatus* (Kalshoven, 1981; Pracaya, 1991; Wattanapongsiri, 1966). Due to the morphological observations that RPW and APW were only distinguishable based on pronotal shape and markings, Wattanapongsiri (1966) concluded there were indeed three species present in Indonesia.

However, we suspected that RRPW and IPW were the same species as APW. The results of morphometric characterization, mating experiments, CyB, and RAPD-PCR analyses support the findings that they were indeed color polymorphic of APW. These suggest that this study there were two species exist: *R. vulneratus* (covering RRPW and APW color phenotypes) and the black palm weevil, *R. bilineatus*. This is in agreement with Rugman-Jones *et al.* (2013) who conclusively proved that *R. ferrugineus* was not present in Indonesia. The ambiguity of species identity of spotted rusty red - palm weevils in Indonesia (Kalshoven, 1981; Pracaya, 1991; DITJENBUN, 2010; Hallett *et al.*, 2004; Santosa, 2016), now has been solved by this study.

The phenomenon of color polymorphism has also been observed in black palm weevil (*R. palmarum*) from Colombia (Lohr *et al.*, 2015). It was found that the black palm weevil was completely black in color, except for nearly 0.3% of the captured weevils have a rusty red color closely similar to RPW. Their morphology and COI data confirmed that these specimens are color polymorphic of *R. palmarum*. The color variations may be a response to nutrition (Vanderplank, 1960), population density (Sword *et al.*, 2000), habitat conditions (Théry *et al.*, 2008), or substrate color (Hochkirch *et al.*, 2008).

The CyB marker has been widely used for species identifications. For examples, it has been used for Atlantic cod (*Gadus morhua*) (Carr and Marshall, 1991), Eurasian species of the genus *Mustela* (Mustelidae: Carnivora) (Kurose *et al.*, 2000), several endangered animals in Taiwan (Hsieh *et al.*, 2001), and the avian parasitic morphospecies *Haemoproteus* (Haemosporida: Haemoproteidae) (Barrientos *et al.*, 2002). This marker has been proved as a sensitive and accurate tool for species identification, even with small amounts of degraded DNA material (Branicki *et al.*, 2003).

Although the RAPD-PCR methods have limitation on the reproducibility (Penner *et al.*, 1993; Micheli *et al.*, 1994; Skroch and Nienhuis, 1995; Jones *et al.*, 1997; Bagley *et al.*, 2001), the analysis suggested that it was a reliable method species identification, with the exception of the individual exhibiting the bilaterally symmetrical pronotum pattern. Beside its limitation, the method has some advantages over the other methods used in this study, as it does not require any prior knowledge on the particular gene in the targeted organism; furthermore, the use of more than one primer might also increase its efficacy (Calvert *et al.*, 2005). It also has the added advantage of being cheaper because it does not require a subsequent sequence analysis.

Abad *et al.* (2014) studied a morphometric of *Rhynchophorus* from Philippines using the length and width of the head, thorax, abdomen, rostrum and antenna, femur, tibia, and tarsus. There were no significant differences reported by these authors. In this study, the multivariate discriminant analysis has shown as an effective method for distinguishing palm weevil species. The use of a single characteristic, or a small number of them, would not be sufficient for identification, especially for cryptic species. We propose that the overall eleven morphometric measurements should be analyzed as a whole for use in species identification.

Additionally, Chong *et al.* (2015) have conducted genetic diversity study of RPW in Terengganu Malaysia using COI marker. It showed that all samples had identical sequences and they suggested that it was originated from a single haplotype introduction from Mediterranean countries. In Malaysia, the RPW was firstly reported as a pest of coconut palm in east coast of the country (Azmi *et al.*, 2013). The separation of APW and RPW as distinct species have been done using cytochrome oxidase sub unit I (COI) (Rugman-Jones *et al.*, 2013; Yong, 2016), and distinguishing *R. ferrugineus* to *R. phoenicis*, *R. cruentatus*, *R. bilineatus*, and *R. palmarum* (Mergawy *et al.*, 2011).

The overall findings of the present study are as follows: 1. The morphometric data and mating experiments provided evidence that RRPW and APW, although variable in color, are a single taxon. 2. The morphological data of Indonesian RRPW and APW color phenotypes, including RPW from the KSA, indicate that RRPW, previously considered *R. ferrugineus*, are actually color phenotypes of *R. vulneratus*. 3. In Indonesia, 32 different pronotal markings representing two palm weevil species, *R. vulneratus* (28 color phenotypes) and *R. bilineatus* (four color phenotypes), have been identified. The number of pronotal markings on male weevils was higher than that on females in both species.

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Size of Interacting Resource-Host-Parasitoid Populations Influences Mass Rearing of *Cotesia vestalis*

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ABSTRACT

The importance of *Cotesia vestalis* (Haliday) has been well documented as one of the most significant biocontrol agents for the diamondback moth, *Plutella xylostella* (L.). The aim of the present study was to optimize the procedure of mass rearing of *C. vestalis*. The effects of space and plant-herbivore-parasitoid biomass on the quality of produced wasps were investigated. In particular, the effects of food and space resources of *P. xylostella* and *C. vestalis* on mass-reared *C. vestalis* were investigated. The results indicated that there was no significant difference between treatments for percentage parasitism, survival rate and larval developmental period of the produced wasps. However, developmental period of pupa, the ratio of female offsprings and sex ratio of the mass-reared wasps were significantly affected by the given treatments. Based on these findings, it can be concluded that the extent of available resources plays a crucial role in mass rearing of *C. vestalis*.

Key words: Mass rearing, optimization, space, density, *Plutella xylostella*.

INTRODUCTION

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera, Plutellidae), is a serious pest of cruciferous plants. To control *P. xylostella* farmers use a large amount of insecticides, which has been created several issues, such as pesticide resistance and eliminations of natural enemies from fields (Talekar and Shelton, 1993; Sarfraz *et al.*, 2005; Afiunizadeh and Karimzadeh, 2015). This overuse of pesticides, which has been resulted in pest resurgence, has led researchers to develop alternative and more sustainable pest management strategies, as an exemplification, biological control (Karimzadeh and Sayyed, 2011; Gulzar *et al.*, 2012; Jafari *et al.*, 2016).

Cotesia vestalis (Haliday) (Hymenoptera: Braconidae), is one of the primary larval endoparasitoid of *P. xylostella* (Sarfraz *et al.*, 2005). The percentage parasitism of *P. xylostella* larvae by *C. vestalis* in the field conditions ranged between 40.0-83.3% in Japan, 3.6-73.2% in Hawaii, 30-50% in South Africa and 5.4-88.7% in Jamaica. In the field experiments and without application of pesticide, parasitism percentage of *P. xylostella* was 90-95% by *C. vestalis* (Kawazu *et al.*, 2011). Afiunizadeh and Karimzadeh (2015) used a more precise method of assessment of percentage parasitism (i.e., recruitment method) to indicate that about 21.0% of field populations of *P. xylostella* were parasitized by *C. vestalis*. In comparison with other larval parasitoids to illustrate, *Diadegma semiclausum* (Hellen) (Hymenoptera, Ichneumonidae), *C. vestalis* has shown the ability to act and establish well at higher temperature (20-30°C). In Taiwan, the integration of *Bacillus thuringiensis* and release of *C. vestalis* has led to a great reduction of diamondback moth populations (Talekar and Shelton, 1993). *C. vestalis* has been subjected to more than 20 classical biological control introductions, of which many have been successful (Sarfraz *et al.*, 2005). In addition, this parasitoid appears to have a wider range of natural distribution compared to *D. semiclausum*. *C. vestalis* has been reported to attack the diamondback moth in many regions to exemplify, Malaysia, Taiwan, Vietnam, China, Japan and Iran with no records of introductions (Afiunizadeh *et al.*, 2011; Furlong *et al.*, 2013; Afiunizadeh and Karimzadeh 2015).

C. vestalis has been reported from Iran on *Simyra dentinosa* Freyer (Lepidoptera, Noctuidae) (Karimpour *et al.*, 2005), and on *P. xylostella* (Golizadehet *et al.*, 2007; Afiunizadeh and Karimzadeh 2010). Afiunizadeh and Karimzadeh (2015) reported *C. vestalis* had the highest distribution and percentage parasitism (21%) in comparison with others larval and pupal parasitoid of *P. xylostella* in central Iran. It has been concluded that the most effective larval parasitoid of *P. xylostella* is *C. vestalis* (Afiunizadeh and Karimzadeh, 2015). The mass rearing and release of this parasitoid, therefore, may establish an ecological and more sustainable strategy for *P. xylostella* management (Afiunizadeh and Karimzadeh, 2010; Afiunizadeh and Karimzadeh, 2015). However, there is little information regarding the mass rearing of *C. vestalis*. Different aspects of rearing of beneficial insects have been discussed by Singh (1982). For most parasitoids there are only limited reports about optimization of mass-rearing conditions. Ramalho *et al.* (2012) evaluated the criteria of optimizing the mass rearing of *Cotesia flavipes* (Cam.) in laboratory. They investigated the optimal density of the host larvae (*Diatraea saccharalis* Fub.) parasitism capacity and adult parasitoid diets.

The Mass Rearing of Cotesia vestalis

In another experiment, Vacari *et al.* (2012) investigated the quality of *C. flavipes* reared on different host densities, and the cost of commercial production. The effects of colour and height of artificial feeding site for *C. vestalis* have been reported by Mitsunaga *et al.* (2004), who found *C. vestalis* was more attracted to yellow compared with others colours. In addition, the parasitoid chooses a feeding site hung 50 cm above ground over the one with 200 cm above ground.

It has been well documented that Chinese cabbage (*Brassica pekinensis*) is the most suitable host plant for rearing of *C. vestalis*, as the greatest parasitism success of *C. vestalis* has been achieved by feeding on *B. pekinensis* (Talekar and Yang, 1991; Liu and Jiang, 2003; Jafary *et al.*, 2012; Karimzadeh *et al.*, 2013; Heidary and Karimzadeh, 2014). Effect of food resource for adults of *C. vestalis* has been investigated in laboratory (Mitsunaga *et al.*, 2004); the highest survival and parasitism rates were obtained when adults of *C. vestalis* fed on a 50% honeybee solution. Also, honey-bee bread has shown a great potential food for adults of *C. vestalis* (Soyelu, 2013). In order to develop a mass rearing program, it would be necessary to optimize the space and resource (food plant and host) density and initial parasitoid density. In a previous work (Rezaei *et al.*, 2014b), we have already indicated that herbivore overcrowding and space limitation have negative effects on mass rearing of *C. vestalis*. The present study aimed to investigate to what extent the enlargement of optimal space and resource-host-parasitoid densities can influence the success of mass rearing of *C. vestalis*.

MATERIALS AND METHODS

Plant and insect rearing

Chinese cabbage, *Brassica pekinensis* (Lour.), was grown in plastic pots (10 cm diameter and 11 cm height) under glasshouse condition (25±5°C, 70±5% RH and L:D 16:8 h) without the application of any pesticide. The cultures of *P. xylostella* and *C. vestalis* were maintained by collection from common cabbage and cauliflower fields in PirBakran region (32°28' 8" N and 51°33' 28" E, at 1,610 m altitude) of Isfahan province (central Iran). The colonies of *P. xylostella* were maintained on 5-week-old Chinese cabbage in ventilated cages (40×40×40 cm). The cultures of *C. vestalis* were then maintained on *P. xylostella* larvae in same cages. All cultures and rearing were kept at standard constant environmental condition (25 ± 2°C, 70 ± 5% RH and L:D 16:8 h). Aqueous honey solutions (40%) (Mitsunaga *et al.*, 2004) were placed in the rearing cages for feeding adults of *P. xylostella* and *C. vestalis* (Karimzadeh *et al.*, 2004; Rezaei *et al.*, 2014a, b).

Experimental design

Experiments were conducted to investigate the effects of enlargement of optimal space and resource-host-parasitoid (RHP) densities on life-history parameters (percentage parasitism, survival rate, sex ratio, larval and pupal developmental periods, and percentage of survived females) of *C. vestalis*. Treatments include 1) one unit of optimal space and RHP densities (a small cage (40×40×40 cm), two plants (5-6-week-old Chinese cabbage), 40 hosts (early 3rd instar larvae of *P. xylostella*) and

5 parasitoid wasps (3-day-old, mated females of *C. vestalis*), 2) three units of optimal space and RHP densities (a median cage (70×55×50 cm), six plants, 120 hosts and 15 parasitoid wasps) and 3) six units of optimal space and RHP densities (a big cage (100×70×55 cm), twelve plants, 240 hosts and 30 parasitoid wasps). To start the experiment, the late 2nd instar larvae of *P. xylostella* were established on 5-6-week-old Chinese cabbages. After 24 h (when the larval population was established), 3-day-old mated females of *C. vestalis* were released in each cage for 24 h. The larvae were then maintained on the host plants until the hosts were pupated or wasp cocoons were formed. Each treatment was daily monitored and the numbers of formed host pupae and wasp cocoons were recorded. The parasitoid cocoons were further monitored for adult emergence and sex ratio. The treatments were replicated four times in a randomized complete block design with four replications and maintained under constant environmental conditions (25 ± 2 °C, $70 \pm 5\%$ and L:D 16:8 h).

Statistical analyses

The percentage parasitism was evaluated as a ratio of the wasp to the sum of host and wasp. The data on percentage parasitism and survival rate were analysed using logistic analysis of deviance. Data on sex ratio was analysed using logistic analysis of deviance (for the difference between the treatments) and exact binomial test (for difference of each treatment with the sex ratio of 1:1). Data on developmental periods were analysed using nested ANOVA. Pair comparisons were done using Tukey's honest significant difference. All statistical analyses were completed in *R. 2.10.0* software (Crawley, 2014).

RESULTS

Percentage parasitism of *P. xylostella* larvae by *C. vestalis*

When percentage parasitism was investigated based on the wasp cocoons, there was no significant ($df=9$, $t\text{-value}=1.374$, $P=0.203$) difference between treatments (Table 1). The mean of percentage parasitism based on the wasp cocoons, varied between treatments from 83.3% (treatment 2) to 90.2% (treatment 1). Also, When percentage parasitism was calculated based on the emerged adult wasps, there was no significant ($df=9$, $t\text{-value}=1.313$, $P=0.222$) difference between treatments (Table 1). The mean of percentage parasitism based on the emerged adult wasps, varied between treatments from 82.1% (treatment 2) to 89.3% (treatment 1).

Survival rate of *C. vestalis*

When survival rate was analysed based on the wasp cocoons, there was no significant ($df=12$, $t\text{-value}=-0.867$, $P=0.402$) difference between treatments (Table 1). The mean of survival rate based on the wasp cocoons, varied between treatments from 68.8% (treatment 1 and 2) to 73.8% (treatment 3). However, when survival rate was evaluated based on the emerged adult wasps, there was no significant ($df=12$, $t\text{-value}=0.911$, $P=0.380$) difference between treatments (Table 1). The mean of survival rate based on the emerged adult wasps, varied between treatments from 62.5% (treatment 1) to 66.5% (treatment 3).

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Table 1. The effects of enlargement of optimal space and resource-host-parasitoid (RHP) densities on parasitism (by *C. vestalis*) and survival of *Plutella xylostella* larvae.

Treatment	Percentage parasitism		Percentage survival	
	Cocoon based	Adult based	Cocoon based	Adult based
1 (one unit of optimal space ¹ and RHP densities ²)	90.2 ± 3.0 a ³	89.3 ± 2.7 a	68.8±3.6 a	62.5±7.2 a
2 (three unit of optimal space and RHP densities)	83.3 ± 2.8 a	82.1 ± 2.9 a	68.8±5.5 a	62.9±4.6 a
3 (six unit of optimal space and RHP densities)	83.7 ± 1.7 a	82.2 ± 2.4 a	73.8±2.4 a	66.5±4.7 a

1 One unit of optimal space denotes a cage of 40×40×40 cm. 2 One unit RHP density denotes two plants (5-6-week-old Chinese cabbage), 40 *P. xylostella* larvae and 5 parasitoid (3-d-old, mated females of *C. vestalis*). 3 Means with same letter in each column are not significantly ($P > 0.05$) different (Tukey).

Developmental period of larvae and cocoons

When developmental period of larvae was evaluated, there was no significant ($F_{2,9}=0.966$, $P=0.417$) difference between treatments (Table 2). The mean of developmental period of larvae varied among treatments from 7.45 days (treatment 1) to 8.02 days (treatment 3). The analysed developmental period of produced wasps cocoons showed significant ($F_{2,9} = 6.922$, $P<0.015$) difference (Table 2) and the mean of developmental period of cocoon varied among treatments from 3.77 days (treatment 1) to 4.00 days (treatment 3). The first treatment has significantly lower developmental period of cocoons in comparison with other treatments.

Table 2. The effects of enlargement of optimal space and resource-host-parasitoid (RHP) densities on pupal and larval developmental period of produced parasitoids wasps, *C. vestalis*.

Treatment	Developmental period	
	Larval	Pupal
1 (one unit of optimal space ¹ and RHP densities ²)	7.45 ± 0.24 a ³	3.77 ± 0.04 b
2 (three unit of optimal space and RHP densities)	7.76 ± 0.18 a	3.96 ± 0.04 a
3 (six unit of optimal space and RHP densities)	8.02 ± 0.41 a	4.00 ± 0.06 a

1 One unit of optimal space denotes a cage of 40×40×40 cm. 2 One unit RHP density denotes two plants (5-6-week-old Chinese cabbage), 40 *P. xylostella* larvae and 5 parasitoid (3-d-old, mated females of *C. vestalis*) 3 Means with same letter in each column are not significantly ($P > 0.05$) different (Tukey)

Sex ratio (the ratio of produced female wasps)

When logistic analysis of deviance was used there were significant ($df=9$, $t\text{-value}=-3.524$, $P<0.001$) differences between treatments (Table 3). The mean of production of female wasps varied between treatments from 0.26 (treatment 1) to 0.45 (treatment 2 and 3). The first treatment produced a lower female sex ratio in comparison to the other treatments. Exact binomial test, however, showed a significant ($P<0.01$) difference between treatments. The first treatment (0.26) has significantly lower sex ratio compare to second and third treatment (0.45).

Percentage of survived female wasps

When the number of surviving female wasps was calculated, there was a significant ($df = 9$, $t\text{-value} = -3.513$, $P<0.001$) difference between treatments (Table 3). The first

treatment (16.2%) produced a significantly lower percentage of surviving female wasps in comparison to the second (28.3%) and third (30.0%) treatments.

Table 3. The effects of enlargement of optimal space and resource-host-parasitoid (RHP) densities on sex ratio and percentage of survived female wasps of *C. vestalis* (success in biological control).

Treatment	Sex ratio	Survived female wasps
1 (one unit of optimal space ¹ and RHP densities ²)	0.26 ± 0.12 a ^{3*}	16.2 ± 5.1 a
2 (three unit of optimal space and RHP densities)	0.45 ± 0.08 b	28.3 ± 7.2 b
3 (six unit of optimal space and RHP densities)	0.45 ± 0.02 b [*]	30.0 ± 1.0 b

1 One unit of optimal space denotes a cage of 40×40×40 cm. 2 One unit RHP density denotes two plants (5-6-week-old Chinese cabbage), 40 *P. xylostella* larvae and 5 parasitoid (3-d-old, mated females of *C. vestalis*). 3 Means with same letter in each column are not significantly ($P > 0.05$) different (Tukey). *A significant difference was obtained when the sex ratio was compared with the sex ratio of 1:1.

CONCLUSIONS AND DISCUSSIONS

The success of biological control programs depend upon releasing huge numbers of biocontrol agents in target areas to reduce pest populations to a lower level, possibly below the economic injury threshold. The main purpose of mass rearing programs is optimal production of beneficial insects, which would be released at target areas. The production of high quality insects has been always received attention (Singh, 1982; Parker, 2005; Watt *et al.*, 2015). The current research attempted to optimize the enlargement of space and resource-host-parasitoid (RHP) biomass for mass rearing of *C. vestalis*. This parasitoid has been subjected to release in many areas for biological control program of *P. xylostella* but there is little information on the optimization of its mass rearing (Talekar and Shelton, 1993; Furlong *et al.*, 2013).

Previous experiments in our laboratory also showed that the different density of *P. xylostella* and *C. vestalis* had influenced the life-history parameters of produced wasps, such as sex ratio, developmental period, percentage parasitism and survival rate; the best results were obtained when a host plant (5-6-week-old Chinese cabbage) containing twenty larvae of the herbivore (the 2nd instar *P. xylostella* larvae) was exposed to five female parasitoid wasp (*C. vestalis* females) in a standard-sized cage (40×40×40 cm) for 24 h (M. Rezaei and J. Karimzadeh, unpublished data). This optimized herbivore density reduced intraspecific competition for food, and resulted in more herbivore survival. In addition, due to low number of *P. xylostella* larvae on each plant there was no need for food plant renewal.

Here, we extend the space and resource-host-parasitoid (RHP) biomass to consider their possible effects on the wasp production. This has clearly a significant influence on the offspring sex ratio, and hence, on the produced female wasps. According to the optimal host and parasitoid densities, for achievement to the high number production of parasitoid wasps we extend the optimal RHP biomass three and six times greater. These cage sizes of second treatment (70×55×50 cm) and third treatment (100×70×55 cm) are not used in any study for rearing of *C. vestalis*. Using the optimal density

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of parasitoid (5 parasitoids for 20 host larvae), superparasitism by *C. vestalis* was prevented. Superparasitism has negative effects on mass rearing system. Li *et al.* (2001) examined the effect of superparasitism on the bionomics *C. vestalis* in which they found that superparasitism can cause physical combat between parasitoid larvae. In comparison to normal single-oviposition parasitism, reduced survival, body size, female sex ratio and parasitic capacity caused by superparasitism, however it stretches out developmental time and adult longevity. Moreover, the negative effects increased with the number of supernumerary parasitoid larvae in the host. Female larvae of parasitoids are mainly better competitors than males under superparasitism conditions (Montoya *et al.*, 2011). In some parasitoids the superparasitism has others effects that it could be impressed some biological factors especially more produced females sex ratio as an exemplification, *D. longicaudata* (Cancino and Montoya, 2008). The negative effects of superparasitism could be reduced by management of exposure time (Montoya *et al.*, 2012; Suarez *et al.*, 2012; Zhang *et al.*, 2016). In the current study, we assumed 24 h for exposure time of *C. vestalis* to host larvae. It is suggested that different exposure time of *C. vestalis* to host larvae would have investigated in further studies. According to the results, durations of egg incubation and larval period varied between treatments from 7.45 to 8.02 days, which are similar to the results of Alizadeh *et al.* (2011). The present study showed that the best outcomes (*i.e.*, the lowest pupal period) resulted from the 1st treatment, which indicates a better suitability of the used space and RHP biomass (or rearing condition).

Parasitoid sex ratio can vary due to various factors as illustration, photoperiod effects, inbreeding, virginity, host size, host quality, superparasitism, numbers of mated females in a foraging patch and rearing conditions (Godfray, 1994; Heimpel and Lundgren, 2000; Montoya *et al.*, 2011). Parasitoid wasps change their produced progeny according to environmental condition. Female parasitoids are of prime importance because females are responsible for population increase via growth rate, and because only females contribute to host (here means pest) mortality (Silva *et al.*, 2014). Many parasitoid species are not commercially available on account of many reasons, one of which might be male-biased sex ratios in mass rearing. Sex ratio, therefore, is considered as an important limiting factor for a number of parasitoid species (Godfray, 1994; Heimpel and Lundgren, 2000). To have a successful mass rearing of parasitoids for field release, it is paramount to determine the factors influence sex ratio (Montoya *et al.*, 2011). The sex ratio of offsprings of *C. vestalis* parasitizing the 2nd, 3rd and 4th instar of *P. xylostella* larvae has shown no significant difference (Kawaguchi and Tanaka, 1999). However, in another key parasitoid of the diamondback moth, *D. semiclausum*, sex ratio of produced wasps in the fourth host instar was significantly different from the first three instars (Yang *et al.*, 1993). The ratio of female progenies and the parasitism rate of *C. vestalis* have shown a decrease with the age of the female wasps (Kawaguchi and Tanaka, 1999). In the current study, a much better sex ratio of offspring wasps was obtained when the space and RHP biomass was extended. According to the local mate competition (LMC) model female parents of parasitoid produce more female offspring when have less contact with other females in the foraging patch (Asante and Danthanarayana,

1993; González-Zamora *et al.*, 2015). It is obvious that the results of this study make scene with this model. This idea has been supported by another study on a related wasp species, *Cotesia flavipes* (Vacari *et al.*, 2012) and it showed by other studies on different parasitoid species as an exemplification, *Spathius galinae* Belokobylskij and Strazenac (Hymenoptera: Braconidae) (Watt *et al.*, 2015). Ghimire and Phillips (2010) were reported different rearing container had any impact on the sex ratio of *Habrobracon hebetor* Say (Hymenoptera: Braconidae) in mass rearing condition. In contrast to the current study, they used of the same host-parasitoid densities for different containers. This variation may be owing to differences in the experimental design. Heimpel and Lundgren (2000) suggested that there was some potential for increasing the female-biased sex ratio of *C. vestalis* in commercial settings. Asante and Danthanarayana (1993) are on this assumption that there are several evolutionary models to predict sex ratio in parasitic wasp as illustration, Local Mate Competition (LMC), host quality models and other factors cases in point, superparasitism, host and parasitoid densities. On the basis of the current study, we reported enlargement of rearing space (with the same proportion of RHP) as another factor that impresses sex ratio of produced wasps. Thus, it is recommended that this factor be investigated for others parasitoids in mass rearing condition. In conclusion, the present study indicated that the second (cage of 70×55×50 cm, with 6 plants, 120 host larvae and 15 parasitoid wasps) and third (cage of 100×70×55 cm, with 12 plants, 240 host larvae and 30 parasitoid wasps) treatments are both optimum for mass rearing of *C. vestalis*. This research has initiated many questions, which need further investigations. It is necessary to weight up the result of this study from economical aspects.

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A Newly Recorded Species, *Chonocephalus depressus* Meijere, 1921 (Diptera: Phoridae), whose Larvae Attacking Oyster Mushroom in China

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ABSTRACT

The scuttle fly *Chonocephalus depressus* Meijere, 1921 (Diptera: Phoridae) is recorded for the first time from China. Its larva attacks the oyster mushrooms *Pleurotus ostreatus* (Jacq.) Kumm, 1871 and *Pleurotus pulmonarius* (Fr.) Quél, 1872. *Chonocephalus depressus* is redescribed and illustrated, and its biological characteristics are briefly outlined.

Key words: Phoridae, taxonomy, new record, China, oyster mushroom.

INTRODUCTION

The cultivated oyster mushrooms are suffered from a range of pests, which include some species of scuttle flies (Diptera, Phoridae). These include mainly larvae of phorids belonging to the genus *Megaselia* Rondani (Diptera: Phoridae) in the Palaearctic (Disney and Evans 1979), Oriental (Mohan *et al.* 1996) and Nearctic Region (Brown and Marshall 1984). Damage to mushrooms is caused through the consumption of mycelium and sporophore (Disney 1994; Disney and Durska 1999). In addition to *Megaselia*, a minor pest, *Chonocephalus rostamani* was reported feeding on oyster mushrooms (Rostaman and Disney 2004).

In this study, the species *Chonocephalus depressus* Meijere, 1912, feeding on the mycelium and fruiting body of *Pleurotus ostreatus* (Jacq.) Kumm, 1871 and *Pleurotus pulmonarius* (Fr.) Quél, 1872, is recorded for the first time from China. The species is redescribed and illustrated, and its biological characteristics are briefly outlined.

MATERIALS AND METHODS

The adults of *Chonocephalus depressus* were collected from infested mushrooms, *Pleurotus pulmonarius*, in Nanning, Guangxi, on 24 December 2014 (ZLL). The specimens were preserved in ethanol (75%). For biological observation, thirty alive males and females were placed in three rearing boxes ($\varnothing=30$ mm, $h=70$ mm), 5 pairs in each box, feeding with fruiting body of oyster mushroom under room conditions ($27\pm1^{\circ}\text{C}$). Their habit and developmental process were observed and recorded daily. All stages of the specimens were sent to one of the authors (GCL) for identification. The specimens were mounted on slides according to the methods of Disney (1994). Species is illustrated using microscope Leica M205A and Leica DM5500B with CCD 450 multi-focus imaging system. The specimens observed are deposited in Shenyang University Museum of Natural History (SUMN), Shenyang, China and College of Agriculture, Guangxi University.

RESULTS AND DISCUSSIONS

Chonocephalus Wandolleck, 1898

Chonocephalus Wandolleck, 1898: 428. Type species: *C. dorsalis* Wandolleck, 1898 by monotype.

Epichonocephalus Schmitz, 1928: 104; synonymized by Disney, 2002: 4. Type species: *E. transversalis* Schmitz.

Description

Male: Frons with median furrow, most frontal bristles not differentiated. Ocelli present. Arista present and articulated with postpedicel. Mesopleuron divided, with one to three small hairs near posterior margin. Tibia without dorsal longitudinal hair palisades and pre-apical isolated bristles. Wing with costa clearly exceeding half

A Newly Recorded Species, Chonocephalus depressus

wing length, vein Rs unforked, base of vein M₁ missing, without axillary bristles and small hair at base of Rs. Hypopygium with short anal tube and at least one gonopod.

Female: Ocelli, wings and halteres absent. Tibia lack dorsal longitudinal hair palisades and pre-apical isolated bristles. Abdomen with well developed tergites I-VI. Sternites 8 and 9 internal, and single spermatheca lightly sclerotised.

***Chonocephalus depressus* Meijere (Fig. 1)**

Material examined. China: Guangxi, Nanning (29.65°N, 95.48°E; 2118 m), 21 December 2014, 5♂♂, 2♀♀, Ziling LI.

Chonocephalus depressus Meijere, 1912: 151 (female only); Disney 1991: 208 (male).

Chonocephalus similis Collin, 1912: 105 (part) nec Brues, 1905. Misidentification.

Chonocephalus dimakae Paulian, 1958: 12. Synonymized by Disney 2005: 406.

Chonocephalus ecitophilus Borgmeier and Schmitz, 1923: 145. Synonymized by Disney 2002: 14.

Chonocephalus japonicus Schmitz, 1941: 82. Synonymized by Disney 2002: 15.

Chonocephalus pallidulus Beyer, 1964 in Hardy and Beyer, 1964: 298. Synonymized by Disney 2002: 15.

Chonocephalus simiolus Beyer, 1964 in Hardy and Beyer, 1964: 300. Synonymized by Disney 2002: 15.

Description

Male: Body generally brown with a pale abdominal venter. Frons brown with a darker ocellar triangle. Only antial and anterolateral bristles clearly differentiated. Postpedicel brown and rounded. Palp strong and about 2 times as long as its greatest breadth. Thorax brownish, dark on top. Scutellum with four fine bristles. Three hairs on upper part of posterior margin of mesopleuron. Legs brown, but all tarsi paler. Front tarsus with a posterodorsal hair palisade on tarsomeres 1-4 and tarsomeres 4 and 5 almost same in length. Mid femur brown, except ventral middle darker. Mid tibia pale white at apical half. Hind leg brown, except femur darker. Wing 1.10-1.20 mm long. Costal index 0.61- 0.62. Thick veins light brown with tip of costa and vein Rs darker. Thin veins grayish brown and membrane tinged brownish gray. Vein Rs with a vesicle at tip and not extends beyond tip of costa. Haltere brown. Abdomen with tergites I-VI brown, with only a few short hairs, almost confined to posterior margins. Venter pale grayish with minute, sparse, pale hairs on segments 3-6. Abdominal tergites with fine hairs, little longer in tergites VI. Venter hairs smaller and finer. Hypopygium mainly brown, with few, short hairs. Left anterior process of epandrium with a small process toward base, right anterior process with two processes, which are with rounded apices.

Female (Fig. 1): Body 1.00-1.19 mm long. Post pedicel yellow brown. Palps paler with a bristle-like apical hair and numerous shorter hairs. Frons with some hairs and microtrichia. Rear of each abdominal tergite with small pits, and with about 20 hairs in row at rear margin. Tergites covered with scatter hairs and microtrichia.

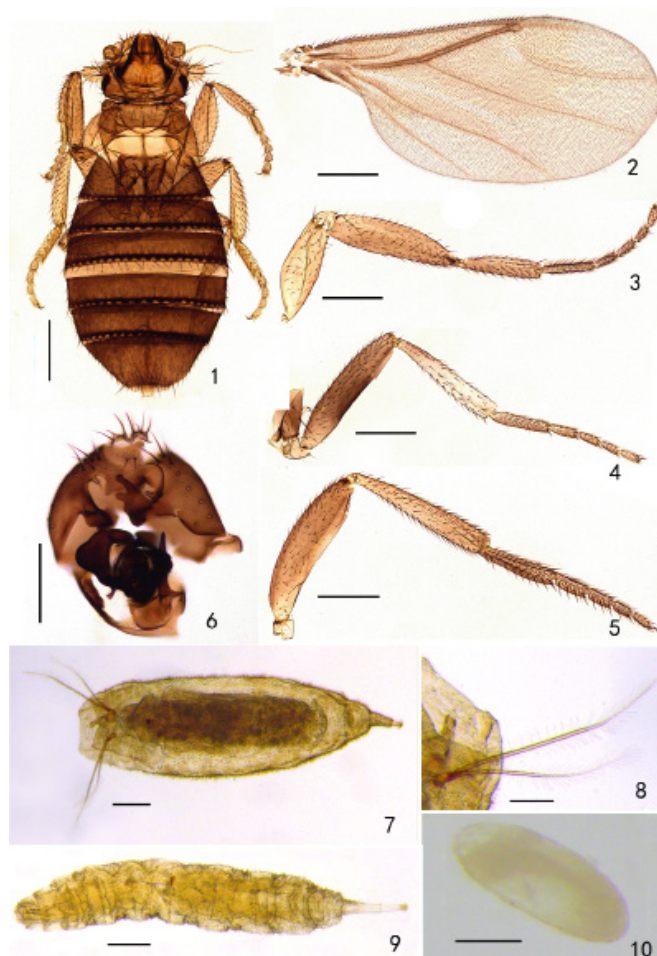


Fig. 1. *Chonocephalus depressus* Meijere. 1. Female; 2-6. Male; 2. Wing; 3. Fore leg; 4. Mid leg; 5. Hind leg; 6. Hypopygium; 7-8. Pupa; 7. Pupa, dorsal view; 8. Pupa, respiratory horns; 9. Larva, dorsal view; 10. Egg. Scale bar: 1,2,3,4,5,7,9=0.2mm; 6, 8,10=0.1mm.

Biological observations

Adults show sexual dimorphism, the male exhibiting typical phorid wing venation, while the female is wingless. The male is actively running and flying. When he finds a female, he suddenly pounces on her. Mating takes place immediately. Female adult period ranges from 3 to 6 days. She lays scattered eggs on the surface of pileus and gill. The egg takes generally 1-2 days to hatch. Larva feeds on fruiting bodies and mycelium of mushroom. The larval period is 4-5 days under 27°C covering 3 instars. Mature larva buries one half in decaying fruiting body and pupariates with the back of head exposed. A pair of breathing horns arise anteriolaterally of the postpronotum, one day after pupariation. The duration of the pupa stage is 6-7 days. The complete life cycle requires about 12-16 days at 27°C.

A Newly Recorded Species, Chonocephalus depressus

The larva mainly feeds on rotten pileus and stipe of oyster mushroom and also can damage the fruiting body. It usually wriggles here and there and invades from lamella into pileus, forming tiny pore canals. The larva also feeds on the mycelium of mushrooms, resulting in destruction of mycelium and fruiting body primordia. The larva infests *P. ostreatus* and *P. pulmonarius*, but no serious damage has been observed.

The genus *Chonocephalus* Wandolleck has been reported from China only recently, with a new species *C. forcipulus* Liu and Chu and a new record *C. fletcheri* Schmitz (Liu and Chu 2016).

Chonocephalus depressus is the third species of *Chonocephalus* observed in China, although this tramp species occurs on the Arabian Peninsula (Disney 2006), in Israel (Mostovski 2016), Indonesia, India, Thailand, Philippines (Disney 2016). A possible invasion route of this species to China may run from southern Asia to China. It may be listed as a quarantine species since it caused significant damages to local cultivated mushrooms.

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Taxonomy and Biology of *Pauropsylla buxtoni* comb. nov. (Hemiptera: Psylloidea) on *Ficus carica* (Moraceae)

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ABSTRACT

A detailed morphological study of adult and immature *Trioza buxtoni* Laing, 1924 (Hemiptera: Psylloidea: Triozidae) shows that the species belongs to the tropical and subtropical genus *Pauropsylla* Rübsaamen, 1899 to which it is transferred. The adult of *Pauropsylla buxtoni* (Laing, 1924) comb. nov. is redescribed and the previously unknown immatures are described. Illustrations are provided for both adults and immatures. Immatures of *P. buxtoni* infest leaves of *Ficus carica* and induce conspicuous galls. The species is a pest on cultivated figs in the Palestinian Territories. Four successive phases in the formation and development of the galls can be recognised in which the five immature instars of *P. buxtoni* develop. The gall size increased significantly when the instar length increased and there were significant differences in the susceptibility of fig cultivars to psyllid infestation. The life cycle of *P. buxtoni* is univoltine with no significant differences between cultivars.

Key words: Triozidae, description, immatures, life cycle, cultivar, susceptibility, gall development.

INTRODUCTION

Psyllids or jumping plant-lice are small phloem-feeding insects that are usually host specific, i.e., they complete their development on one or few related plant species (Burckhardt *et al.*, 2014). Often closely related psyllid species are restricted to one plant taxon such as the species of Homotomidae which all develop on Moraceae (Hollis and Broomfield, 1989). Moraceae, and *Ficus* species in particular, are utilised as hosts also by psyllids from other groups such as *Pauropsylla* (Trioizidae) (Hollis, 1984) or some species of *Paurocephala* (Liviidae) (Mifsud and Burckhardt, 2002) and *Trioza* (Trioizidae) (Ouvrard, 2017).

Two species, viz. *Homotoma ficus* (Linnaeus) (Homotomidae) and *Pauropsylla buxtoni* (Laing) (Trioizidae) are associated with cultivated fig, *Ficus carica* L., in the west Palaearctic realm. The former is widely distributed throughout the Mediterranean region, West and Central Europe, the Black Sea region, the Caucasus, the Middle East to Iran and, introduced, in North America (Ouvrard, 2017). It is monophagous on its host with free living immatures, usually on the underside of the leaves, which do not induce galls (Prodanovic, 2011). *Pauropsylla buxtoni*, the second species, in contrast, develops on *F. carica*, *F. palmata* Forssk. and *F. cf. exasperata* Vahl on which their immatures induce very conspicuous leaf galls (Figs. 1-4). *Pauropsylla buxtoni* was originally described by Laing (1924) as *Trioza* and reported by Buxton (1924) as a serious pest on *Ficus carica* trees in Jericho (Palestinian Territories) and Lod (as Lydda) (Israel). The species was listed later from Israel on *F. carica* and *F. palmata* (Bodenheimer, 1937; Halperin *et al.*, 1982; Spodek *et al.*, 2017), from Egypt on *F. palmata* (Halperin *et al.*, 1982), from Saudi Arabia on *F. cf. exasperata* and *F. palmata* (Burckhardt, 1986) and from Jordan on *F. carica* (Al-Khawaldeh *et al.*, 1997). The last authors list the species also from Syria but do not provide a source for this record. Apart from the original description of the adult and the few distributional records not much is known about *P. buxtoni*. In particular, several taxonomically important characters were not mentioned in the original description, the immatures have not been described nor have the phylogenetic relationships of the species been previously studied. Laing (1924) stated that *P. buxtoni* resembles *Colopelma thomasi* (Löw) (cited as *Trioza thomasi* (sic)) in the shape of the genal processes and the rounded apex of the forewing but differs in coloration. He did not mention if the characters shared between the two species indicate close phylogenetic relationship.

Common fig, *F. carica*, is an important fruit tree in the Palestinian Territories where many local cultivars are grown. These are characterised by having large fruits with a sweet taste, in addition to their adaptation to Mediterranean climate (Shtayeh *et al.*, 1991). Basheer-Salimia *et al.* (2013) characterised the genetic diversity in relation to the genotypes of 12 local Palestinian varieties of *F. carica* defined by pomological and morphological descriptors. Their results indicated that there were four clusters: the first cluster consisted of one genotype (cultivar Khidari), the second of four (Ghzali, Biadi, Shami and Himari), the third of three (Mowazi, Moozi and Ruzzi) and the fourth of four genotypes (Aswad, Sewadi, Khurtmani and Smari), respectively. Similar results on genetic diversity of Palestinian fig cultivars were reported by Ali-Shtayeh *et al.* (2014).

In recent years, heavy infestations of fig trees by *P. buxtoni* were observed in the northern part of the Palestinian Territories. The psyllids induce large, elongate, pocket-shaped galls, often clustered in large groups on the upper leaf surface (Figs. 1-6).

The objectives of the present paper are to redescribe the adults and describe the immatures of *P. buxtoni*, to examine the phylogenetic relationships and the life cycle of the species, to describe the gall development, to quantify growth and development of immatures relative to gall development and to study the susceptibility of fig cultivars to psyllid infestation.

MATERIAL AND METHODS

Material

For each of the fig varieties Biadi, Himari, Khidari, Khurtmani and Mowazi 100 leaves of the infested with galls of *P. buxtoni* were collected in *F. carica* orchards at different locations in the Tulkarm district in the northern part of the Palestinian Territories (N32.3125° E35.021111°). The mean annual temperature in the sampled area is 17.6 °C (5-38 °C) and the average annual relative humidity is 60.8 % (45-98 %) (PMA, 2015). Samples were taken from different fig cultivars. The collected galls were used either for describing the growth and development of galls or for dissecting them to extract the immatures living inside. Immatures were extracted from dissected galls and preserved in 70 % ethanol and adults were reared from the galls and preserved dry or in 70 % ethanol. Voucher specimens are preserved in the collections of the Naturhistorisches Museum Basel (NHMB). Additional material was examined from the collections of the NHMB, the Natural History Museum, London, UK (BMNH), the Muséum d'histoire naturelle, Geneva (MHNG) and the Naturhistorisches Museum, Vienna, Austria (NHMV).

Morphological terminology follows White and Hodkinson (1982), Hollis (1984, 2004), Ossiannilsson (1992) and Yang *et al.* (2009). The nomenclature of psyllid names accords with Ouvrard (2017) and the psyllid classification with Burckhardt and Ouvrard (2012).

Development of galls and immatures

For studying the development of galls and immatures, the galls were cut open under a dissecting microscope and the enclosed immatures extracted. Then the instar was determined and the length of the specimens measured using an eyepiece reticule mounted on the microscope. The gall size was quantified by cutting the gall longitudinally into two halves and then measuring the gall length, the distance between the base and apex of the gall (Figs. 5, 6) using a ruler. For each instar, 100 galls and the enclosed immature were measured, each gall containing one immature.

Susceptibility of local fig cultivars

Samples of fig leaves infested with galls of *P. buxtoni* were randomly chosen from trees of the five local cultivars (Biadi, Himari, Khidari, Khurtmani and Mowazi) for testing their susceptibility to psyllid infestation. The sampled trees were located in

neighbouring orchards in the Tulkarm district with similar meteorological conditions. For each cultivar, 50 galled leaves were randomly chosen and the number of galls on each leaf, singly or in clusters (Figs. 1-3), was counted.

Life cycle duration and number of generations per year

The duration of life cycle of *P. buxtoni* was studied on the five local fig cultivars (Biadi Himari, Khidari, Khurtmani and Sewadi) from 1 March 2015 to 30 April 2016. The life cycle duration, which represents the time from egg laying to adult emergence, was measured as follows: a pair of newly emerged male and female of *P. buxtoni* was put into a small cage (3.5 cm diameter x 3.0 cm height) fixed onto non infested young fig leaves on trees of the tested cultivars. Each cage consisted of two identical top and bottom parts made of transparent fiberglass with spongy margins to ensure a perfect fit when assembled. Each part had a dish shape with a circular diameter of 3.5 cm and a height of 1.5 cm. The bottom of each part consisted of a tightly fixed fine muslin mesh to allow aeration of the chamber. The cages were fixed onto the leaves using a clamp so that the insects could access a circular part of the leaf (Fig. 7). For each cultivar, 50 young leaves with 50 pairs of *P. buxtoni* were studied. After egg laying by the confined females, the leaves were labelled and the time to adult emergence was recorded.



Figs. 1-7. Galls induced by *Pauropsylla buxtoni* on leaves of *Ficus carica*, 1. Galls densely clustered on dorsal leaf surface, 2. Galls spread over the leaf surface, 3. Detail of clustered galls, 4. Lower leaf surface with slit-like openings of galls (arrows), 5. Gall enlarged, outer, hairy surface, 6. Gall enlarged, inner surface with one immature (arrows) per chamber; red lines indicate gall length, 7. Lateral view of the small cage used for confining one pair of *P. buxtoni* on young leaves of *F. carica* for the study of life cycle duration.

Statistics

The mean and range values of the length of galls and enclosed immatures, the number of galls per leaf and cultivar, as well as the life cycle duration on different cultivars were calculated. Analysis of Variance (ANOVA) and means separation by Tukey's HSD test were used to compare the gall size during the gall development as well as length of the enclosed immatures and for testing differences in the number of galls of *P. buxtoni* as well as life cycle duration of the psyllid between cultivars.

RESULTS

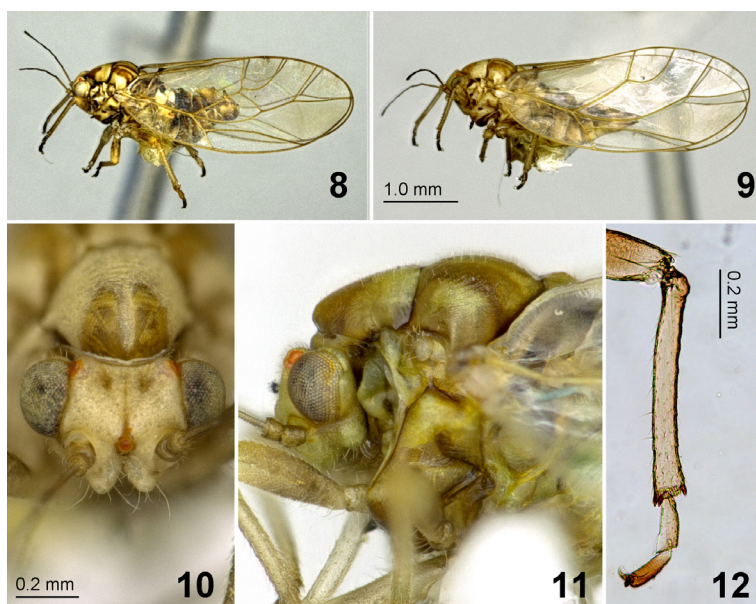
Pauropsylla buxtoni (Laing, 1924), comb. nov. (Figs. 8-25)

Trioza buxtoni Laing, 1924: 247; lectotype ♂: 'Type, *Trioza buxtoni*', 'Making thickenings on fig leaves', 'Palestine, Ludd (Lydda = Lod), ix.1921, P.A. Buxton', 'Pres. by, Imperial Bureau of Entomology, British Museum 1923-289', *Trioza buxtoni* Lg., Det. F. Laing., 'BMNH(E)_1266347' (BMNH, examined), here designated.

Description. Adult (Figs. 8, 9). Colour. Body greenish, yellow or ochreous. Median suture, lateral margins of vertex and foveae brown; ocelli red; clypeus brown; antenna dark brown to black, segments 1-3 ochreous to brown. Mesopraescutum with two submedian patches anteriorly; mesoscutum with four submedian longitudinal brown stripes; mesothorax brown ventrally. Forewings transparent with light brown veins; hindwings transparent. Legs with dark brown tarsi, except for basimetatarsus which is ochreous. Abdomen with dark brown sclerites and yellowish membranes; terminalia partly ochreous. Younger specimens with less expanded dark pattern.

Structure. With generic characters as given by Hollis (1984). Integument granular, sparsely covered with short setae. Median suture of vertex weak near occiput, becoming stronger towards median ocellus; area around lateral ocelli hardly raised; frons triangular, small, about as long as diameter of median ocellus; vertex anteriorly rounded down to genae which are produced into conical processes, subacute apically, about half as long as vertex along mid-line (Fig. 10); with big suborbital lobe (Fig. 11). Clypeus slightly tubular, with a pair of setae; ultimate rostral segment with or without a pair of long basal setae. Antenna 10-segmented, 1.71-2.00 times as long as head width; with a simple subapical rhinarium on each of segments 4, 6, 8 and 9; terminal setae about three quarters and half as long as antennal segment 10. Forewing (Fig. 9) oval, widest in the middle, narrowly rounded apically; 2.07-2.33 times as long as wide, 4.43-5.67 as long as head width; sparsely clothed in setae which are about as long as diameter of veins; vein R+M+Cu trifurcating into veins R, M and Cu; m_1 cell value 1.35-1.44. Hind wing with costal margin bearing 1-2 setae proximal to costal break, setae distal to costal break divided into two distinct groups of 2-3 setae each; vein R+M+Cu indistinctly splitting into veins R and M+Cu. Procoxa lacking ventro-apical spur; basal and apical tarsal segments subequal; metacoxa with large, conical, subacute meracanthus; metafemur with a transverse row of 4-6 subapical bristles; metatibia 1.00-1.33 times as long as head width, with 1+2 apical metatibial spurs

(Fig. 12). Abdomen with lateral setae on tergite 2♂♂ and 3♀♀. Male proctiger (Figs. 13, 18) flask-shaped; outer surface covered with long setae in apical half; in profile, with weak posterior expansions which bear moderately long, simple setae on their inner surface (Fig. 14). Subgenital plate (Figs. 13, 18) subglobular, sparsely covered with moderately long setae posteriorly and ventrally. Paramere (Figs. 13, 18, 19) narrowly lamellar, in profile, weakly tapering towards apex and hardly curved to the rear; outer surface with long setae mostly along apical half of foremargin and along entire hind margin; inner surface (Fig. 19) covered with moderately long setae, in basal portion longer and thicker than near apex; apex strongly sclerotised, somewhat blunt in profile, sharply truncate and with each a small anterior and posterior point in dorsal view. Distal portion of aedeagus (Fig. 20) simple, weakly expanded and rounded apically; sclerotised end tube of ductus ejaculatorius short, weakly sinuous. Female terminalia (Fig. 15) cuneate. Female proctiger 1.08-1.33 times as long as head width, dorsal margin, in profile, almost straight, apex subacute; covered in moderately long setae, in apical third with two submedian longitudinal rows of long setae, and some sparse peg setae in apical fifth, laterally; circumanal ring oval, very short, consisting of two unequal rows of oval pores (Fig. 16). Dorsal valvulae cuneate, lacking teeth; ventral valvulae styliform, straight, apically narrowly rounded, lacking teeth (Fig. 17).



Figs. 8-12. Adult *Pauropsylla buxtoni*, 8. Male, 9. Female, 10. Head, dorsal view, 11. Head and thorax, lateral view, 12. Metatibia and metatarsus. Scale 1.0 mm (Figs. 8, 9), 0.2 mm (Figs. 10, 11), 0.2 mm (Fig. 12).

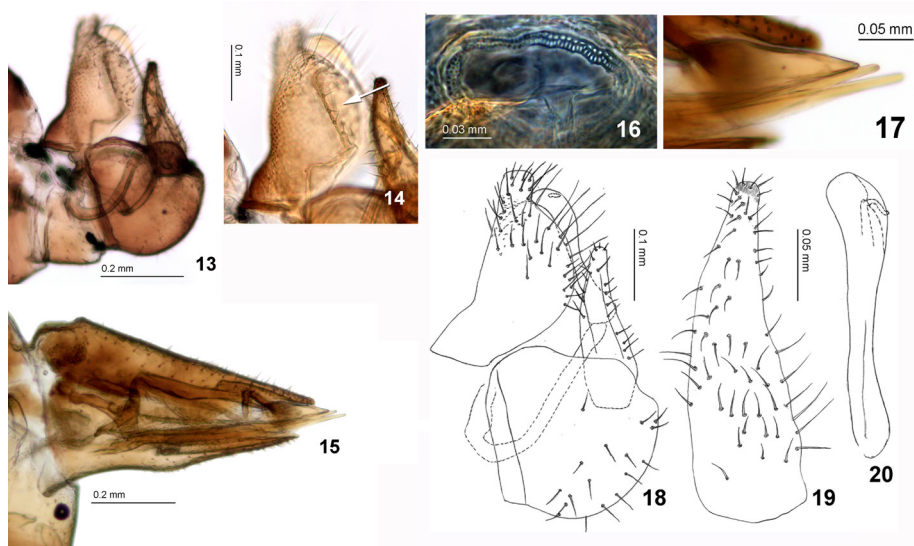
Measurements (in mm, range, mean \pm SD taken from 6♂♂, 6♀♀, dry mounted specimens). Body length ♂ 3.5-3.8 (3.65 \pm 0.11), ♀ 3.7-4.2 (3.92 \pm 0.17); head width ♂ 0.6-0.7 (0.62 \pm 0.04), ♀ 0.6-0.7 (0.62 \pm 0.05); antenna length ♂ 1.1-1.1 (1.15 \pm 0.06), ♀ 1.1-1.3 (1.18 \pm 0.08); forewing length ♂ 2.9-3.1 (2.95 \pm 0.08), ♀ 3.1-3.5 (3.30 \pm 0.17);

Taxonomy and Biology of Pauropsylla buxtoni

metatibia length ♂ 0.7-0.8 (0.77 ± 0.05), ♀ 0.7-0.9 (0.78 ± 0.06); proctiger length ♀ 0.7-0.8 (0.75 ± 0.06).

Immature. The five instars differ in body size (Table 1). Fifth instar (Figs. 21-25). Colour. Whitish, yellowish. Antennae and legs brown. Thorax and abdomen with a longitudinal row of submedian brown dots (Fig. 25).

Structure. Body (Fig. 21) elongate, oval, 1.83-1.92 times as long as wide. Antenna (Fig. 22) 7-segmented, segment 7 sometimes indistinctly subdivided. Forewing pads with humeral lobes weakly developed, rounded, not reaching posterior eye margin anteriorly. Caudal plate (Figs. 24, 25) 0.73-0.82 times as long as wide; circumanal ring ventral; distance between hind margins of circumanal ring and caudal plate as distance between fore and hind margin of circumanal ring; transversely elongate, small, consisting of a single row of pores. Head anteriorly with densely spaced very slender sectasetae and short normal setae, distance between setae shorter than length of setae; forewing pad with irregularly spaced very slender pointed marginal and submarginal sectasetae and normal setae, distance between setae usually larger than length of setae; hindwing pad with a few slender pointed marginal and submarginal sectasetae, distance between setae about as long as setae; postocular seta absent, dorsal sectasetae absent from head, thorax and wing pads; caudal plate marginally, submarginally and dorsally with relatively thick pointed sectasetae, distance between setae shorter than setae (Fig. 24). Tarsal arolium sessile, slightly shorter than claws, fan-shaped (Fig. 23).



Figs. 13-20. Terminalia of *Pauropsylla buxtoni*, 13, 18. Male terminalia, lateral view, 14. Male proctiger showing setae on inner surface (arrow), 15. Female terminalia, lateral view, 16. Female circumanal ring, dorsal view, 17. Valvulae, lateral view. 19. Paramere, inner surface, 20. Distal portion of aedeagus. Scale 0.2 mm (Figs. 13, 15), 0.1 mm (Fig. 14), 0.03 mm (Fig. 16), 0.05 mm (Fig. 17) 0.1 mm (Fig. 18), 0.05 mm (Figs. 19, 20).

Table 1. Length of galls and instars of *Pauropsylla buxtoni* on fig leaves in the Palestinian Territories. Mean and range values of gall length and length of associated instar (in mm) (n=100 galls/instar) on cultivar Khidari.

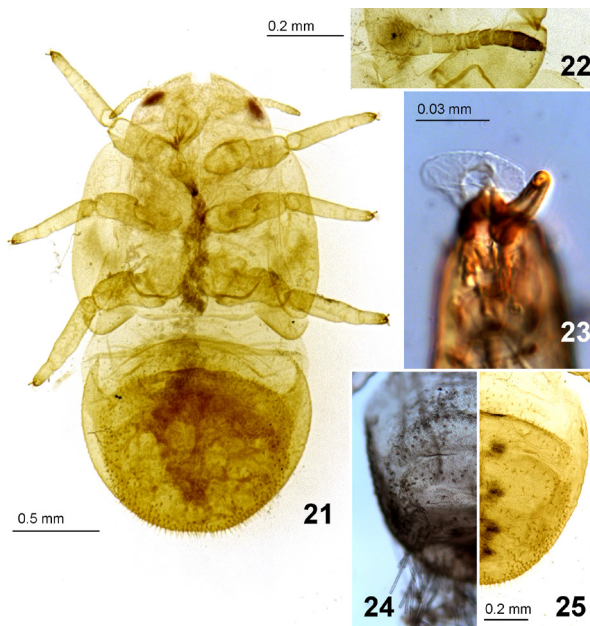
Instar	Gall length		Instar length	
	mean \pm SEM ¹	range	mean \pm SEM ¹	range
First	1.05 \pm 0.14 a	0.7-1.3	0.21 \pm 0.05 a	0.18-0.33
Second	2.97 \pm 0.66 b	2.3-3.6	0.41 \pm 0.07 b	0.35-0.53
Third	4.98 \pm 1.27 c	4.0-6.1	0.65 \pm 0.12 c	0.61-0.85
Fourth	6.60 \pm 0.36 d	6.2-7.3	0.98 \pm 0.09 d	0.89-1.08
Fifth	8.75 \pm 1.90 e	8.0-9.8	1.52 \pm 0.28 e	1.21-1.68

¹Means of gall length and instar length within the same column followed by different letters are significantly different at $p < 0.05$ according to ANOVA and Tukey's HSD test for means separation.

Measurements (in mm, taken from 4 slide mounted specimens). Body length 2.2-2.3 (2.25 \pm 0.06); forewing pad length 0.7-1.0 (0.85 \pm 0.01); caudal plate length 0.8-0.9 (0.85 \pm 0.06).

Egg. Yellowish. Oval, 0.15 mm long, with short pedicel which is inserted into leaf tissue.

Material examined: Israel: Bet Dagan; Kfar Yedidya; Lod; Tel Aviv (BMNH, MHNG, NHMB). Jordan: Al-Hammah (NHMB). Palestinian Territories: Tulkarm (BMNH, NHMB). Saudi Arabia: Abha/Jawsan; Bisha (MHNG, NHMB).



Figs. 21-25. Fifth instar immature of *Pauropsylla buxtoni*. 21. Habitus, dorsal view, 22. Antenna, 23. Apex of tarsus with tarsal arolium and claws, 24. Caudal plate showing setal pattern and wax strands, dorsal view, 25. Caudal plate showing setal pattern and brown submedian dots, dorsal view. Scale 0.5 mm (Fig. 21), 0.2 mm (Fig. 22), 0.03 mm (Fig. 23), 0.2 mm (Figs. 24, 25).

Phylogenetic relationships

Adult *P. buxtoni* fit the description of *Pauropsylla* Rübsaamen provided by Hollis (1984), e. g., the basally weak median suture of the vertex, the broad apically rounded forewings, the 1+2 apical metatibial spurs and its host association with *Ficus* spp. It differs from other *Pauropsylla* species in the presence of short conical genal processes and the absence of lateral setae on abdominal tergite 3 in males and on tergite 4 in females. Presence or absence, size and shape of genal processes can vary within trioizid genera such as *Bactericera* Puton (Burckhardt and Lauterer, 1997) or *Leuronota* Crawford (Burckhardt and Couturier, 1994) and the distribution of lateral setae on the abdominal tergites may vary between closely related species, e.g. in the *Trioza berberidis*-group (Burckhardt, 1988). These characters are hence not of generic importance and we transfer *Trioza buxtoni* to *Pauropsylla* as *Pauropsylla buxtoni* (Laing, 1924), comb. nov. The immatures of *P. buxtoni* resemble those of other *Pauropsylla* spp. (e.g. Hollis, 2004) but we could not find any putative synapomorphies defining *Pauropsylla*.

Pauropsylla is a tropical and subtropical genus with 25 described species from the Old World (Hollis, 1984; Ouvrard, 2017) and two undescribed species from the New World (Brown and Hodkinson, 1988). Most species develop, as far as known, on *Ficus* species. Within *Pauropsylla*, Hollis (1984) defined three species groups, in addition to several ungrouped species. *Pauropsylla buxtoni* does not fit any of them. It resembles two Afrotropical species: *P. trigemma* Hollis in the relatively narrow forewings and the simple apical segment of the aedeagus, and *P. longipes* Hollis in the lamellar paramere and the long, apically pointed female terminalia. However, these are probably superficial similarities, not indicating close phylogenetic relationships.

When describing *P. buxtoni*, Laing (1924) suggested that it is 'near to *T. thomasi*, Lw. [= *Colopelma thomasi*], in the genal cones and the rounded apex of the tegmen, but differing in the coloration.' *C. thomasi*, of which we have examined syntypes from Ratzes, Alto Adige, Italy (NHMW), has similar forewings and relatively long female terminalia. It differs, however, in the absence of genal processes and 1+3 apical metatibial spurs. The resemblance between the two species is superficial and they are probably not closely related.

Gall development

The galls induced by *P. buxtoni* are on the upper leaf surface of fig leaves either solitary or in clusters (Figs. 1-3). The galls are unilocular and contain only one immature each. The development of the gall passes through four successive phases.

Folding phase. The gall formation starts after the first instar hatches from the egg which is inserted with the pedicel into leaf tissue and begins to suck plant sap. The area around the immature on the upper leaf epidermis starts folding upwards.

Swelling phase. With the growth of the first instar the surrounding tissue swells and forms a small nipple-shaped gall containing the insect. Within the gall, the insect grows, passing through five instars. The gall grows simultaneously and increases in size by a factor of 100, becoming more pocket-shaped.

Dehiscence phase. It starts when the third instar immature completes its development and the fourth instar is ready to hatch. This phase is characterised by the development of a small slit-like opening at the bottom of the gall on the lower leaf surface (Fig. 4). This opening is used by the full-grown fifth instar immature for leaving the gall. Once outside, the adult will hatch.

Senescence phase. It begins after the fully developed fifth instar leaves the gall. The gall turns greyish and black apically, followed by the blackening and desiccation of the rest of the gall. While uninfested leaves are usually shed in autumn, the ones bearing mature galls with insects persist on the tree throughout winter until new flush appears in spring coinciding with the hatching of adults which will re-infest the new leaves.

Development of galls and immatures

Our study of gall size in relation to that of the enclosed immature shows that the size increase of the two is correlated. The most significant increase in gall size was observed during the swelling phase. On the cultivar Khidari, e.g., the body length of the immature from the first to the fifth instar increased significantly (at $p < 0.05$) from 0.21 to 1.52 mm during the development (Table 1). At the same time, the mean values of gall length (as an indicator of gall size) increased significantly (at $p < 0.05$) from 1.05 to 8.75 mm (Table 1). This implies that there was a significant relationship between the gall size and the body length of the instars developing inside the galls.

Susceptibility of local fig cultivars to psyllid infestation

The results indicate that there are significant differences (at $p < 0.05$) between the means of gall number per leaf of the cultivars Khurtmani, Himari and Biadi but not of Khidari, Mowasi and Biadi (Table 2). Himari is the most susceptible to *P. buxtoni* infestation with the highest mean number of galls per leaf (150.3), whereas Khurtmani is the least susceptible with the lowest mean number of galls per leaf (96.3). The other tested cultivars (Biadi, Khidari and Mowasi) are intermediate.

Table 2. Number of galls induced by *Pauropsylla buxtoni* on fig leaves of local cultivars grown in the Palestinian Territories. Mean and range values of gall numbers per randomly chosen leaf were used as indicator of varietal susceptibility to psyllid infestation (n=50 leaves/variety).

Fig cultivars	Mean \pm SEM ¹	Range
Biadi	125.8 \pm 8.89 b	98-142
Himari	150.3 \pm 7.12 c	135-206
Khidari	142.3 \pm 8.49 bc	103-194
Khurtmani	96.3 \pm 3.71 a	81-120
Mowazi	110.5 \pm 9.39 ab	90-151

¹Means of gall number followed by different letters are significantly different at $p < 0.05$ according to ANOVA and Tukey's HSD test for means separation.

Life cycle duration and number of yearly generations

The life cycle of *P. buxtoni* lasted from 298 to 324 days. The comparison of the means of life cycle duration revealed no significant differences between the five tested fig cultivars with the means ranging from 305.3 to 320.6 days (Table 3). *P. buxtoni* is hence univoltine, independent on which cultivar it develops.

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Table 3. Mean of life cycle duration (in days) of *Pauropsylla buxtoni* on fig leaves of five cultivars grown in the Palestinian Territories (n=50 leaves/variety).

Fig cultivars	Mean \pm SEM ¹	Range
Biadi	315.4 \pm 0.39 a	312-320
Himari	320.6 \pm 0.35 a	315-324
Khidari	305.3 \pm 0.15 a	298-310
Khurtmani	310.5 \pm 0.71 a	302-315
Sewadi	318.1 \pm 0.89 a	314-322

¹ Means of the life cycle duration followed by the same letter are not significantly different at $p < 0.05$ according to ANOVA and Tukey's HSD test for means separation.

The newly hatched adults become active after bud break and the appearance of young leaves of the fig trees from early March to late April. After mating the females lay eggs inserting the egg pedicel into the leaf tissue with the ovipositor. The first instar immatures hatch in late spring and the following instars develop during summer and autumn. The fifth instar immatures hibernate inside the gall and leave it in early spring when the adult emerges. Contrary to uninfested leaves, which are shed in autumn, those bearing galls persist on the plants during winter until next spring. Then, the last instar leaves the gall and the adult hatches.

DISCUSSION

Gall induction is widespread amongst psyllids and Triozidae in particular (Hodkinson, 1984; Burckhardt, 2005). *Pauropsylla* species are gall inducing on *Ficus* spp. as far as hosts are confirmed (Hollis and Broomfield, 1989; Percy *et al.*, 2016). Our results indicate that the increase in gall size of *P. buxtoni* is correlated with the increase of size of the immature living inside. Similar observations were reported from other gall-inducing psyllids such as *Pachypsylla venusta* (Osten-Sacken) on *Celtis occidentalis* (Cannabaceae) (Yang and Mitter, 1994) or *Glycaspis* (*Synglycaspis*) *cameloides* Moore, *Schedotrioza marginata* Taylor and *S. multitudinea* (Maskell) on *Eucalyptus obliqua* (Myrtaceae) (Taylor, 1987). The galls induced by *P. buxtoni* can cover most of the leaves leading to serious distortion. In the Palestinian Territories high gall density reduces the yield of cultivated figs up to 5 % (Batta, unpublished data) leading to economic losses.

Our observations regarding the prolonged attachment to the tree of leaves bearing *P. buxtoni* galls through autumn and winter until spring and the synchronisation between the adult emergence and fig phenology are in agreement with reports on other gall-inducing psyllids such as *Trioza simplifica* Mathur on *Terminalia travancorensis* (Combretaceae), *T. gigantea* Crawford on *Vaccinium neilgherrense* (Ericaceae) or *Trioza tabebuiae* Burckhardt and Santana on *Handroanthus* spp. (Bignoniaceae) (Hodkinson, 1984, 2009; Santana and Burckhardt, 2001; Burckhardt, 2005). *Pauropsylla* species are variable with respect to voltinism. *P. buxtoni* and *P. longispiculata* Mathur are univoltine, *P. depressa* Crawford and *P. purpurescens* Mathur have 1-2 and 3 generations per year, respectively, and *P. trichaeta* Petty and *P. udei* R bsaamen are polyvoltine (Hodkinson, 2009).

It is known that different cultivars can influence psyllid populations (Asadi *et al.*, 2011; Camargo *et al.*, 2014) which was also observed on fig. Himari and Khidari were the most susceptible to *P. buxtoni* infestation among the tested cultivars with the highest number of galls per leaf (mean = 150.3 and 140.3, respectively) compared to Khurtmani and Mowazi with the lowest number (mean = 96.3 and 110.5, respectively). As the climatic conditions within the study area are similar, the higher susceptibility of certain local fig cultivars can be attributed to genetic differences between the tested cultivars. Himari, the most susceptible to *P. buxtoni* infestation, belongs to a different genotype cluster than Khurtmani, the most resistant cultivar (Basheer-Salimia *et al.*, 2013). According to these authors, the genetic distances (UPGMA=Unweighted Pair Group Method with Arithmetic Mean) between these genotype clusters ranged from 0.517-0.863 (mean=0.690) implying that Himari and Khurtmani cultivars are genetically distant and thus may respond differently to the infestation of *P. buxtoni*. Probably the same is true for Khidari and Mowazi.

CONCLUSION

The detailed morphological study of adult and immature *Trioza buxtoni* suggests that the species is congeneric with *Pauropsylla udei*, the type species of *Pauropsylla*, rather than similar to *Colopelma thomasi* as was suggested by Laing (1924). For this reason the species is transferred here to *Pauropsylla*. *P. buxtoni* differs from other *Pauropsylla* species in the presence of short, though distinct genal processes.

For a successful management of *P. buxtoni* on fig trees in the Palestinian Territories, information on its life history is crucial. In view of sustainable fig production, the most resistant cultivar should be planted and in autumn the gall bearing leaves which persist on the trees should be removed and burned for reducing the psyllid population. Future research should investigate the role of natural enemies, such as predators and in particular parasitoids, about which nothing is known to date and which may be useful in the management of this psyllid pest.

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Tachinid Fauna of Serbia and Montenegro Updated with New Findings (Diptera: Tachinidae)

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ABSTRACT

During 2012-2016 period, Tachinids were reared in Serbia and Montenegro. Nineteen species were identified. The genus *Buquetia* and five species are recorded for the first time in Serbia. A checklist based on literature data and internet sources is given here. In total, there are 295 tachinid species known from the territory of Serbia and Montenegro.

Key words: Tachinidae, tachinid flies, checklist, Serbia, Montenegro.

INTRODUCTION

The family Tachinidae is one of the most diverse families of all flies (Diptera). Currently there are about 10000 species from about 1520 genera worldwide (Irwin *et al.*, 2003). The family is usually divided into four subfamilies: Exoristinae, Dexiinae, Phasiinae and Tachininae (Herting and Dely-Draskovits, 1993). All tachinids are almost exclusive parasitoids of other insects, with some exceptions which parasitize centipedes, scorpions and spiders (Vincent, 1985; Williams *et al.*, 1990). Most tachinid species parasitize Lepidoptera caterpillars, of which a large number represent important pests in crop fields and food storages (O'Hara, 2008). The investigation of tachinids as biological control agents dates from the beginning of the 20th century when *Comptosia concinnata* (Meigen, 1824) was used to control the gypsy moth *Lymantria dispar* (Linnaeus, 1758) in USA (De Bach and Rosen, 1991). Afterwards, tachinids were used in many other biological control programs with more or less success (Alam *et al.*, 1971; Embree, 1971; Ferrer, 2001). Knowing tachinids of some specific region is a premise for further research and application. With 877 registered species, the European tachinid fauna is relatively well known (Hubenov, 1992; Tschorsnig and Herting, 1994; Andersen, 1996) supplemented by Fauna Europaea Internet Database (Tschorsnig, 2013). According to the same source, Fauna Europaea, there are 242 species listed for the territories of Serbia and Montenegro. The earliest work on tachinids in Serbia begun in 1900s (Strobl, 1902), later Baranov (1926a, 1926b, 1927b, 1929a) the greatest contributions were provided by Sisojević (1953a, 1953b, 1955, 1975) Sisojević and Čepelák (1983, 1987, 1998a, 1998b, 1998c) and Sisojević *et al.* (1991). The biggest contribution on the knowledge of the Serbian tachinid fauna is revealed Hubenov (2008a, 2008b) reporting 288 species in total. The latest work on the diversity of tachinids for the territories of Serbia and Montenegro was provided by Stanković *et al.* (2014), including two more species as new for the investigated territories.

Since the literature and current databases, such as Fauna Europaea (Tschorsnig, 2013), do not comprise the full scope of the tachinid fauna in Serbia and Montenegro, our main task was to update the checklists of this group. Additionally, we report some data of the reared species.

MATERIAL AND METHODS

The tachinid specimens were reared during the period 2012-2016. Different instars of caterpillars were collected with the plant material and then put into plastic boxes which were covered by muslin cloth to enable sufficient ventilation. The material was kept under laboratory conditions until the emergence of the flies. All emerged adults were preserved in vials with 96% ethanol. The tachinid flies were identified by the last co-author. Remarks on the tachinid/host couples are based on Tschorsnig (2017). Most of the localities originate from the mountain areas in Serbia: Kopaonik (1700 m a.s.l.), Radan (800 m a.s.l.), Tara (580 m a.s.l.), Vlasina plateau (1250 m a.s.l.); Zlatibor (1045 m a.s.l.) and Montenegro: Durmitor (2000 m a.s.l.) and Visitor (950 m a.s.l.). The material is stored at the Department of Biology and Ecology, Faculty of

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Science and Mathematics, University of Niš, Serbia. The breeders and collectors are Saša Stanković (SS) and Vladimir Žikić (VŽ), and to a minor extent Boženka Hric (BH), Marijana Ilić Milošević (MIM), and Mihailo Vujić (MV). Besides the original data, this work also includes all available literature data from the articles listed in the references.

Subfamilies and species are alphabetically arranged. Species new to the investigated territories are marked with one asterisk (*).

RESULTS

A list of reared species with their hosts is given below. In total 240 tachinid specimens were reared from more than 500 caterpillars of Lepidoptera which we collected during the investigation period of (2012-2016).

List of reared species

Subfamily Dexiinae

**Thelaira solivaga* (Harris, 1780)

Material examined: Serbia, Vrčin, 150 m, 07.02.2015, 1♀, leg. MV, from *Phragmatobia fuliginosa*, Linnaeus (Noctuidae, Arctiinae).

Remark: A parasitoid of Arctiinae, often reared from *P. fuliginosa* (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

Voria ruralis (Fallén, 1810)

Material examined: Serbia, Stara Pazova, 80 m, 12.03.2014, 2♀♀, leg. BH; Serbia, Vrčin, 150 m, 26.03.2015, 1♀, leg. MV, from *Autographa gamma* Linnaeus (Noctuidae).

Remark: A common parasitoid of Noctuidae-Plusiinae, with *A. gamma* as a main host (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

Subfamily Exoristinae

Bessa parallela (Meigen, 1824)

Material examined: Serbia, Niš, Niška banja, 300 m, 24.06.2016, 1♂, leg. VŽ, from *Yponomeuta cagnagella* Hübner (Yponomeutidae). Serbia, Radan mt., 800 m, 12.06.2016, 1♀, leg. VŽ.

Host: *Yponomeuta malinellus* Zeller.

Remark: Mostly a parasitoid of "Microlepidoptera". Commonly reared from both mentioned hosts (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

**Buquetia musca* Robineau-Desvoidy, 1847

Material examined: Serbia, Sićeavačka klisura (gorge), 230 m, 17.07.2013, 3♂♂, 2♀♀, leg. SS, from *Papilio machaon* Linnaeus (Papilionidae).

Remark: Specific tachinid of this host, commonly reared (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

***Carcelia gnava* (Meigen, 1824)**

Material examined: Montenegro, Visitor, 950 m, 11.07.2013, 1♂, leg. VŽ, from *Malacosoma neustria* Linnaeus (Lasiocampidae).

Remark: *Malacosoma neustria* is a common host of *Carcelia gnava* which mainly parasitizes Lasiocampidae and Noctuidae-Lymantriinae (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

***Carcelia lucorum* (Meigen, 1824)**

Material examined: Serbia, Vlasina Lake, 1250 m, 22.05.2016, 1♂, 1♀, leg. VŽ, from *Melitaea arduinna* Esper (Nymphalidae).

Remark: *Melitaea arduinna* is an atypical host for the common arctiid parasitoid *Carcelia lucorum*. An indication that it is not a usual host might be the dwarf-like body length (5 mm) of both tachinid specimens. Up to the present, there were no confirmed records of nymphalid hosts for the large genus *Carcelia* (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

***Ceromasia rubrifrons* (Macquart, 1834)**

Material examined: Serbia, Sopot, Guberevac, 230 m, 24.05.2014, 1♂, 1♀, leg. BH, from *Aporia crataegi* Linnaeus pupa (Pieridae).

Remark: The host/parasitoid couple was already recorded by Stanković et al. (2014). This is another record from the year 2014.

***Chetogena filipalpis* Rondani, 1859**

Material examined: Serbia, Tara mt., 580 m, 23.06.2016, 1♂, leg. VŽ, from *Megalophanes viciella* Denis and Schiffermüller (Psychidae).

Remark: *Chetogena filipalpis* is a specific parasitoid of Psychidae. The host *Megalophanes viciella* was already recorded by Herting (1960).

***Epicampocera succincta* (Meigen, 1824)**

Material examined: Serbia, Vršina, 150 m, 04.02.2015, 1 ♀, leg. MV, from *Pieris rapae* Linnaeus pupa (Pieridae).

Remark: Common parasitoid of Pieridae, often reared from *Pieris rapae* (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

****Erycia festinans* (Meigen, 1824)**

Material examined: Serbia, Vlasina Lake, 1250 m, 05.06.2014, 1♀, leg. VŽ, from *Melitaea phoebe* Denis and Schiffermüller pupa (Nymphalidae).

Remark: Specific parasitoid of the nymphalid tribe Melitaeini. The host *Melitaea phoebe* became already known from Hungary by T. Szentiványi two years ago (tachinid identified after photos by the last author) (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

***Eurysthaea scutellaris* (Robineau-Desvoidy, 1848)**

Material examined: Serbia, Sićeavačka klisura (gorge), 230 m, 15.05.2013, 1♀, leg. SS, from indet. Tortricidae (on *Populus tremula*). Serbia, Niš, Niška banja, 300 m, 24.06.2016, 2♂♂, 2♀♀, leg. VŽ,

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from *Yponomeuta cagnagella* (Hübner) (Yponomeutidae). Serbia, Radan mt., 800 m, 06.06.2015 and 12.06.2016, 57♂♂, 63♀♀, leg. VŽ, from *Yponomeuta malinellus* Zeller. Serbia, Radan mt., 800 m, 06.06.2015, 11♂♂, 14♀♀, leg. VŽ, from *Yponomeuta padella* (Linnaeus). Serbia, Radan mt., 800 m, 06.06.2015, 10♂♂, 18♀♀, leg. VŽ, from *Yponomeuta* sp. (on *Malus domestica*).

Remark: Common parasitoid of Yponomeutidae and other "Microlepidoptera". Often reared from the recorded hosts (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

****Exorista deligata* Pandellé, 1896**

Material examined: Serbia, Zlatibor mt, Čavlovac, 1045 m, 27.06.2015, 1♂, 1♀, leg. MIM, from indet. Psychidae.

Remark: A rare species which is known as reared from several Psychidae (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

***Pales pavid* (Meigen, 1824)**

Material examined: Serbia, Tara, Derвента, 580 m, 25.06.2015, 1♂, 1♀, leg. VŽ, from *Cucullia* sp. (Noctuidae).

Remark: Common unspecific parasitoid of many Lepidoptera. Occasionally reared from *Cucullia* species (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

***Phryxe hirta* (Bigot, 1880)**

Material examined: Serbia, Vlasina Lake, 1250 m, 05.06.2014, 1♀, leg. VŽ; 06.06.2014, 1♀ leg. SS, from *Heterogynis sondereggeri* De Freina (Heterogynidae).

Remark: Already recorded by Stanković *et al.* (2014) from the same host and locality (reared 2013).

***Phryxe nemea* (Meigen, 1824)**

Material examined: Serbia, Vlasina Lake, 1250 m, 28.06.2012, 1♂, leg. VŽ, from *Zygaena filipendulae* Linnaeus (Zygaenidae).

Remark: Common parasitoid of many Lepidoptera. The host/parasitoid couple *Phryxe nemea* ex *Zygaena filipendulae* was already recorded by Audcent (1942).

***Phryxe vulgaris* (Fallén, 1810)**

Material examined: Serbia, Niš, Pantelej, 240 m, 21.04.2014, 1♀, leg. VŽ, from *Issoria lathonia* Linnaeus (Nymphalidae). Serbia, Kopaonik mt., 1700 m, 12.06.2016, 1♀, leg. VŽ, from *Zygaena lonicerae* Scheven (Zygaenidae).

Remark: *Phryxe vulgaris* is a common parasitoid of many Lepidoptera. The hosts *Issoria lathonia* and *Zygaena lonicerae* were already recorded by Brauer and Bergenstamm (1894) (revised by Herting (1960)) and Edelsten (1933) respectively.

***Sturmia bella* (Meigen, 1824)**

Material examined: Montenegro, Durmitor mt., Ledena pećina, 2000 m, 16.07.2015, 9♂♂, 14♀♀, leg. SS, from *Aglaia urticae* Linnaeus (Nymphalidae). Serbia, Vranjski Priboj, 300 m, 10.06.2014, 5♂♂, 4♀♀, leg. VŽ, from *Inachis io* Linnaeus (Nymphalidae).

Remark: *Sturmia bella* is a common parasitoid of Nymphalidae. The recorded two species are well-known as usual hosts (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

****Zenillia dolosa* (Meigen, 1824)**

Material examined: Serbia, Sićevačka klisura (gorge), 230 m, 24.05.2015, 1♀, leg. SS, from *Pleuroptya ruralis* Scopoli (syn. *Patania ruralis* (Scopoli)) pupa (Crambidae).

Remark: Common parasitoid of mainly "Microlepidoptera". *Pleuroptya ruralis* is a well-known host (Tschorsnig and Herting, 1994; Tschorsnig, 2017).

Subfamily Tachininae

***Tachina* sp. indet.**

Material examined: Serbia, Radan mt., 800 m, 06.06.2015, 1♀, leg. VŽ, from *Cucullia verbasci* Linnaeus (Noctuidae).

Remark: A species of the unrevised *Tachina magnicornis* agg. with the abdomen without dark median stripe.

Besides the list of reared tachinid species, we gathered the literature data and presented them as a checklist (Table 1). As the main data source, we used the Fauna Europaea Internet Database (Tschorsnig, 2013). However, the species list of the mentioned database should be updated with the 48 species which were additionally recorded in Serbia and Montenegro faunas.

DISCUSSION

The majority of the reared species belongs to the subfamily Exoristinae (16) while the subfamilies Dexinae and Tachininae are represented with two and one species respectively. This is not surprising, because Exoristinae includes the most often reared tachinid species. From this subfamily, the genus *Phryxe* is the most commonly reared, three species are recorded. In this investigation, there were no species from the subfamily Phasiinae, which is due to the fact that Heteroptera are only reared by breeders which are specialised on this insect order. The ermine moths from the genus *Yponomeuta* with its two identified species, *Y. cagnagella* and *Y. malinellus* served as hosts for two tachinids, *Eurystaea scutellaris* and *Bessa parallela*, thus making *Yponomeuta* species in our investigation the most common hosts from which parasitoids were reared. Moreover, *Eurystaea scutellaris* was the most abundant species with 178 individuals reared from both ermine moths. Out of the five newly reported species for the investigated territory, four species are usually relatively common (*Thelaira solivaga*, *Buquetia musca*, *Erycia festinans* and *Zenillia dolosa*). It is interesting that *Buquetia musca* has not been reported previously for the Serbian and Montenegrin fauna by any author, having in mind that the species is often reared in other European countries (Tschorsnig, 2017).

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Table 1. Literature records and findings of tachinid species new for Serbia and Montenegro. The ratio represents the number of recorded species for the investigated territory in relation to the number of species in whole Europe (according to Fauna Europaea). (*) Ratio between species found in Serbia and Montenegro/species found in Europe.

SUBFAMILY	GENUS	SPECIES	*SPECIES EXPLORATION
Dexiinae	<i>Athrycia</i>	<i>A. impressa</i> (van der Wulp, 1869), <i>A. trepida</i> (Meigen, 1824)	2/3
	<i>Billaea</i>	<i>B. adelpha</i> (Loew, 1873), <i>B. irrorata</i> (Meigen, 1826), <i>B. maritima</i> (Schiner, 1862), <i>B. pectinata</i> (Meigen, 1826), <i>B. triangulifera</i> (Zetterstedt, 1844)	5/13
	<i>Campylocheta</i>	<i>C. praecox</i> (Meigen, 1824)	1/9
	<i>Cyrtophleba</i>	<i>C. ruricola</i> (Meigen, 1824)	1/2
	<i>Dexia</i>	<i>D. rustica</i> (Fabricius, 1775)	1/2
	<i>Dinera</i>	<i>D. carinifrons</i> (Fallen, 1817), <i>D. ferina</i> (Fallen, 1817), <i>D. griseus</i> (Fallen, 1817)	3/3
	<i>Dufouria</i>	<i>D. chalybeata</i> (Meigen, 1824)	1/4
	<i>Eriothrix</i>	<i>E. apenninus</i> (Rondani, 1862), <i>E. argyreatus</i> (Meigen, 1824), <i>E. prolixa</i> (Meigen, 1824), <i>E. rufomaculatus</i> (De Geer, 1776)	4/9
	<i>Estheria</i>	<i>E. bohemani</i> (Rondani, 1862), <i>E. petiolata</i> (Bonsdorff, 1866), <i>E. picta</i> (Meigen, 1826)	3/12
	<i>Kirbya</i>	<i>K. moerens</i> (Meigen, 1830)	1/2
	<i>Klugia</i>	<i>K. marginata</i> (Meigen, 1824)	1/1
	<i>Periscepsia</i>	<i>P. carbonaria</i> (Panzer, 1798)	1/2
	<i>Phyllomya</i>	<i>P. volvulus</i> (Fabricius, 1794)	1/2
	<i>Plagiomima</i>	<i>P. hilfi</i> (Strobl, 1902)	1/2
	<i>Prosenia</i>	<i>P. siberita</i> (Fabricius, 1775)	1/1
	<i>Rhamphina</i>	<i>R. pedemontana</i> (Meigen, 1824)	1/2
	<i>Stomina</i>	<i>S. tachinoides</i> (Fallen, 1817)	1/4
	<i>Thelaira</i>	<i>T. leucozona</i> (Panzer, 1809), <i>T. nigripes</i> (Fabricius, 1794) <i>T. solivaga</i> (Harris, 1780)	3/3
	<i>Trafoia</i>	<i>T. monticola</i> Brauer & Bergenstamm, 1893	1/3
	<i>Trixa</i>	<i>T. conspersa</i> (Harris, 1776)	1/5
	<i>Voria</i>	<i>V. ruralis</i> (Fallen, 1810)	1/1
	<i>Wagneria</i>	<i>W. gagatea</i> Robineau-Desvoidy, 1830	1/9
	<i>Zeuxia</i>	<i>Z. aberrans</i> (Loew, 1847), <i>Z. cinerea</i> Meigen, 1826	2/13
Exoristinae	<i>Acemya</i>	<i>A. rufitibia</i> (von Roser, 1840)	1/3
	<i>Admontia</i>	<i>A. maculisquama</i> (Zetterstedt, 1859)	1/9
	<i>Aplomya</i>	<i>A. confinis</i> (Fallen, 1820)	1/1
	<i>Atylomyia</i>	<i>A. loewi</i> Brauer, 1898	1/3

Table 1. Continued.

SUBFAMILY	GENUS	SPECIES	*SPECIES EXPLORATION
Exoristinae	<i>Bactromyia</i>	<i>B. aurulenta</i> (Meigen, 1824)	1/1
	<i>Baumhaueria</i>	<i>B. goniaeformis</i> (Meigen, 1824), <i>B. microps</i> Mesnil, 1963	2/3
	<i>Bessa</i>	<i>B. parallela</i> (Meigen, 1824)	1/2
	<i>Blepharipa</i>	<i>B. pratensis</i> (Meigen, 1824), <i>B. schineri</i> (Mesnil, 1939)	2/2
	<i>Blondelia</i>	<i>B. inclusa</i> (Hartig, 1838), <i>B. nigripes</i> (Fallen, 1810)	2/2
	<i>Bothria</i>	<i>B. frontosa</i> (Meigen, 1824)	1/2
	<i>Brachicheta</i>	<i>B. strigata</i> (Meigen, 1824)	1/1
	<i>Buquetia</i>	<i>B. musca</i> Robineau-Desvoidy 1847	1/1
	<i>Cadurcia</i>	<i>C. casta</i> (Rondani, 1861)	1/1
	<i>Carcellia</i>	<i>C. atricosta</i> Herting, 1961, <i>C. bombylans</i> Robineau-Desvoidy, 1830, <i>C. falenaria</i> (Rondani, 1859), <i>C. gnava</i> (Meigen, 1824), <i>C. lucorum</i> (Meigen, 1824), <i>C. puberula</i> Mesnil, 1941, <i>C. rasa</i> (Macquart, 1849), <i>C. rasella</i> Baranov, 1931	8/16
	<i>Catagonia</i>	<i>C. aberrans</i> (Rondani, 1859)	1/1
	<i>Ceratochaetops</i>	<i>C. triseta</i> (Villeneuve, 1922)	1/2
	<i>Ceromasia</i>	<i>C. rubrifrons</i> (Macquart, 1834)	1/1
	<i>Chetogena</i>	<i>C. acuminata</i> Rondani, 1859, <i>C. filipalpis</i> Rondani, 1859, <i>C. media</i> Rondani, 1859, <i>C. nigrofasciata</i> (Strobl, 1902)	4/13
	<i>Clemelis</i>	<i>C. pullata</i> (Meigen, 1824)	1/5
	<i>Compsilura</i>	<i>C. concinnata</i> (Meigen, 1824)	1/1
	<i>Cyzenis</i>	<i>C. albicans</i> (Fallén, 1810)	1/2
	<i>Diplostichus</i>	<i>D. janitrix</i> (Hartig, 1838)	1/1
	<i>Drino</i>	<i>D. atropivora</i> (Robineau-Desvoidy, 1830), <i>D. galii</i> (Brauer & Bergenstamm, 1891), <i>D. inconspicua</i> (Meigen, 1830), <i>D. lota</i> (Meigen, 1824), <i>D. vicina</i> (Zetterstedt, 1849)	5/9
	<i>Elodia</i>	<i>E. morio</i> (Fallen, 1820)	1/3
	<i>Epicampocera</i>	<i>E. succincta</i> (Meigen, 1824)	1/1
	<i>Erycia</i>	<i>E. fasciata</i> Villeneuve, 1924, <i>E. fatua</i> (Meigen, 1824), <i>E. festinans</i> (Meigen, 1824)	3/4
	<i>Erynniopsis</i>	<i>E. antennata</i> (Rondani, 1861)	1/1
	<i>Erythroceras</i>	<i>E. nigripes</i> (Robineau-Desvoidy, 1830)	1/1
	<i>Ethilla</i>	<i>E. aemula</i> (Meigen, 1824)	1/1
	<i>Eumea</i>	<i>E. linearicornis</i> (Zetterstedt, 1844), <i>E. mitis</i> (Meigen, 1824)	2/2
	<i>Eurysthaea</i>	<i>E. scutellaris</i> (Robineau-Desvoidy, 1848)	1/1

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Table 1. Continued.

SUBFAMILY	GENUS	SPECIES	*SPECIES EXPLORATION
Exoristinae	<i>Exorista</i>	<i>E. civilis</i> (Rondani, 1859), <i>E. deligata</i> Pandelle, 1896, <i>E. fasciata</i> (Fallen, 1820), <i>E. florentina</i> Herting, 1975, <i>E. grandis</i> (Zetterstedt, 1844), <i>E. larvarum</i> (Linnaeus, 1758), <i>E. mimula</i> (Meigen, 1824), <i>E. nymphaeum</i> (Rondani, 1859), <i>E. rustica</i> (Fallen, 1810), <i>E. segregata</i> (Rondani, 1859), <i>E. sorbillans</i> (Wiedemann, 1830), <i>E. tubulosa</i> Herting, 1967, <i>E. xanthaspis</i> (Wiedemann, 1830)	13/23
	<i>Gaedia</i>	<i>G. connexa</i> (Meigen, 1824), <i>G. distincta</i> Egger, 1861	2/3
	<i>Gastrolepta</i>	<i>G. anthracina</i> (Meigen, 1826)	1/1
	<i>Gonia</i>	<i>G. bimaculata</i> Wiedemann, 1819, <i>G. capitata</i> (De Geer, 1776), <i>G. distinguenda</i> Herting, 1963, <i>G. divisa</i> Meigen, 1826, <i>G. ornata</i> Meigen, 1826, <i>G. picea</i> (Robineau-Desvoidy, 1830), <i>G. vacua</i> Meigen, 1826	7/14
	<i>Huebneria</i>	<i>H. affinis</i> (Fallen, 1810)	1/1
	<i>Istocheta</i>	<i>I. cinerea</i> (Macquart, 1850), <i>I. longicornis</i> (Fallen, 1810)	2/6
	<i>Lecanipa</i>	<i>L. bicincta</i> (Meigen, 1824), <i>L. leucomelas</i> (Meigen, 1824)	2/2
	<i>Leiophora</i>	<i>L. innoxia</i> (Meigen, 1824)	1/1
	<i>Ligeria</i>	<i>L. angusticornis</i> (Loew, 1847)	1/2
	<i>Ligeriella</i>	<i>L. aristata</i> (Villeneuve, 1911)	1/1
	<i>Lydella</i>	<i>L. thompsoni</i> Herting, 1959	1/6
	<i>Masicera</i>	<i>M. silvatica</i> (Fallen, 1810), <i>M. sphingivora</i> (Robineau-Desvoidy, 1830)	2/3
	<i>Medina</i>	<i>M. luctuosa</i> (Meigen, 1824), <i>M. multispina</i> (Herting, 1966)	2/5
	<i>Meigenia</i>	<i>M. dorsalis</i> (Meigen, 1824), <i>M. majuscula</i> (Rondani, 1859), <i>M. mutabilis</i> (Fallen, 1810), <i>M. uncinata</i> Mesnil, 1967	4/7
	<i>Myxexoristops</i>	<i>M. bicolor</i> (Villeneuve, 1908), <i>M. blondeli</i> (Robineau-Desvoidy, 1830)	2/6
	<i>Nemorilla</i>	<i>N. floralis</i> (Fallen, 1810), <i>N. maculosa</i> (Meigen, 1824)	2/2
	<i>Nilea</i>	<i>N. hortulana</i> (Meigen, 1824), <i>N. innoxia</i> Robineau-Desvoidy, 1863	2/4
	<i>Ocytata</i>	<i>O. pallipes</i> (Fallen, 1820)	1/1
	<i>Oswaldia</i>	<i>O. spectabilis</i> (Meigen, 1824)	1/5
	<i>Pales</i>	<i>P. pavida</i> (Meigen, 1824)	1/6
	<i>Parasetigena</i>	<i>P. silvestris</i> (Robineau-Desvoidy, 1863)	1/1
	<i>Paratryphera</i>	<i>P. barbatula</i> (Rondani, 1859), <i>P. bisetosa</i> (Brauer & Bergenstamm, 1891), <i>P. palpalis</i> (Rondani, 1859)	3/4
	<i>Periarchiclops</i>	<i>P. scutellaris</i> (Fallen, 1820)	1/1
	<i>Phebellia</i>	<i>P. glauca</i> (Meigen, 1824), <i>P. nigripalpis</i> (Robineau-Desvoidy, 1847), <i>P. stulta</i> (Zetterstedt, 1844)	3/11
	<i>Phonomyia</i>	<i>P. aristata</i> (Rondani, 1861)	1/1

Table 1. Continued.

SUBFAMILY	GENUS	SPECIES	*SPECIES EXPLORATION
Exoristinae	<i>Phorinia</i>	<i>P. aurifrons</i> Robineau-Desvoidy, 1830	1/1
	<i>Phorocera</i>	<i>P. assimilis</i> (Fallen, 1810), <i>P. obscura</i> (Fallen, 1810)	2/4
	<i>Phryxe</i>	<i>P. erythrostoma</i> (Hartig, 1838), <i>P. hirta</i> (Bigot 1880), <i>P. magnicornis</i> (Zetterstedt, 1838), <i>P. nemea</i> (Meigen, 1824), <i>P. prima</i> (Brauer & Bergenstamm, 1889), <i>P. vulgaris</i> (Fallen, 1810)	6/12
	<i>Picconia</i>	<i>P. incurva</i> (Zetterstedt, 1844)	1/1
	<i>Platymya</i>	<i>P. fimbriata</i> (Meigen, 1824)	1/2
	<i>Policheta</i>	<i>P. unicolor</i> (Fallén, 1820)	1/1
	<i>Prosopaea</i>	<i>P. nigricans</i> (Egger, 1861)	1/1
	<i>Pseudogonia</i>	<i>P. parisiaca</i> (Robineau-Desvoidy, 1851), <i>P. rufifrons</i> (Wiedemann, 1830)	2/3
	<i>Pseudoperichaeta</i>	<i>P. nigrolineata</i> (Walker, 1853), <i>P. palesoidea</i> (Robineau-Desvoidy, 1830)	2/2
	<i>Rhaphiochaeta</i>	<i>R. breviseta</i> (Zetterstedt, 1838)	1/1
	<i>Senometopia</i>	<i>S. confundens</i> (Rondani, 1859), <i>S. excisa</i> (Fallen, 1820), <i>S. pollinosa</i> (Mesnil, 1941), <i>S. separata</i> (Rondani, 1859), <i>S. susurrans</i> (Rondani, 1859)	5/8
	<i>Smidtia</i>	<i>S. amoena</i> (Meigen, 1824)	1/4
	<i>Spallanzania</i>	<i>S. hebes</i> (Fallen, 1820), <i>S. multisetosa</i> (Rondani, 1859)	2/5
	<i>Sturmia</i>	<i>S. bella</i> (Meigen, 1824)	1/1
	<i>Thecocarcelia</i>	<i>T. acutangulata</i> (Macquart, 1850), <i>T. trichops</i> Herting, 1967	2/2
	<i>Townsendiellomyia</i>	<i>T. nidicola</i> (Townsend, 1908)	1/1
	<i>Tryphera</i>	<i>T. lugubris</i> (Meigen, 1824)	1/1
	<i>Vibrissina</i>	<i>V. turrita</i> (Meigen, 1824)	1/2
	<i>Winthemia</i>	<i>W. erythrura</i> (Meigen, 1838), <i>W. quadripustulata</i> (Fabricius, 1794), <i>W. rufiventris</i> (Macquart, 1849), <i>W. variegata</i> (Meigen, 1824), <i>W. venusta</i> (Meigen, 1824)	5/11
	<i>Zaira</i>	<i>Z. cinerea</i> (Fallen, 1810)	1/1
	<i>Zenillia</i>	<i>Z. dolosa</i> (Meigen 1824), <i>Z. libatrix</i> (Panzer, 1798)	2/2
Phasiinae	<i>Besseria</i>	<i>B. dimidiata</i> (Zetterstedt, 1844)	1/6
	<i>Cistogaster</i>	<i>C. globosa</i> (Fabricius, 1775)	1/2
	<i>Clytiomya</i>	<i>C. continua</i> (Panzer, 1798)	1/4
	<i>Cylindromyia</i>	<i>C. auriceps</i> (Meigen, 1838), <i>C. bicolor</i> (Olivier, 1812), <i>C. brassicaria</i> (Fabricius, 1775), <i>C. brevicornis</i> (Loew, 1844), <i>C. intermedia</i> (Meigen, 1824), <i>C. interrupta</i> (Meigen, 1824), <i>C. pilipes</i> (Loew, 1844), <i>C. rufipes</i> (Meigen, 1824)	8/17
	<i>Dionaea</i>	<i>D. aurifrons</i> (Meigen, 1824)	1/3
	<i>Ectophasia</i>	<i>E. crassipennis</i> (Fabricius, 1794)	1/3

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Table 1. Continued.

SUBFAMILY	GENUS	SPECIES	*SPECIES EXPLORATION
Phasiinae	<i>Elomya</i>	<i>E. lateralis</i> (Meigen, 1824)	1/1
	<i>Gymnosoma</i>	<i>G. clavatum</i> (Rohdendorf, 1947), <i>G. costatum</i> (Panzer, 1800), <i>G. desertorum</i> (Rohdendorf, 1947), <i>G. inornatum</i> Zimin, 1966, <i>G. nudifrons</i> Herting, 1966, <i>G. rotundatum</i> (Linnaeus, 1758)	6/13
	<i>Hemyda</i>	<i>H. obscuripennis</i> (Meigen, 1824)	1/2
	<i>Labigastera</i>	<i>L. forcipata</i> (Meigen, 1824)	1/4
	<i>Leucostoma</i>	<i>L. simplex</i> (Fallen, 1815), <i>L. tetraptera</i> (Meigen, 1824)	2/12
	<i>Lophosia</i>	<i>L. fasciata</i> Meigen, 1824	1/1
	<i>Opesia</i>	<i>O. cana</i> (Meigen, 1824), <i>O. grandis</i> (Egger, 1860)	2/3
	<i>Phania</i>	<i>P. funesta</i> (Meigen, 1824), <i>P. incrassata</i> Pandellé, 1894, <i>P. speculifrons</i> (Villeneuve, 1919)	3/6
	<i>Phasia</i>	<i>P. aurigera</i> (Egger, 1860), <i>P. hemiptera</i> (Fabricius, 1794), <i>P. obesa</i> (Fabricius, 1798), <i>P. pusilla</i> Meigen, 1824	4/14
	<i>Redtenbacheria</i>	<i>R. insignis</i> Egger, 1861	1/1
Tachininae	<i>Xysta</i>	<i>X. holosericea</i> (Fabricius, 1805)	1/1
	<i>Actia</i>	<i>A. crassicornis</i> (Meigen, 1824), <i>A. infantula</i> (Zetterstedt, 1844), <i>A. pilipennis</i> (Fallen, 1810)	3/7
	<i>Aphria</i>	<i>A. longilingua</i> Rondani, 1861, <i>A. longirostris</i> (Meigen, 1824)	2/4
	<i>Bithia</i>	<i>B. demotica</i> (Egger, 1861), <i>B. immaculata</i> (Herting, 1971) <i>B. modesta</i> (Meigen, 1824)	3/12
	<i>Chrysosomopsis</i>	<i>C. aurata</i> (Fallén, 1820)	1/1
	<i>Demoticus</i>	<i>D. plebejus</i> (Fallen, 1810)	1/3
	<i>Entomophaga</i>	<i>E. exoleta</i> (Meigen, 1824)	1/3
	<i>Eurithia</i>	<i>E. anthophila</i> (Robineau-Desvoidy, 1830), <i>E. caesia</i> (Fallen, 1810), <i>E. connivens</i> (Zetterstedt, 1844), <i>E. consobrina</i> (Meigen, 1824), <i>E. cristata</i> (Villeneuve, 1920), <i>E. gemina</i> (Mensil, 1972), <i>E. intermedia</i> (Zetterstedt, 1844), <i>E. vivida</i> (Zetterstedt, 1838)	8/14
	<i>Germania</i>	<i>G. ruficeps</i> (Fallén, 1820)	1/5
	<i>Linnaemya</i>	<i>L. comta</i> (Fallen, 1810), <i>L. frater</i> (Rondani, 1859), <i>L. helvetica</i> Herting, 1963, <i>L. impudica</i> (Rondani, 1859), <i>L. lithosiophaga</i> (Rondani, 1859), <i>L. picta</i> (Meigen, 1824), <i>L. rossica</i> Zimin, 1954, <i>L. tessellans</i> (Robineau-Desvoidy, 1830), <i>L. vulpina</i> (Fallén, 1810), <i>L. zachvatkini</i> Zimin, 1954	10/21
	<i>Loewia</i>	<i>L. setibarba</i> Egger, 1856	1/11
	<i>Lydina</i>	<i>L. aenea</i> (Meigen, 1824)	1/1

Table 1. Continued.

SUBFAMILY	GENUS	SPECIES	*SPECIES EXPLORATION
Tachininae	<i>Macquartia</i>	<i>M. chalconota</i> (Meigen, 1824), <i>M. dispar</i> (Fallen, 1820), <i>M. grisea</i> (Fallen, 1810), <i>M. nudigena</i> Mesnil, 1972, <i>M. tenebricosa</i> (Meigen, 1824), <i>M. tessellum</i> (Meigen, 1824), <i>M. viridana</i> Robineau-Desvoidy, 1863	7/11
	<i>Microphthalma</i>	<i>M. europaea</i> Egger, 1860	1/1
	<i>Mintho</i>	<i>M. rufiventris</i> (Fallen, 1817)	1/2
	<i>Nemoraëa</i>	<i>N. pellucida</i> (Meigen, 1824)	1/1
	<i>Nowickia</i>	<i>N. atripalpis</i> (Robineau-Desvoidy, 1863), <i>N. ferox</i> (Panzer, 1809), <i>N. reducta</i> Mesnil, 1970	3/8
	<i>Panzeria</i>	<i>P. argentifera</i> (Meigen, 1824), <i>P. puparum</i> (Fabricius, 1794), <i>P. rudis</i> (Fallen, 1810)	3/5
	<i>Pelatachina</i>	<i>P. tibialis</i> (Fallen, 1810)	1/1
	<i>Peleteria</i>	<i>P. rubescens</i> (Robineau-Desvoidy, 1830), <i>P. ruficornis</i> (Macquart, 1835), <i>P. varia</i> (Fabricius, 1794)	3/9
	<i>Peribaea</i>	<i>P. apicalis</i> Robineau-Desvoidy, 1863, <i>P. setinervis</i> (Thomson, 1869), <i>P. tibialis</i> (Robineau-Desvoidy, 1851)	3/6
	<i>Siphona</i>	<i>S. collini</i> Mesnil, 1960, <i>S. flavifrons</i> Stæger, 1849, <i>S. geniculata</i> (De Geer, 1776)	3/22
	<i>Solieria</i>	<i>S. fenestrata</i> (Meigen, 1824), <i>S. pacifica</i> (Meigen, 1824)	2/5
	<i>Tachina</i>	<i>T. casta</i> (Rondani, 1859), <i>T. fera</i> (Linnaeus, 1761), <i>T. grossa</i> (Linnaeus, 1758), <i>T. lurida</i> (Fabricius, 1781), <i>T. magnicornis</i> (Zetterstedt, 1844), <i>T. nupta</i> (Rondani, 1859), <i>T. praeceps</i> Meigen, 1824, <i>T. ursina</i> Meigen, 1824	8/12
	<i>Trichactia</i>	<i>T. pictiventris</i> (Zetterstedt, 1855)	1/2
	<i>Zophomyia</i>	<i>Z. temula</i> (Scopoli, 1763)	1/1

Most of the updated species in the summarized table 1 were taken from Hubenov (2008a), 46 species, while two species were taken from Stanković et al. (2014). We give a list of 295 tachinid species for the fauna of Serbia and Montenegro along with new findings. Almost half of the species belong to Exoristinae subfamily being the most diverse one, and with the most diverse genus *Exorista* with 12 species recorded. The second most numerous subfamily is Tachininae comprising of 70 species in total, while the other two Dexinae and Phasiinae are represented with 38 species. The genera richness for the European fauna are also well represented on the investigated territory. For example, *Exorista*, *Gonia*, *Cylindromyia*, *Eurithia*, *Linnaemya* and *Tachina* comprise of a half or more than a half of the total European species from the mentioned genera. However, genera such as *Leucostoma* and especially *Siphona* are actually poorly represented in comparison to the whole European fauna (see Table 1).

This faunistic survey represents a good starting point for further tachinid investigation and possibly integrated pest management.

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First Record of *Termatophylum orientale* Poppius (Hemiptera: Miridae: Deraeocorinae) from India with Biological Note

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ABSTRACT

Termatophylum orientale Poppius is being reported for the first time from India. It was collected from *Mangifera indica* (Mango, Anacardiaceae), *Carica papaya* (Papaya, Caricaceae) and *Peltophorum pterocarpum* (Copperpod, Fabaceae) where it shares niche along with other predators like anthocorids, geocorids and pests like thrips, mites and lepidopteran larvae. For the first time rearing protocol and biology has been given for this mirid.

Key words: Mango, Miridae, Deraeocorinae, Termatophylini, *Termatophylum orientale*, Thrips.

INTRODUCTION

Mirid bugs of the tribe Teratophylini are known to inhabit inflorescences, moth larval galleries or rolled bark. They are known to feed on thrips, besides feeding on nectar and pollen (Cassis, 1995; Cassis *et al.*, 2011; Yasunaga *et al.*, 2001). Three species of the genus *Teratophylidea* Reuter and Poppius were reported to attack on the cacao thrips, occupying the niche shared by anthocorids. It is assumed that these mirids are obligate predators and feed exclusively on thrips. Both immature stages and adults of *Teratophylidea maculate* Usinger were reported to feed on cacao thrips present on the underside of the leaves of cacao and cashew (Callan, 1975). However, biology of *T. maculate* is unknown.

From India, so far two species of *Teratophylum* Reuter and one species of *Teratophylina* Carvalho have been reported in the tribe Teratophylini of the subfamily Dereacorinae (Cassis, 1995). So far fifteen species of *Teratophylum* have been reported worldwide (online catalogue Schuh, 2002-2013). During recent field work, *Teratophylum orientale* Poppius was collected from mango (Anacardiaceae), papaya (Caricaceae) and flowers of copper pod tree (*Peltophorum pterocarpum*) (Fabaceae) wherein it was found associated with thrips and other predators. Earlier it was reported from Taiwan and later from Japan by Nakatani (1997). Current study reports this species for the first time from India. The present note provides the host plants details, diagnosis of the species with illustrations of genitalia, rearing protocol and biology.

MATERIAL AND METHODS

Mirids were collected from the above mentioned host plants and brought to the laboratory. The specimens were studied under stereo zoom binocular microscope. Illustrations of male genitalia were drawn using a Leica DM2000 attached to a camera lucida. Photographs of different nymphal instars and adult were taken using a Leica M205C. Attempts were made to rear and study its biology in the laboratory on eggs of *Corcyra cephalonica* (Stainton) (Lepidoptera, Pyralidae) (National Accession number: NBAIL-MP-PYR-01).

Rearing protocol

Teratophylum orientale was reared in the laboratory on bean pods and UV-irradiated *C. cephalonica* eggs. Pearlpet® plastic containers (500 ml capacity) were used for multiplication. The floor of containers was provided with tissue paper. In each container, 5-6 pieces of bean pods were placed as oviposition substrate for egg laying along with cotton lint (to avoid cannibalism) and *C. cephalonica* eggs were sprinkled on it. A swab of cotton soaked in water was stuck to the wall of the container. One container could hold up to 30 adults.

Adult bugs laid their eggs inserted into the sides of the bean pods in small groups (usually 3-4) or singly, with only the operculum of the eggs visible. After 24 hours, the bean pods with eggs were removed and placed in small, round, ventilated plastic containers (diameter 6.5 cm and height 2.5 cm). After 3-5 days when the nymphs

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hatched, they were shifted to pearlpet® jars provided with *Corcyra cephalonica* eggs on bean pods. *C. cephalonica* eggs were provided for feeding on every alternate days till the nymphs developed into adults. Once they developed into adults the freshly moulted adults were shifted to the jars for mating and egg laying with pieces of bean pods for oviposition. Containers were kept in an incubator at 25°C, 60-70% RH, and a photoperiod of 12:12 (L: D) h.

Species, *T. insigne* Reuter from Egypt was reared in the laboratory on larvae of *Tribolium confusum* Jacquelin du Val, *Lasioderma serricornae* (Fabricius) and *Stegobium paniceum* (Linnaeus) (Awadallah *et al.*, 1986). They found a higher reproductive potential on *T. confusum* than on other storage pests.

Biological parameters and morphometry

Termatophylum orientale, with the above discussed rearing method or technique, was reared successfully and continuously till date of manuscript submission. To study biology of this mirid, one pair of adult was released into each pearlpet plastic container (200 ml) covered with black cloth. Ten such sets were maintained. Each container was provided with UV-irradiated *C. cephalonica* eggs as feeding and bean pieces (2-3) for egg laying. After every 24 h, bean pods were collected and observed under microscope to record number of eggs laid. Eggs were collected, counted and beans with eggs were placed in small, round, ventilated plastic boxes (diameter 6.5 cm and height 2.5 cm) for hatching separately.

The number of nymphs, which hatched from total eggs collected from each container, was counted for calculating per cent hatching. Ten freshly hatched nymphs per set were kept individually in plastic boxes provided with UV-irradiated *C. cephalonica* eggs. Observations were recorded on total number of instars, duration of each instar and total nymphal period. When adults were formed, they were collected and observed under microscope to differentiate the sex. Per cent adults formed was calculated based on the number of healthy adults developed from the total number of nymphs which hatched in each set. Longevity of adult *T. orientale* was recorded. Morphometrics of nymphal and adult stages were measured by using ocular and stage micrometers. The measurements indicated in the text are expressed in millimeters.

Taxonomy

Tribe Termatophylini Reuter, 1884

Genus *Termatophylum* Reuter, 1884

***Termatophylum* Reuter, 1884: 218. Type species: *Termatophylum insigne* Reuter, 1884.**

***Termatophylum orientale* Poppius 1915 (Figs. 1A, 1B and 1C)**

Key diagnostic characters

Easily recognized by dark brown to black coloration of dorsum, large eyes and strongly projecting apex of head and a longitudinal depression on the vertex. Male

genitalia with left paramere blade like; right paramere reduced or vestigial; vesica without sclerites.

Brief description

Colouration: Body dark brown to black; head, pronotum, scutellum dark brown; membrane smoke grey; antennal segment III, IV, labium and legs pale yellow; embolium and apical half of cuneus tinged with red. Body complete shining with dorsum covered with evenly distributed elongate shining golden yellow setae. Head moderately produced in front with frons and vertex moderately punctuate, vertex with a shallow longitudinal impression; eyes large occupying entire height of the head in lateral view. Antennal segment I tubular, slightly longer than the tylus; segment II basally narrow and rest portion enlarged or tubular, slightly shorter or subequal to length of segment II and IV together; segments III and IV narrow, subequal in length; labium long reaching mesocoxae. Pronotum with narrow anterior and posterior regions, with a prominent neck as wide as width of vertex; anterior pronotal region with a prominently marked calli, posterior pronotal margin nearly straight, lateral margins weak sinuate; scutellum short broadly triangular; scent gland with a prominent peritreme. Hemelytra short, broad, with prominent embolium, cuneus prominent, wider and broadly triangular; membrane with a single large primary cell; legs elongate, all femora cylindrical, all tibia elongate, fore legs widely separated from middle legs; mid and hind legs very close, claws cleft at apex.

Male genitalia: Genital capsule short and broad (Fig. 1A); left paramere with a well developed sensory lobe with elongate setae, hypophysis flattened, blade like towards the apex (Figs. 1B and C), right paramere vestigial, vesica membranous without any spicules.

Distribution: Japan, Taiwan, and India (new record).

Material examined: INDIA: Karnataka: Kanakapura, 4 ♂♂, 4 ♀♀, 16.11.2016, Richa Varshney, on Mango tree; Bagalur, Richa Varshney, on Copperpod tree (*Peltophorum pterocarpum*).

All specimens have been deposited in the insect museum of ICAR- National Bureau of Agricultural Insect Resources, Bangalore, India.

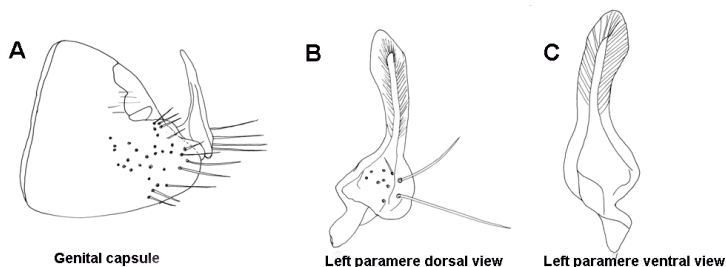


Fig. 1. Male genitalia of *Teratophylum orientale* Poppius A) Genital capsule B) Left paramere (dorsal view) C) Left paramere (ventral view).

Host Plants: In mango, it was collected from leaf web made by leaf webber *Orthaga exvinacea* (Hampson) larvae where it was found to feed on thrips and other arthropods. However its feeding on neonates of mango leaf webber is not clear. The same niche

First Record of Teratophylum orientale Poppius

is shared by other predatory hemipterans viz. anthocorid, geocorids, etc. Immature stages of this mirid were found inside the inflorescence and leaf webs.

In mango, population of this mirid was observed during December and continues to multiply till March in dried inflorescence. Similar observations were made by Rafeeq and Ranjini (2013) on two species of mirids i.e. *Teratophylina indiana* Carvalho (Dearaeorinae) and *Charagochilus* sp. (Mirinae) in dried larval web formed by *Orthaga exvinacea* in Mango.

In *Peltophorum* (copperpod tree, Fabaceae), *T. orientale* along with anthocorid, *Blaptostethus pallescens* Poppius was observed in the yellow inflorescence infested with thrips. In papaya it was found to be associated with other anthocorids along with papaya mealy bug *Paracoccus marginatus* Williams and Granara de Willink (Pseudococcidae).

Biology: It was observed that this mirid on an average laid 45-50 eggs singly or in group of 3 and 4 eggs embedded on the side of bean pod. Eggs are white, ovoid with red opercula exposed on the surface. Two silvery white thread like structures are present at both the side of operculum (Figs. 2A and B). After 3-5 days nymphs start emerging. The newly hatched nymphs when fed on UV -irradiated eggs of *Corcyra cephalonica*, undergo five nymphal instars and became adults in about 16-21 days.

First instar: Total length-0.97, width-0.34, antennal length-0.41, labium-0.43, pronotum width-0.22.

Newly hatched nymph is light orange in colour. Immediately after hatching, for few hours they feed on beans. After 1 -2 days nymph turns to brown red. Antennae are 4 segmented. First two segments are dark brown and swollen, remaining two segments are thread like. Two chocolaty brown shields appear on thorax on third day. Abdomen is swollen. Duration of first nymphal instar is 3-5 days (Fig. 2C).

Second instar: Total length-1.05, width-0.47, antennal length-0.51, labium-0.51, pronotum width-0.28.

Second instar nymph is dark red in colour. Eyes become more prominent and occupy mid lower portion. Coxae and femur are brownish red. Duration of second instar is 2-3 days (Fig. 2D).

Third instar: Total length-1.59, width-0.54, antennal length-0.59, labium-0.65, pronotum width-0.36.

Wing pad starts developing and occupying upper 1/3rd area of abdomen. The average duration of third instar nymph is 2.6 days (Fig. 2E).

Fourth instar: Total length-1.95, width-0.70, antennal length-0.72, labium-0.77, pronotum width-0.44.

Wing pads become prominent. After 2-4 days 4th instar nymph moults into 5th instar.

Fifth Instar: Total length-2.57, width-0.93, antennal length-0.90, labium-0.85, pronotum width-0.55. The fifth instar moults to become an adult with full black body and fully developed wings in 3-4 days. Average nymphal and developmental period for this mirid were 15.2 and 18.8 days, respectively when reared on *C. cephalonica* eggs. Average percent hatchability was 67.2.

Adult male: Total length-2.67, width-0.98, antennal length-0.99, labium-0.93, pronotum width-0.81; Adult female: Total length-3.06, width-1.16, antennal length-1.00, labium-0.97, pronotum width-0.97 (Fig. 2F).

Mating occurs after 1-2 days of emergence. Female continues to lay eggs till the death. Adult longevity is 15-20 days (Table 1). Sex ratio was 1.25:1.00 (female:male) indicating balanced sex ratio in the laboratory and thus as ideal candidate for mass rearing in insectaries. Female is larger in size than male.

The study indicated that this insect can be intensively cultured, without encountering problems of cannibalism and excessive handling. Furthermore investigation is being carried out to understand its feeding preferences and its interactions with other predators and pests which share same niche. Their behavior, whether they are obligatory or facultative predator, needs to be investigated.

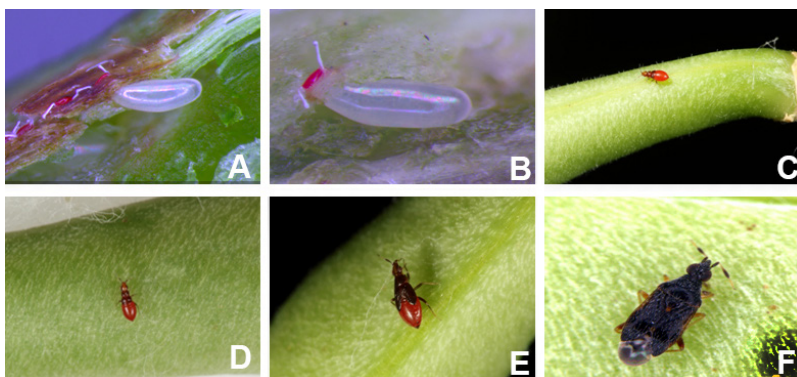


Fig. 2. Different stages of *Teratophylum orientale* Poppius. a Eggs laid in row with operculum exposed b. Larger view of egg c. First instar d. Second instar e. Third Instar f. Adult.

Table 1. Biology of *Teratophylum orientale* reared on *Corcyra cephalonica* eggs.

Stage	Mean \pm SE	Range
Incubation period	3.6 \pm 0.4	3-5
First instar	3.8 \pm 0.37	3-5
Second instar	2.6 \pm 0.24	2-3
Third instar	2.6 \pm 0.24	2-3
Fourth instar	2.8 \pm 0.37	2-4
Fifth instar	3.4 \pm 0.24	3-4
Total nymphal duration	15.2 \pm 0.86	13-18
Total developmental period	18.8 \pm 0.96	16-21
Adult longevity	17.8 \pm 1.77	15-20
Eggs/female	47.2 \pm 0.97	45-50
Per cent eggs hatched	67.2 \pm 1.85	62-72

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Conflict of Interest: The authors declare that they have no conflict of interest because this paper is a part of project. The collection, rearing and biological study was done by first author. Second author made the description of the species.

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Two New Species of *Bathypogon*: *gerhardi* and *monartoensis* (Insecta: Diptera: Asilidae: Bathypogoninae) from Monarto Zoological Park, South Australia, with Notes on Their Behaviour and Seasonal Distribution

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ABSTRACT

Two new species of robber flies of the genus *Bathypogon*, studied in the Monarto Zoological Park, South Australia, are described based on an examination of specimens of both sexes. *Bathypogon gerhardi* is a late autumn species and *B. monartoensis* is an early summer species; both occur in Mallee scrub and other dry environments. Copulation of both species takes place in the 'tail-to-tail' position with the male and female facing in opposite directions.

Key words: Biology, seasonal distribution, new species, South Australia, taxonomy, behavior.

INTRODUCTION

The genus *Bathypogon*, described in 1851 by Loew as a subgenus, and recently placed in the subfamily Bathypogoninae (Dikov, 2009), is confined to continental Australia. A large number of species apparently have evolved on the continent, but few have been described (Hull, 1962). The expansion of our knowledge of the genus was due largely to the work of Hull (1956a, b; 1958a, b, c, 1959), who was the first to recognize the importance of the structural modifications of the hypandrial plate for separating species. Since 1959, the only new species of *Bathypogon* to be described were *B. danielsi* Lavigne in 2006 and *B. glatzi* Lavigne in 2013.

MATERIALS AND METHODS

Methods and procedures have been covered in detail in several other papers (Lavigne and McAllister, 2012a; 2012b; 2015) and the author feels that there is no need to repeat them here other than to state that the seasonal data is based on a transect in Area 1 which consisted of the investigator following a circular convoluted path through the Mallee scrub over a two hour period on multiple dates in Monarto Zoological Park (GPS: 35°06'45"S, 139°08'51"E). *Bathypogon danielsi* also has been collected on site as well as a yet undescribed species collected only in ant traps. Most of the *Bathypogon* type material from South Australia resides in the Entomology collection of the South Australian Museum (Lavigne, 2004) so that comparisons of Monarto specimens to identified material was simplified.

RESULTS AND DISCUSSION

Biology

Habitat

In Monarto Zoological Park there occurs a mixture of original and revegetated Mallee scrub (WSW of Visitors Centre) (Fig. 1). The primary vegetation on Area 1 through which *B. gerhardi*, had to manoeuvre is as follows: *Enchylaena tomentosa* - Ruby Saltbush; *Heliotropium europaeum* - Common Heliotrope; *Lomandra effusa* - Scented Mat-rush; *Maireana brevifolia* - Bluebush; *Salsola kali* - Prickly Saltwort; *Sclerolaena* sp. - Bindyi. The vegetation at the Monarto Conservation Park nearby is the same. In December, species of wild flowers bloom profusely in the Park, but not in the study area where only weeds bloom.

Seasonal Abundance

Adults of *B. gerhardi* (recorded as *Bathypogon* species 1) were present on the Monarto Zoological Park site from late December 2003 to the beginning of January 2004 and from mid November 2004 to the end of January 2005 (Lavigne, 2005). Those of *B. monartoensis* recorded as *Bathypogon* sp. 2) were present from the beginning of January to the beginning of May, 2004 and from the beginning of February to beginning of May, 2005 (Fig. 2).

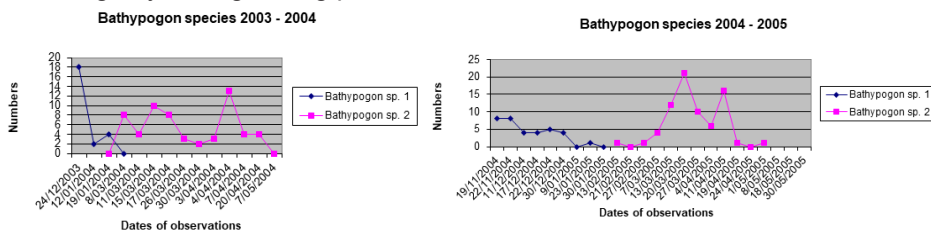
Two New Species of Bathypogon: gerhardi and monartoensis



Fig. 1. Site in Monarto Zoological Park, S.A. where *Bathypogon gerhardi* and *B. monartoensis* were studied. Photo: R. Lavigne.

***Bathypogon gerhardi* Lavigne, sp. nov.**

Diagnosis: *Bathypogon gerhardi* is a medium sized asilid characterized by having the third antennal segment short and broad, a cream coloured mystax, hind femora black and the posterior margin of sternite 7 expanded with a single row of backward directed elongate setae. The genitalia are compact, the hypandrium being smooth, and lacking any distinguishing protrusions.



black, broad, short (0.28 mm), 4th segment short (0.10 mm), narrow, black, elongate with very short apical spine. Face strongly white pruinose. Facial gibbosity yellowish, almost reaching base of antennae; mystax contains 14-16 stout cream coloured setae. Frons and vertex dull whitish pruinose; single row of white setae laterally on frons; 4 ocellar bristles elongate, proclinate, brown. Occiput: narrow white pollinose stripe bordering eye with row of white setae, otherwise black; dorsal black, 5-6 postocular setae behind each eye. Palpi shining black covered with elongate setae; one segmented. Proboscis shiny black with pale short white setae at apex and longer white setae below for 2/3rd length from base, downwardly directed.

Thorax: Mesonotum brownish, but bordered dorsolaterally by yellowish grey pruinosity from humerus to base of scutellum. Mesonotum largely bare dorsally except for brownish medium pollinose stripe upon which are two parallel rows of tiny black setae. Antepronotum with row of stout brownish white bristles centrally. Postpronotum with multiple very thin setae. Humerus brownish grey pruinose with short reddish brown very thin setae. The elongate dorsal thoracic bristles are disposed on each side as follows: Three pairs of elongate black, dorsocentral bristles not extending anterior to transverse suture. Mesonotal bristles stout, elongate, black and curved at tips: 1 posthumeral bristle, 2 presutural bristles, 1 supra-alar bristle, 2 post-alar bristles. Katatergite with 20+ thin brownish white setae crinkled at apex. Apex of mesonotum squared off. Pleura mostly grey pollinose, bicoloured, darkened on anterior side of anepisternum and katepisternum; patch of thin white setae on posterior corner of katepisternum. Scutellar disk with dense yellowish grey tomentum; 4 long, apically curved black marginal setae the interior pair crossing at tips.

Wings: Hyaline, discal x-vein at 3/4th length of discal cell, wing tips usually not extending beyond apex of 4th abdominal segment; Haltere brown, knob brown.

Legs: Legs covered with dense short, white pile. Fore and mid coxae with dense patch of stout dirty white bristles intermixed with elongate thin, white setae on anterior surface; hind coxae with 2 stout white bristles. Fore and mid femora black anteriorly, brown posteriorly, hind femora black; fore femur with 1 stout, dirty reddish brown bristle laterally at 5/8 distance from base and 2 subapically; middle femur with 2 stout, reddish brown, subapical bristles anteriorly; hind femur with 4 stout white bristles in a row laterally on outer surface and 2 subapically. Fore and mid tibia black anteriorly, brown posteriorly, hind tibiae black; tibiae with 3 rows of 3-4 stout bristles plus 2 white subapical setae; bristles are mixed white and reddish brown. Tarsi: basal tarsal segment elongate, subequal to segments 2-4 combined; tarsal segment 5 elongate; stout, both short and long setae ventrally on tarsal segments 1-5. Claws bicolored, mostly black, brown only at base; pulvilli, cream coloured, and empodia, brown, as long as claws.

Abdomen: Tergites brown dorsally, grey tomentum laterally on segments 1-4. Tergite 1 with patch of 3-4 stout, dirty white bristles amidst elongate, thin hairs dorsolaterally and multiple dorsomedial straight dirty white backwardly directed setae/hairs. Brownish white short setae widely distributed on tergites 1-7. Posterior margin of sternite 7 expanded with a single row of backward directed elongate setae. Hairs on sternites 2-4 elongate, white.

Two New Species of Bathypogon: gerhardi and monartoensis

Genitalia: (Figs. 5-6) Epandria black, elongate, pear-shaped, dorsally with multiple backwardly directed curved elongate light brown setae that are almost as long as the epandrium, double row of stout setae at point where epandria expands. Cerci not protruding but held between epandria, Gonocoxite broadly triangular, pointed apically, gonostylus narrow, knife-like at apex, inwardly directed but protruding beyond tip of gonocoxite. Hypandrium simple with no enhancements. Aedeagus single tubed, short.



Fig. 5. Lateral view of male terminalia of *Bathypogon gerhardi*, illustrating the flattened circular epandrium (SAMA 29-001213). Photo: A. McArthur and R. Lavigne.

Females: Body length: Range 13.5-15 mm. Average = 14.23 mm (n = 4). Similar to the male except that 3rd segment of antennae more elongate; visible 7th tergite 2 ½ times length of visible 8th tergite, grey pollinose, bearing 5 visible pairs of long apically rounded brown acanthophorite spurs; pads beneath acanthophorite spurs with dense sensory hairs (Fig. 7).

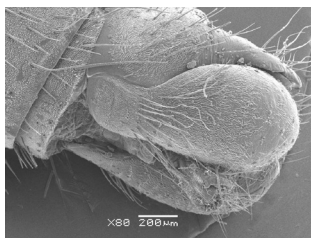


Fig. 6. SEM photo of genitalia of *Bathypogon gerhardi* illustrating shape of epandrium and the double row of setae near the base. Photo: Dr. Z. Suludere, Gazi University, Ankara, Turkey



Fig. 7. Lateral view of female *Bathypogon gerhardi* sp. nov. (SAMA 29-001847) Photo: G. Weber and R. Lavigne.

Material examined: Type data: Holotype male. Body length: 12.4 mm (Fig. 3). South Australia. Monarto/ Zoological Park/ 35°06'45"S, 139°08'51"E/ 22/12/04/ Mallee scrub R. J. Lavigne, Coll., SAMA Database No. 29-001214.

Paratypes: 1M S. Aust. Monarto/ Zoological Park/ 35°06'45"S, 139°08'51"E/ 17/12/04/ Mallee scrub R. J. Lavigne, Coll., SAMA Database No. 29-001213. 1F: same data as 1st paratype, SAMA Database No. 29-001216; 1M: same data as 1st paratype, except 22/11/04, SAMA Database No. 29-001215; 1F:

same data as 1st paratype, except 19/11/04, SAMA Database No. 29-001218; 1M: same data as 1st paratype, except 05/11/05, SAMA Database No. 29-001846; 1F: same data as 1st paratype, except 22/11/05, SAMA Database No. 29-001847.

Etymology: The species is named after Mr. Gerhard Weber, an ardent observer of Asilid behavior, amateur photographer, and occasional co-author who has generously given of his time to produce large numbers of photographs of asilids belonging to the South Australian Museum over the past several years.

Depository: The holotype and 7 paratypes of *B. gerhardi* are deposited in the South Australian Museum (SAMA) Entomology Collection in Adelaide.

***Bathypogon gerhardi* Lavigne, sp.nov**

Distribution: This new species has only been studied in Area 1 (GPS: 35°06'45"S, 139°08'51"E) in Monarto Zoological Park, but has been found also at a farm in Loxton SA, at Point Davenport SA on Yorke Peninsula and at Vivonne Bay on Kangaroo Island. Additionally it has been recorded one km E of One Tree Hill, Elizabeth SA [as sp. A] by G. Daniels based on material collected by the author in 1979.

Resting Behavior: Based on 118 observations, specimens of *B. gerhardi* were noted resting on sand 19% of the time, on small rocks 36% of the time, on dead twigs 23% of the time, on low growing vegetation 14% of the time and on litter 8% of the time. Specimens rested on these substrates at heights ranging from 2.5-15 cm, where temperatures varied from 33 to 41° C.

Perch sites, from which *B. gerhardi* launched attacks on potential prey, varied: from sand 19%; from small rocks (3x3x3 cm to 8x10x2.5cm) 36%, from litter 8%; from dead twigs 23%; and from vegetation 14% (Fig. 8).

Grooming: Cleaning of body parts occurred intermittently and was accomplished primarily with the fore tarsi. However, cleaning usually followed feeding.

Flight patterns: Flight heights varied presumably depending on the pending activity and ranged from 4-30 cm (mean - 11.5 cm)

Orientation Flights: Orientation flights were flights of 15-60 cm involving flitting from rock to rock and usually occurred when the asilid was disturbed, primarily by ants.

Foraging Flights: While resting on foraging sites, the flies were largely quiescent, except when potential prey flew within their field of vision. The asilid would then turn its whole body to face the organism. One can assume that such postural changes increase range of vision and place the asilid in a suitable position to make a direct forage flight (Dennis and Lavigne, 1975). All forage flights were directed at insects that were air borne. Distances covered in forage flights ranged from 7.6 to 71 cm (mean 25.6 cm). Flights which resulted in prey capture ranged in length from 7.6 to 45.7 cm.

Prey Selection: Collected prey of *Bathypogon gerhardi*

Prey were usually collected by the author at the cessation of feeding. All of the collected prey were less than half the size of their predator. Because of the small size of many of the prey selected, many could not be collected before the wind blew

Two New Species of Bathypogon: gerhardi and monartoensis

them away. Prey were varied. Four orders and 6 families of insects were represented in this very small sample suggesting that *B. gerhardi* is an opportunistic predator.

Prey selected: Diptera: Muscidae: bush fly [*Musca vetustissima* Walker] ♀, ♀, ♂ (Fig. 9), Coleoptera: Curculionidae: *Sitona discoideus* Gyll., Coleoptera: Scarabaeidae: *Liparetrus rothei* Blackburn, Coleoptera: Scarabaeidae: *Liparetrus laeticulus* Blackburn (2), Hemiptera: Lygaeidae: *Nysius vinitor* Bergroth (2), Hymenoptera: Tiny wasp -unidentified, Additional prey: Tiny prey (7) (unknown - blew away)



Figs. 8-9. *Bathypogon gerhardi* S. A. Photos: R. Lavigne 8. Male resting on debris in Monarto Zoological Park. 9. Female with bush fly, *Musca vetustissima* Walker, as prey in Monarto Zoological Park.

Manipulation: During feeding, *B. gerhardi* may manipulate prey. In this process, the robber fly raises its head and thorax and adjusts the prey with its front and hind tarsi using the mid legs as stabilizers similar to the manner of *Lasiopogon cinereus* (Cole) [Lavigne and Holland 1969] and *B. monartoensis*. Manipulation of prey was observed five times during the study.

Mating Behavior: The position taken by mated pairs was 'tail to tail'; initiation of mating was not observed, but is presumed to be similar to that carried out by *B. monartoensis* Lavigne noted later in this paper. Mated pairs of *B. gerhardi* were observed three times in 2004 and twice in 2005 (22/11/04; 11/12/04; 30/12/04; 22/11/05; 07/12/05) (Fig. 10) when temperatures on the substrate were 32-35° C. In two instances the female was feeding on prey.



Figs. 10-11. *Bathypogon gerhardi* in Monarto Zoological Park, S. A. Photo: R. Lavigne 10. Mated pair resting on small rock 11. Female ovipositing in soil.

Oviposition Behavior: The female tests potential oviposition sites by tapping the tip of her abdomen on the surface of the soil prior to inserting her abdomen into the soil. Eggs are deposited singly but glued together. Once the eggs are deposited in a clutch she removes her abdomen and sweeps dirt into the hole. Three of the observations of female ovipositions (Fig. 11) were made in a single day [dates of occurrence: 19/11/04; 07/12/05 (3)] when temperatures on the substrate were 32-35°

C. In one instance eggs were recovered; eggs were cemented together in a packet of seven (Fig. 12); soil temperature was 46° C.



Fig. 12. Egg packet deposited by female *Bathypogon gerhardi* in Monarto Zoological Park, S. A. Photo: R. Lavigne.

***Bathypogon monartoensis* Lavigne**

Diagnosis: *Bathypogon monartoensis* is a medium sized asilid characterized by having the third antennal segment broad, a mystax with black bristles centrally, hind femora bi-coloured and the genitalia with multiple backwardly directed curved elongate light brown setae, that are almost as long as the epandrium. The hypandrium is yellowish brown, with paired ventromedial spurs.

Description: *Bathypogon monartoensis* Lavigne (Figs. 13-14) Monarto/ Zoological Park/ 35°06'21"S 139°08'51"E. Length of Holotype Male - 13.9 mm.



Figs. 13-14. Male *Bathypogon monartoensis* sp. nov. (SAMA 29-001226) Photo: G. Weber and R. Lavigne. 13. Lateral view. 14. Dorsal view.

Range for additional ♂ s: 13 - 15 mm. Average = 14.02 mm (n= 6).

Head: Antennae bi-colored, 4 segmented with apical spine: scape orange with multiple short white setae below, pedicel brown with two white setae above and a three or four white setae below, scape, elongate (0.26 mm), pedicel darker orange (0.14 mm) , 3rd segment black, elongate (0.50 mm), 4th segment short (0.06 mm), narrow, black, elongate with very short apical spine. Face strongly white pruinose. Facial gibbosity orange basally reaching $\frac{3}{4}$ distance to base of antennae; mystax contains 7-8 central dark brown slightly curved bristles surrounded by 20+ slender curved whitish setae. Frons and vertex greyish pruinose; single row of black setae laterally on frons; 2 ocellar bristles elongate, erect, black. Occiput: yellowish grey pollinose; 10 dorsal black, postocular setae behind each eye. Palpi shining black with multiple whitish setae at apex; one segmented. Proboscis shiny black with pale short white setae at apex and longer white setae below on basal half, downwardly directed. Beard white haired.

Two New Species of Bathypogon: gerhardi and monartoensis

Thorax: Mesonotum brownish, but bordered dorsolaterally by whitish pruinosity from humerus to base of scutellum. Mesonotum with pair of central narrow dark brownish stripes; central portion of mesonotum covered with tiny black setae. Antepronotum with row of stout dark brown bristles centrally. Postpronotum bare, black with fairly dense dirty white hair. Humerus greyish whitish pruinose with very short mixed black and whitish setae. Proepisternum with 2 stout white bristles amidst white hair. The elongate dorsal thoracic bristles are disposed on each side as follows: Four pair of elongate black, dorsocentral bristles, not extending anterior to transverse suture. Mesonotal bristles stout, elongate, black and curved at tips: 1 posthumeral bristle, 2 presutural bristles, 1 supra-alar bristle, 2 strongly curved post-alar bristles. Pleura mixed grey and brown pollinose, anepimeron with patch of thin white setae on posterior half. Scutellar disk with whitish tomentum on corners, darkened in middle with multiple long white hairs; 4 long, apically curved black marginal setae, the interior pair crossing midway.

Wings: Hyaline except microtrichiae occur along veins giving wings a brown aspect, discal x-vein at approximately 3/4th length of discal cell; wing tips extending to apex of 5th abdominal segment. Haltere stem light brown, knob light brown.

Legs: Legs covered with dense short, white pile. Fore and mid coxae with dense patch of stout dirty white bristles intermixed with elongate thin, white setae on anterior surface; hind coxae with 2 stout white bristles. Femora black anteriorly, brown posteriorly; fore femur with 1 stout, dirty reddish brown bristle laterally at 5/8 distance from base and 1 subapically; middle femur with 3 stout, reddish brown, subapical bristles; hind femur with 4 stout brown bristles in a row laterally on outer surface and 2 subapically. Fore and middle tibia light brown with 10-12 mixed light and dark brown setae plus subapical setae. Hind tibia dark brown with 3 rows of 3 stout reddish brown bristles plus 2 white and 1 brown subapical setae. Tarsi: basal tarsal segment elongate, subequal to segments 2-4 combined; tarsal segment 5 elongate; short white setae with multiple elongate mixed reddish brown and white bristles ventrally on tarsal segments 1-5. Claws bicolored, mostly black, brown only at base; pulvilli, cream coloured, and empodia, brown, nearly as long as claws.

Abdomen: Thin pollinose line dorsolaterally on segments 1-6. Tergite 1 with 3-4 stout, dirty white setae dorsolaterally and a row of straight dirty white backwardly directed setae/hairs dorsomedially. Short cream coloured setae widely distributed on tergites 1-7. Hairs on sternites 2-4 elongate, white. Patch of white setae laterally on sternite 7.

Genitalia (Fig. 15-16): Epandria blackish, reddish brown at base, broadly rounded, dorsally with multiple backwardly directed curved elongate light brown setae, that are almost as long as the epandrium; black setae at apex exceptionally long. Cerci elongate protruding between epandria and heavily setose apically. Gonocoxite broadly triangular, apically becoming a short finger-like structure, gonostylus narrow, knife-like at apex, inwardly directed but protruding beyond tip of gonocoxite. Hypandrium yellowish brown, with paired ventromedial spurs. Aedeagus single tubed, short.

Similar to the male except that: a/ 8th tergite prominent, grey pollinose, slightly longer than 7th, bearing 4 visible pairs of long apically rounded brown acanthophorite spurs; pads beneath acanthophorite spurs with dense marginal hairs.



Fig. 15. Lateral view of male terminalia of *Bathypogon monartoensis*, illustrating the elongate curved setae arising from the epandrium (SAMA 29-001226). Photo: A. McArthur and R. Lavigne.

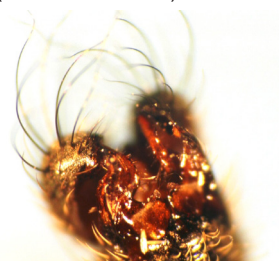


Fig. 16. Ventral view of male terminalia of *Bathypogon monartoensis* illustrating one type of physical enhancement on the hypandrium. Photo: A. McArthur and R. Lavigne.

Females: Body length: Range 13.8-16.2 mm. Average = 14.9 mm (n = 5). (Fig. 17)



Fig. 17. Lateral view of female *Bathypogon monartoensis* sp. nov. . (SAMA 29-001225) Photo: G. Weber and R. Lavigne.

Material Examined

Type data: *Holotype* male. Body length: 13.9 mm (Fig. 13). S. AUST. Monarto/ Zoological Park/ 35°06'45"S 139°08'51"E / 04/04/04 /R. J. Lavigne, Coll, 2nd label: SAMA Database No 29-001226.

Paratypes: 1M S. AUST. Monarto/ Zoological Park/ 35°06'45"S 139°08'51"E/ 08/03/04/ Mallee scrub R. J. Lavigne, Coll., SAMA Database No 29-001219; 1M: same data as 1st paratype, except 26/03/04 SAMA Database No 29-001220; 1M: same data as 1st paratype, except 20/04/04, SAMA Database No 29-001221; 1F: same data as 1st paratype, except 11/03/04, SAMA Database No 29-00122; 1F: same data as 1st paratype, except 30/03/04, SAMA Database No 29-001223; 2F: same data as 1st paratype, except 04/04/04, SAMA Database No 29-001224, No 29-001225; 1M: same data as 1st paratype, except 04/04/04, SAMA Database No 29-001226; 1F: same data as 1st paratype, except 03/04/05, SAMA Database No 29-001227; 2M: same data as 1st paratype, except 04/04/05, SAMA Database No 29-001228, No 29-001229; 1M: same data as 1st paratype, except 13/02/05, SAMA Database No 29-001230; 1M: same data as 1st paratype, except 11/04/05, SAMA Database No 29-001231; 1M: same data as 1st paratype, except 30/03/04, SAMA Database No 29-001232 (genitalia in glycerine).

Etymology: The species is named after Monarto Zoological Park where the new species was studied.

Two New Species of Bathypogon: gerhardi and monartoensis

Depository: The holotype and 12 paratypes of *B. monartoensis* are deposited in the South Australian Museum (SAMA) Entomology Collection in Adelaide.

Distribution: This new species has been studied only in Area 1 (GPS: 35°06'45"S, 139°08'51"E) in Monarto Zoological Park; in the same area as *B. gerhardi*, but occurs in the early months of the year.

Habitat: Same as for *Bathypogon gerhardi*.

Resting Behavior: Based on 184 observations, specimens of *B. monartoensis* were noted resting on sand 53% of the time, on small rocks 21% of the time, on dead twigs 23% of the time, on low growing succulents 7% of the time, on litter 14% of the time and on dead branches 3% (Fig. 18). Specimens rested on substrates at heights ranging from 2.5-15 cm.

½ hour observation of female *Bathypogon monartoensis* 08/03/04: "- 1:34 pm - ♀ *Bathypogon* (sp. 2) id OK landed on dead branch 2" above soil - broadside to sun; 1:35 reacted to tiny ant scurrying (5" away) and turned to face it; 30 sec later it turned back (terminal segments reddish brown - same color as red sand); 1:41 - reacted to flying insect 3' away - turned to face and then back, but up on extended legs; 1:44 turned 180° - still with legs extended (slight breeze); 1:49 - turned to face fast flying insect 1' above and then took original position broadside to sun - ignores moving shade provided by leaves in tree being blown by wind; 1:52 - crouched facing away from the sun; 1:53 - up and down (in position); 1:56 - now completely in shade provided by tree leaves overhead, turned and aligned self in direction of length of branch; 2:02 mostly in shade - turned broadside to sun; 2:05 (collected female) - temp at height in shade -92 °F, 33 °C."

Flight patterns: Orientation flights varied in length from 1-3m.

Foraging Flights performed by this species were on a diagonal tract ranging in length from 10 cm to 1.5m. When movement is observed, the whole body is turned to face potential prey.

They tended to make rapid semi-circular flights of 1m when disturbed.

Prey Selection: Prey were usually collected by the author at the cessation of feeding. Most of the collected prey were less than half the size of their predator. Because of the small size of many of the prey selected, many could not be collected before the wind blew them away. Prey were varied. Five orders of insects were represented in this small sample suggesting that *B. monartoensis* is an opportunistic predator.

Collected prey of *B. monartoensis*: Coleoptera: Carabidae: *Adelotopus* sp., Diptera: Asilidae: ♂ *Stichopogon davfergusi* Lavigne and McAlister, Muscidae: *Musca vetustissima* Walker; Tabanidae: unidentified, Hymenoptera: Formicidae: *Iridomyrmex* sp. (winged reproductives -3): tiny flying ant, tiny flying ant or wasp, tiny black wasp, tiny wasp, about size of asilid's thorax (4), Hemiptera: Lygaeidae: *Nysius vinitor* Bergroth (2); Pentatomidae: *Kapunda troughtoni* (Distant), Lepidoptera: microlepidoptera (5) (Fig. 19), Tiny unidentified prey (5).



Figs. 18-19. *Bathypogon monartoensis* in Monarto Zoological Park, S. A. Photo: R. Lavigne. 18. resting on sand 19. Female with microlepidoptera as prey.

On two occasions, a *B. monartoensis* flew towards a honey bee, but turned away before making contact. While feeding, the asilids were constantly disturbed by foraging ants and moved to new locations.

Manipulation: During feeding, *B. monartoensis* may manipulate the prey. In this process, the robber fly raises its head and thorax and adjusts the prey with its front and hind tarsi using the mid legs as stabilizers in a manner similar to that exhibited by *Lasiopogon cinereus* (Cole) [Lavigne and Holland 1969] and *B. gerhardi*. Manipulation of prey was observed 12 times during the study.

Mating Behavior: Matings of *B. monartoensis* are initiated in air when a male encounters a female and the pair land on the substrate. Matings are short. Four complete matings were observed [11:53-12:06, 1:21-1:29, 1:35-1:43, 1:50-1:57] (Fig. 20) when temperatures on the substrate were 31-38° C. Additional partial mating times were 6 min., 9 min. and 8 minutes. The earliest mating observed was at 9:48 am and the latest at 2:58 pm. Upon completion of mating the male releases his claspers and flies off. In two instances a mated female was observed to have prey. In one instance a second male attacked a mated pair. Male to male encounters were observed twice suggesting that males are unable to recognize females until contact is made.



Fig. 20. *Bathypogon monartoensis* mated pair resting on small rocks in Monarto Zoological Park, Monarto, S. A. Photo: R. Lavigne

Oviposition Behavior: Pre-oviposition by *B. monartoensis* was only observed twice, once on 15/03/2004 and again on 20/03/2005. Pre-oviposition behavior was the same as that for *B. gerhardi*. Based on pre-oviposition behavior, eggs are deposited in the soil although no eggs of this species were recovered. In preparation for oviposition a female would test the soil with her ovipositor, moving from one spot to another over an area approximating 900 square cm. Upon finding a 'suitable' site, the female would

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sink her ovipositor into the soil to a depth of 5-6 abdominal segments. If not disturbed, her ovipositor would remain embedded in the soil for approximately 7-8 min. Once she completed oviposition, she would remove her ovipositor and sweep soil into the hole with the tip of her ovipositor.

Cleaning Behavior: Cleaning behavior occurred following feeding; sometimes a feeding asilid would go through a cleaning sequence. However asilids would often exhibit cleaning behavior while resting on a substrate or while in copula. The sequence varied, but usually began with the cleaning of the eyes with the fore tarsi or the rubbing together of the fore tarsi. A typical sequence occurred as follows: 20/04/04 "12:01pm: sp.2 after feeding cleaned fore tarsi, then hind tarsi; fore tarsi, fore tarsi, eyes, fore tarsi; eyes, fore tarsi; facing sun while sitting on sand straddling former prey; cleaned fore tarsi again; 12:02 - cleaned fore tarsi again while still crouched over prey; cleaned fore tarsi again."

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Influence of Dietary Titanium Dioxide Nanoparticles on the Biology and Antioxidant System of Model Insect, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae)

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ABSTRACT

The potential toxic effects of the widespread use of titanium dioxide (TiO₂) nanoparticles (NPs) on insects have been brought into question as their presence in the ecosystem is unavoidable. Hence, the toxic effects of the different TiO₂ NPs should be investigated by establishing experimental model insects. Here, we examined the effects of different concentrations of TiO₂ NPs (100, 500, 1000, 3000 and 5000 ppm) on the biological parameters and total protein amount, antioxidant enzyme activities, malondialdehyde (MDA) amounts in the hemolymph of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). We found that larval and pupal developmental times significantly increased at 100, 500, 1000 and 3000 ppm when compared with control and highest doses of TiO₂ NPs. However, adult longevity time was shortened at low concentrations of dietary TiO₂ NPs (100, 500 and 1000 ppm). Exposure with dietary TiO₂ NPs caused a significant increase in the total protein amount and content of MDA and glutathione S-transferase activity in the hemolymph at 100, 500 and 1000 ppm compared with control and other doses of TiO₂ NPs. While the activity of catalase increased by 1000, 3000, and 5000 ppm and superoxide dismutase activity increased at all doses of TiO₂ NPs when compared with control. Our results indicated that TiO₂ NPs has a dose-dependent toxic effects on the *G. mellonella* larvae and can enhance the stress resistant capacity of insects at low concentrations.

Key words: Titanium dioxide nanoparticles, *Galleria mellonella*, nanotoxicology, antioxidant enzymes, model insect.

INTRODUCTION

The use of synthetic nanoparticles (NPs) in science and industry has been growing enormously in various commercially products because of their biological, chemical, and physical aspects which enable them to be an indispensable element for the technology. The most fascinating feature of these particles lies in their relatively smaller size and larger surface area. The word “nano”, derived from the Greek nanos which means dwarf, includes natural or manmade materials that are between 1 to 100 nm and NPs occur naturally in the environment (Buzea *et al.*, 2007). NPs exhibit different behaviors than the materials that have the same composition, but a larger size. Two primary factors, such as surface and quantum effects, cause NPs to behave differently than bulk materials (Roduner, 2006; Buzea *et al.*, 2007; Handy *et al.*, 2008). These factors affect the material’s chemical reactivity along with mechanic, optic, electric, and magnetic properties (Buzea *et al.*, 2007). However, the potential toxic effects of the widespread use of synthetic NPs on living organisms have been brought into question as their presence in the ecosystem is inevitable given their extensive use in science and industry. Thus, this situation led to the emergence of the new term “nanotoxicity” in recent decades. Studies into the toxicological effects of NPs on living organisms (Klaper *et al.*, 2009; Chakravarthy *et al.*, 2012; Karthigarani and Navaraj, 2012; Li *et al.*, 2012a, b; 2016; Pelclova *et al.*, 2017) have been published but are still needed to continue for insects.

Here, our concern is about titanium dioxide (TiO₂) NPs, which exists in the form of white powders and are widely used in electronics (Robertson *et al.*, 2010), cosmetics (Hu *et al.*, 2010), water treatment (Lachheb *et al.*, 2002), antibacterial products (Yu *et al.*, 2007), self-cleaning (Carneiro *et al.*, 2007), and air cleaning applications (Yu *et al.*, 2007). TiO₂ NPs also used as a white pigment because it has a high refractive index (Ortlieb, 2010). According to particle size, purity, surface area and characteristics, crystalline shape, and chemical reactivity, several TiO₂ NPs are being produced today. Hence, adverse effects of TiO₂ NPs should be investigated by establishing experimental models to study their toxicity to environmentally relevant species. Some studies have been conducted relating to *in vivo* and *in vitro* effects of TiO₂ NPs on human and animals (Hussain *et al.*, 2005; Kannan *et al.*, 2011; Chakravarthy *et al.*, 2012; Dalai *et al.*, 2013; Memarizadeh *et al.*, 2014; Pelclova *et al.*, 2017). In brief, these studies showed that TiO₂ NPs caused adverse effects such as cytotoxicity, inflammation, and oxidative stress. However, TiO₂ NPs can be toxic or nontoxic depend on high or low concentrations on bio-organisms in particular insect (Hussain *et al.*, 2005; Li *et al.*, 2012a, b, 2014, 2016; Dalai *et al.* 2013; Zhang *et al.*, 2014; Wang *et al.*, 2015). Wang *et al.* (2007) showed that different particle sizes of TiO₂ NPs (25 and 80 nm) accumulated in the liver, spleen, kidney, and lungs in mice and caused oxidative stress due to oral administration. Zhang *et al.* (2014) determined that feeding silkworm *B. mori* with TiO₂ NPs at low concentrations increased the feed efficiency of larval stage and increased the activities of trehalase, protease, and lipase. However a recent study found that high concentrations of TiO₂ NPs were toxic on the silkworm (Li *et al.*, 2016). Li *et al.* (2012a) investigated *Bombyx mori* L. (Lepidoptera: Bombycidae)

nuclear polyhedrosis virus (BmNPV) resistance in insects after TiO₂ NPs exposure. Interestingly, TiO₂ NPs provided a decrease in reactive oxygen species (ROS) and caused an accumulation of nitric oxide (NO). In another study, Li *et al.*, (2012b) reported that TiO₂ NPs added to the diet at 5mg L⁻¹, significantly decreased biochemical dysfunctions in the hemolymph of fifth instar larvae of silkworms following exposure to phoxim insecticide. Similarly, Wang *et al.* (2015) showed that pretreatment with nano-TiO₂ attenuated the phoxim-induced midgut injury, increased body weight and survival, and decreased oxidative stress in the midgut of *B. mori*. On the other hand, Dalai *et al.* (2013) examined the toxicity of TiO₂ NPs (<25 nm) on *Ceriodaphnia dubia* (Cladocera: Daphniidae) and they reported that mortality increased at high doses. Furthermore, the expressions of the genes, including superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) were increased.

The latter results formed the basis of our current study concerning the biological and biochemical effects of TiO₂ NPs on the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). *G. mellonella* is found almost everywhere on earth and it is a pest for beekeeping. It is also used as a host for rearing biological control agents and evaluated in many different physiologic and toxicology studies owing to the various advantages of *G. mellonella* larvae for providing a model system in the laboratory (Dere *et al.*, 2015; Altuntaş *et al.*, 2016; Maguire *et al.*, 2016). It is also known that *G. mellonella* is an excellent model organism which can be used instead of mammalian species for *in vivo* toxicity and pathogenicity studies (Desbois and Coote, 2011; Maguire *et al.*, 2016). For this reason, the nanotoxicity studies that are conducted on *G. mellonella* will help determine the possible effects on ecosystem and humans. Therefore, we aimed to determine the effects of dietary TiO₂ NPs on the life parameters, total protein amount, CAT, SOD and GST activities, and the content of malondialdehyde (MDA) of the *G. mellonella*.

MATERIALS AND METHODS

Insect culture

Galleria mellonella culture was prepared in glass jars (1 L) with honeycomb in the laboratory at 27 ± 1°C, 60 ± 5% RH, and constant darkness. Continuity of the culture was provided to emerge adult insects and newly hatched larvae.

Characterization and preparation of TiO₂ NPs

The sizes of TiO₂ NPs (Sigma-Aldrich Co.) were tested at The Scientific and Technological Research Council of Turkey (TUBITAK) by using a high resolution transmission electron microscope (model no: HRTEM, JEOL 2100) and scanning electron microscope (model no: JEOL/JSM-6510LV-INCA/EDS). The Zeta potential value of the particles was determined by using ZetaSizer Nano ZS (Malvern Instruments Inc., UK). Different concentrations of TiO₂ NPs (100, 500, 1000, 3000, and 5000 ppm) were added into double distilled water and treated with an ultrasonic homogenizer (amp: 60%, 15 min, Bandelin Sonoplus, HD 3200, Berlin, Germany) for the dispersion, stabilization, and preparation of the stock solution.

Biological assay

For bioassays, 1 ml of TiO_2 NPs at different concentrations of 100, 500, 1000, 3000, and 5000 ppm was sprayed into 1 gr of powderized honeycomb as a diet. Thus, TiO_2 NPs were easily attached on the surface of honeycomb. Then, the diet was left to dry for 1 day. Honeycomb in double distilled water was used for the control group. First instars of *G. mellonella* larvae were transferred individually into each sterile petri dishes (90 x 15 mm) with diet containing TiO_2 NPs, whereas diet without TiO_2 NPs served as a control. Development of first instar larvae was monitored daily until the last instars to determine the larval developmental time. Each last instars were also observed daily until pupation to determine the pupal developmental time. The time required for completion of pupal stage was recorded as the time of adult emergence per pupae. Adult longevity time (day) was also recorded in treated with different doses of dietary TiO_2 NPs and untreated groups. Each biological assay was replicated three times with twenty first instar larvae, selected from different populations at different times.

Hemolymph collection

The amount of total protein, MDA and the activity of antioxidant enzymes were determined in the treated with different doses of dietary TiO_2 NPs and untreated larval hemolymph. For this purpose, hemolymph samples were collected from the last instars of *G. mellonella*. Before the hemolymph collection procedure, larvae were anaesthetized on ice for 10 minutes and sterilized with cotton including 70% ethanol. Subsequently, ten microliters of hemolymph from each individual larva were collected with a 10- μL glass micro capillary tube (Sigma, St. Louis, MO) and transferred to a micro centrifuge tube (1.5 ml) placed on ice. Hemolymph samples were immediately mixed with a cold homogenization buffer (1:2 v/v) and 0.001 mg 1-phenyl-2-thiourea was added to each tube to avoid hemocyte aggregation. The samples were immediately stored at -80°C until assays. Before all analyses, samples were homogenized according to Dere *et al.*, (2015). Ten *G. mellonella* larvae (0.16 ± 0.01 g) were evaluated for each experimental and control assays in four replicates ($n = 40$ per treatment).

Total protein and MDA amount

The total protein amount in larval hemolymph was determined according to Bradford's method (Bradford, 1976) using bovine serum albumin for the standard curve and analyzed with a microtiter plate (SpectraMax M2) at 595 nm. The MDA content in larval hemolymph was determined by using a commercial test kit (Cayman Chemicals Co., 10009055, USA). This test was assayed according to Dere *et al.*, (2015). In this method, 25 μL sodium dodecyl sulfate and coloring reagent, (TBA acetic acid, TBA sodium hydroxide), were added to 25 μL hemolymph and the mixture was boiled for 1 hour. It was then left on ice for 10 min. After cooling, the mixture was centrifuged at 1600xg, 10 min, 4°C . 150 μL supernatant was used to determine the content of MDA. Absorbance was measured at 532 nm with microtiter plate (SpectraMax M2) and the content of MDA was expressed as nmol/mg protein by using the extinction coefficient $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

CAT activity

CAT (EC 1.11.1.6) activity in hemolymph was assayed using Dere *et al.*, (2015) method. In this method, a certain amount of phosphate buffer and hydrogen peroxide (H_2O_2) were added to hemolysate and analyzed for 3 min at 240 nm. Absorbance values were detected with an ultraviolet-visible spectrophotometer (Shimadzu UV-1601, Tokyo, Japan). Specific CAT activity was determined as the amount of decomposition of 1 mmol of H_2O_2 to water and oxygen per min per mg protein using the extinction coefficient value ($\epsilon_{240} = 0.0394 \text{ mM}^{-1}\text{cm}^{-1}$).

SOD activity

SOD (EC 1.15.1.1.) activity was analyzed according to assay kit, following the manufacturer's protocols (Cayman Chemical Co., 706002, USA). According to the protocol, SOD activity was determined by 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) reacting with superoxide radicals, using xanthine and xanthine oxidase (XOD) at 450 nm (Dere *et al.*, 2015). Absorbance was read by a 96-well microtiter plate (Spectra Max M2). SOD enzyme activity was expressed as U/mg protein.

GST activity

GST (EC 2.5.1.18) activity (cytosolic and microsomal) was assayed with a commercial assay kit (Cayman Chemical, 703302). According to kit protocol, an increase absorbance was detected continuously at 340 nm for 5 min related to the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (Dere *et al.*, 2015). Analyses were performed in a 96-well microtiter plate, and enzyme activities were also determined as nmol /min/mg protein using the extinction coefficient $\epsilon_{340 \text{ nm}} = 9.6 \text{ mM}^{-1}\text{cm}^{-1}$.

Statistical analysis

First, all means of analyses were checked against the normality of data distribution. Then one-way analysis of variance (ANOVA) was performed to compare means because the data were normally distributed. In addition, significant differences of means were defined using Tukey's Honestly Significant Difference (HSD) or Tamhane T2 post hoc tests according to the homogeneity of variances. SPSS software program (SPSS, version 18.0 for Windows, SPSS Science, Chicago, IL) was used for these analyses. Means were considered statistically significant when $p < 0.05$.

RESULTS**Characterization of TiO_2 NPs**

TiO_2 NPs have an anatase crystalline structure according to the X-ray diffraction pattern. The particle size of TiO_2 NPs was calculated by using the Scherrer equation. We found that the size of TiO_2 NPs is about 25 nm based on this equation. The Zeta potential value, which is a measurement of the pushing or pulling of particles on each other in aqueous medium, was also calculated for TiO_2 NPs as +26.1 millivolt

(mV). The result showed that the stability of the TiO₂ NPs are less. This value is used for the calculation of the electro-kinetic properties of metal oxide NPs in aqueous suspension. It is expressed as negative or positive mV. The value is directly affected by suspension stability, aggregation of NPs and the surface morphology of the particle. The morphology of TiO₂ NPs was also observed by using HRTEM. These analyses showed that the structural shapes of TiO₂ NPs were hexagonal.

Effects on biological parameters

Larval and pupal developmental time prolonged at all concentrations of dietary TiO₂ NPs except for doses of 5000 ppm when compared with untreated groups ($\chi^2= 8.040$, df= 5, 340, P= 0.015; $\chi^2= 16.421$, df= 5, 301, P= 0.006). However, adult longevity time was shortened at doses 100 and 500 ppm TiO₂ NPs when compared to untreated larvae. On the other hand, adult longevity did not change in higher doses of TiO₂ NPs with respect to control ($\chi^2= 13.356$, df= 5, 279, P= 0.02, Table 1).

Table 1. Biological effects of TiO₂ NPs on the *G. mellonella*.

TiO ₂ NPs (ppm)	Larval developmental time (day)	Pupal Developmental Time (day)	Adult Longevity Time (day)
0	28.2 ± 0.36c	7.5 ± 0.31c	20.3 ± 0.65 ac
100	44.8 ± 2.97a	22.4 ± 3.06a	16.9 ± 1.18 b
500	44.9 ± 3.25a	19.9 ± 2.65a	18.8 ± 0.89 bc
1000	39.0 ± 2.63b	15.8 ± 2.19b	19.0 ± 1.02 bc
3000	40.7 ± 3.03b	16.6 ± 2.73b	22.1 ± 0.81 a
5000	31.8 ± 1.75c	11.3 ± 1.62bc	19.6 ± 0.79 ac

*Average for three assays. each with 20 larvae per treatment. Means within a column followed by the same letter are not significantly different (P≥0.05. 2-independent samples test: Mann-Whitney U).

Effects on total protein and MDA amount

The total protein amount in larval hemolymph of *G. mellonella* fed with dietary different doses of TiO₂ NPs is given in Fig. 1. The amount of total protein in control was 46.66 µg/µL and did not change significantly at 3000 and 5000 ppm. The total protein amount of larval hemolymph was showed a significant increase at 100, 500 and 1000 ppm of TiO₂ NPs exposure (F= 3.870; df= 5, 18; P= 0.015) when compared with untreated larval hemolymph. The effects of dietary TiO₂ NPs on the MDA amount in larval hemolymph are also represented in Fig. 1. The amount of MDA in control was 3.05 µM. TiO₂ NPs treatment had the most significant effect on MDA level a greater than two-fold increase at 100 ppm when compared to untreated larvae (F= 29.274; df= 5, 18; P= 0.000). The same trend is also evident at 500 and 1000 ppm doses when compared to that of control. However, exposure to TiO₂ NPs in diet did not significantly change the amount of MDA in larval hemolymph at 3000 and 5000 ppm doses when compared with control.

Effects on antioxidant anzyme activities

Results relating CAT activity in hemolymph of the last instars after exposure to TiO₂ NPs in diet is given in Fig. 2. CAT activity in the hemolymph of untreated larvae

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was detected as 0.11 mmol/min/mg protein. CAT activity increased at 1000, 3000, and 5000 ppm doses whereas no statistically difference in activity were observed at 100 and 500 ppm doses of TiO₂ NPs exposure when compared with untreated larvae. Exposure to the highest dose of 5000 ppm of TiO₂ NPs in diet result in greater than four-fold increase in CAT activity in larval hemolymph when compared with control (F= 261.638; df= 5, 18; P= 0.000).

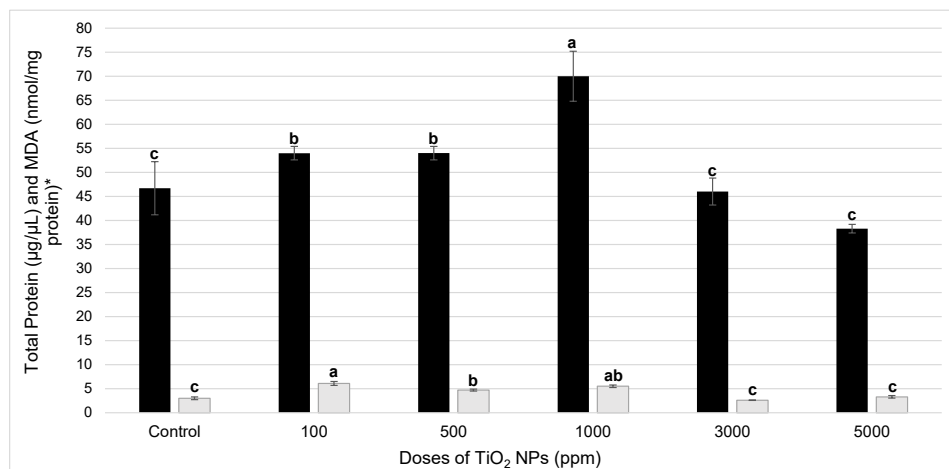


Fig. 1. Effects of TiO₂ NPs on the total protein and MDA amount in hemolymph of last instars of *G. mellonella*. *Values in the figure are Mean + SE from four replicates with 10 larvae per treatment. Different letters (a-c) indicate statistically significant differences (P<0.05; Tukey-HSD test). Black bars represent the total protein amount, grey bars represents the MDA amount.

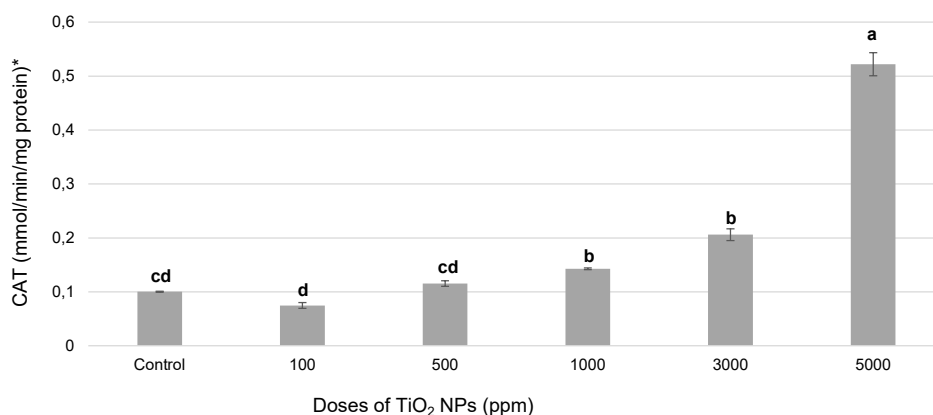


Fig. 2. Effects of TiO₂ NPs on CAT activity in hemolymph of last instars of *G. mellonella*. *Values in the figure are Mean + SE from four replicates with 10 larvae per treatment. Different letters (a-d) indicate statistically significant differences (P<0.05; Tukey-HSD test).

SOD activities associated with the exposure of dietary TiO₂ NPs doses in hemolymph of the last instar *G. mellonella* larvae is showed in Fig. 3. These results

revealed that SOD activity in larval hemolymph increased at all doses of TiO_2 NPs when compared to the untreated group. In particular, a great increase of SOD activity was determined in the hemolymph of larvae at 3000 ppm doses of TiO_2 NPs when compared with control and other doses. In contrast, a drastic decrease in SOD activity was observed in the highest dose of TiO_2 NPs with respect to other treatment doses ($F= 61.594$; $df= 5, 18$; $P= 0.000$).

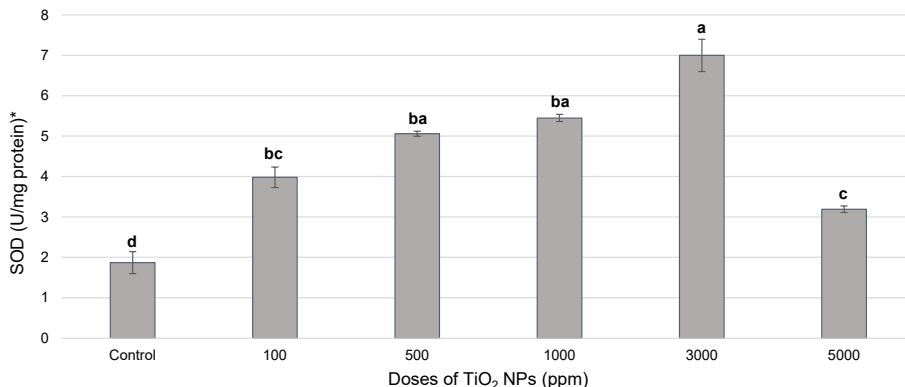


Fig. 3. Effects of TiO_2 NPs on SOD activity in hemolymph of last instars of *G. mellonella*. *Values in the figure are Mean + SE from four replicates with 10 larvae per treatment. Different letters (a-d) indicate statistically significant differences ($P<0.05$; Tukey-HSD test).

The influence of dietary TiO_2 NPs showed similar changes in total protein amount and GST activity in hemolymph of larvae (Fig. 4.). Therefore, a significant increase of GST activity was determined at 100, 500 and 1000 ppm when compared to control. However, GST activity did not change statistically in higher doses of TiO_2 NPs in larval diet when compared with untreated larvae ($F= 15.375$; $df= 5, 18$; $P= 0.000$).

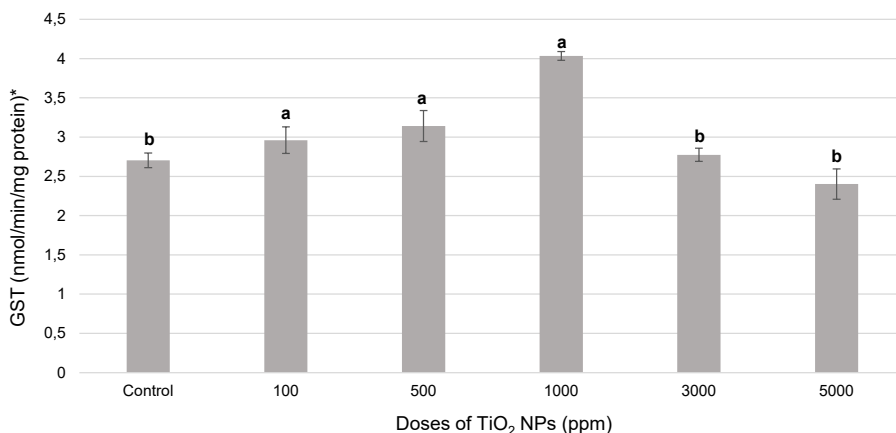


Fig. 4. Effects of TiO_2 NPs on GST activity in hemolymph of last instars of *G. mellonella*. *Values in the figure are Mean + SE from four replicates with 10 larvae per treatment. Different letters (a-c) indicate statistically significant differences ($P<0.05$; Tukey-HSD test).

DISCUSSION

Nanotoxicology has become the revolutionary subbranch of toxicology and has been gaining importance in agriculture, particularly in pest management recently. Sizes of NPs are the most effective factor for their toxic properties (Buzea *et al.*, 2007; Handy *et al.*, 2008). In principle, smaller sizes of NPs easily penetrate into the cells (Panariti *et al.*, 2012) and this effect could then cause toxicity by generating reactive oxygen species (ROS) in organisms (Gurr *et al.*, 2005; Bhattacharya *et al.*, 2008; Shukla *et al.*, 2011; Unnithan *et al.*, 2011). Reports on the toxicity of nanoparticles are now continuously growing in number (Ghosh *et al.*, 2010; Chakravarthy *et al.*, 2012; Karthigarani and Navaraj, 2012; Pelclova *et al.*, 2017). Generally, NPs show greater toxicity than their larger counterparts because of their specific particle number and surface area per unit mass (Buzea *et al.*, 2007). TiO₂ has also been classified as a possible carcinogenic by the World Health Organization (WHO) (IARC, 2010) in spite of the fact that previous studies about silkworm reported that TiO₂ was non-toxic at low doses of application into diet (Zhang *et al.*, 2014; Wang *et al.*, 2015; Li *et al.*, 2016). Zhu *et al.* (2009) also indicated that TiO₂ NPs toxicity changed in a dose-wise manner in *Daphnia magna* Straus (Cladocera: Daphniidae). Two recent studies revealed that silkworm (*B. mori*) growth and developmental time were promoted and silkworm ecdysteroidogenesis was stimulated at low doses of dietary TiO₂ NPs (Shi *et al.*, 2017). However, in the same study it was reported that high concentrations of TiO₂ NPs showed negative biological effects in *B. mori* with inhibited growth and development (Li *et al.*, 2016). Contrary to this previous study, in our study showed that low doses of dietary TiO₂ NPs prolonged larval and pupal period and shortened the adult longevity in *G. mellonella*. Also, this biological parameters of *G. mellonella* did not change at higher doses. Our findings indicated that biological effects of TiO₂ NPs may be depend on digestion and absorption process of nutrition in the midgut of *G. mellonella* larvae.

In the previous toxicity studies, high concentrations of TiO₂ NPs caused the generation of ROS in various human cells (Gurr *et al.*, 2005; Bhattacharya *et al.*, 2008; Shukla *et al.*, 2011), reproductive toxicity on the various freshwater organisms (Zhu *et al.*, 2009). Whereas in some studies showed that low concentrations of TiO₂ NPs induced protein and carbohydrate metabolisms (Li *et al.* 2012a, b, 2014) and stimulated the important resistance genes (GPx, SOD, heat shock protein 21 (HSP21)), and induced AChE activity to the support the antioxidant response (Li *et al.*, 2012a). Therefore, these previous findings are consistent with our data that the amount of total protein, MDA level, and the activities of CAT, SOD, and GST enzymes in larval hemolymph of *G. mellonella* varied depending on the concentrations of TiO₂ NPs. These results also may be related to some factors of TiO₂ NPs, such as size, surface area, particle chemistry, and crystallite structure (Buzea *et al.*, 2007). In particular the toxicity of TiO₂ NPs may be depend on their bulk form's property. Because aggregates increase the size of the NPs and cause them to exhibit microparticulate properties (Buzea *et al.*, 2007). For this reason, the zeta (ζ) potential value of TiO₂ NP is 26.1 mV indicating that the stability of the NPs in the aqueous solution can be weak and it strengthens the possibility that our material can aggregate.

Previous studies showed that low or optimum concentration of dietary TiO₂ NPs increased protein synthesis, midgut protease activity and improved the absorption and utilization of amino acids of fifth instar larva of silkworm *B. mori* (Zhang *et al.*, 2005; Li *et al.*, 2012b, 2016). Similarly, in the present study, the highest amount of protein in larval hemolymph was detected as 69,7 µg/µl at 1000 ppm of TiO₂ NPs exposure, then an insignificant decrease was detected at the two highest concentrations of 3000 and 5000 ppm when compared to control (Fig. 1). This case may be related to the adaptive response of the organism against the toxicity of TiO₂ NPs and it is likely that the amount of various proteins, such as metal binding proteins, heat shock proteins (HSPs), and metallothioneins (MTs), may increase to block toxicity. However, the conservancy in the amount of total protein at doses higher than 1000 ppm may be due to the lipoproteins that are used for healing the antioxidant damage which is created by toxic materials. It is also likely that TiO₂ NPs may cause an increase in the intracellular calcium (Ca²⁺) concentrations and this fact may in turn result in oxidative stress in organisms (Panariti *et al.*, 2012). The increase of Ca²⁺ binding proteins that regulate the activities of enzymes and structural proteins depends on Ca²⁺ releases (Lee *et al.*, 2002), which is an adaptive response for toxicity. Therefore, the increase of Ca²⁺-binding proteins may affect the increase in the total protein amount in the hemolymph of *G. mellonella* at 1000 ppm dose of TiO₂ NPs. Another scenario is that the increase in free Ca²⁺ in response to cellular stress may promote autophagy that damages proteins and organelles (Panariti *et al.*, 2012). In our study, the main reason for the decrease in the total protein amount at 3000 and 5000 ppm doses of TiO₂ NPs might depend on protein damage by the autophagy process.

Lipid peroxidation in hemolymph was estimated by measuring the amount of an active aldehyde that is known as MDA which is of great importance for toxicity studies in non-metabolized form. Increasing the MDA level is a very important factor for toxicity studies (Ghosh *et al.*, 2010; Karthigarani and Navaraj, 2012). Previous studies revealed that exposure to TiO₂ NPs increased the MDA level in human bronchial epithelial cells and rats (Gurr *et al.*, 2005; Unnithan *et al.*, 2011). In our study, we also observed that TiO₂ NPs induced MDA content in hemolymph of the model organism, *G. mellonella*, and an increase was evident at doses 100 to 1000 ppm (Fig. 3) following a decrease again at 3000 and 5000 ppm. Undoubtedly, higher doses of TiO₂ NPs were more effective on *G. mellonella* than lower doses. This might be a response of the organism against the toxicity of TiO₂ NPs and may also be related to the activity of the antioxidant enzymes. It is likely that MDA content elevated by the increase of H₂O₂ initially, then later began to decline with the activity of CAT at especially 1000, 3000, and 5000 ppm doses of TiO₂ NPs, because CAT is an important antioxidant enzyme which responds to scavenge H₂O₂ concentration (Dorval *et al.*, 2003). It is well known that CAT is found in nearly all living organisms and catalyzes to separate H₂O₂ to water and molecular oxygen (Chelikani *et al.*, 2004). The most striking effect observed here was a tremendous increase in CAT activity when last instars of *G. mellonella* were treated with 5000 ppm dose of TiO₂ NP. This activity of CAT at the highest dose treatment might be related to an adaptive response of the larvae to

an increase in H_2O_2 , because CAT regulates H_2O_2 concentration in living organisms during oxidative stress conditions (Fornazier *et al.*, 2002).

SOD is phase I enzyme that is found in nearly all cells exposed to oxygen (Bayir, 2005) and catalysis the dismutation of superoxide radicals to O_2 and H_2O_2 (Karthigarani and Navaraj, 2012). Li *et al.* (2012a) reported that adding TiO_2 NPs to an artificial diet at optimal doses significantly promotes SOD, CAT, and GPx expression in *B. mori* larvae under BmNPV infection. The results of this study support previous study that a dose-wise correlation was evident in SOD activity up to 3000 ppm of TiO_2 NPs exposure, with a tremendous decrease then at 5000 ppm. This increase may be related to the over production of ROS at lower doses. Otherwise, the cause of decreased SOD activity in higher doses may be related to increased CAT activity at 5000 ppm of TiO_2 NPs exposure. SOD activity is also used as a bio-indicator of toxicity showing the scavenging ability of ROS and the overwhelming of the antioxidant defense system (Vander *et al.*, 2003). Thus, the decrease in SOD activity at 5000 ppm dose of TiO_2 NPs might be related to an overproduction of ROS and decreasing the defensive ability of the antioxidant system (Karthigarani and Navaraj, 2012). It is a well-known fact that CAT and SOD are the main enzymes, which prevent cells from oxidative stress (Ayar-Kayali and Tarhan, 2004). Collectively, our results indicate that CAT activity is an effective antioxidant enzyme against nanoparticle toxicity.

GST, which is also responsible for the radical detoxification mechanism in organisms (Fournier *et al.*, 1992), is the major antioxidant enzyme which provides cells and tissue protection against the adverse effects of ROS. GST activity at the highest doses of 3000 and 5000 ppm did not differ from those estimated in control in our study. A relationship also appeared between MDA content and GST activity at the latter doses which may be an adaptive response of the organism against toxicity. It is expected that the activity of GST may increase at these doses depending on the MDA content, because GST is the major detoxifying enzyme synthesized in response to catalyze the conjugation of GSH during oxidative stress (Barbehenn *et al.*, 2013). As a physiological response, this activity provides the detoxification of endogenous compounds such as peroxidized lipids (Oakley, 2011). Similar to SOD, GST activity might also be affected by the overproduction of ROS and inhibited at higher doses of TiO_2 NPs. Long *et al.* (2006) also reported that free radical species which are generated by TiO_2 NPs reduce antioxidant enzymes including GST. Likewise, it has been declared that other NPs such as cerium oxide, single-wall carbon nanotubes (SWCNTs), and semiconductor quantum dots (QDs), considerably reduce the level of antioxidants (Park *et al.*, 2008). Similar to our results, Klaper *et al.* (2009) suggested that GST and CAT activities can be used to determine the physiological effects and toxicity of NPs. On the other hand, considerable elevations in the activity of GST at 100, 500, and 1000 ppm may be an attempt to counteract the elevation of MDA level as a defense mechanism against the accumulation of lipid peroxidation products in cells and physiological response mechanism against TiO_2 NPs toxicity for cellular detoxification. Connecting all these results we suggested that low doses of TiO_2 NPs can improve the antioxidant capacity to provide of physiological resistance in insects. The most striking difference in our

study was also in CAT activity, particularly at 5000 ppm dose of TiO₂ NPs. Collectively, we can emphasize that antioxidant enzyme activities, particularly CAT activity, can be used as a bio-indicator to evaluate NPs induced-oxidative stress in insects.

CONCLUSION

In conclusion, this paper is the first study that investigates the biological effects of TiO₂ NPs on biology and antioxidant capacity of *G. mellonella*'s. Therefore, our study provides important information about nanotoxicity on the insect. In general, biological data show that *G. mellonella* larvae are significant targets for TiO₂ NPs exposure and need to be included when evaluating the toxicological impact of NPs chemicals in the environment. Our study also suggests that TiO₂ NPs caused alterations to the total protein amount, the content of MDA, and the enzyme activity of *G. mellonella*. For this reason, we recommend common use of this insect as a model test organism for environmental effects of nanomaterials.

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Diversity and Distribution of Odonates of the Meriç Delta Wetland in Turkish Thrace, with a New Record for the Region

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ABSTRACT

This study was performed in the Meriç Delta located in the Edirne province of the Turkish Thrace Region in order to reveal the diversity and distributions of odonates in the delta wetlands. Samplings were performed from spring to autumn in 2014 and 2015 in different wetland localities represented by lagoons, lakes and a river. A total of 30 Odonata species were recorded during the study, of which *Libellula quadrimaculata* is a new record for both the study area and Turkish Thrace. In addition, *Calopteryx splendens* (Harris, 1780), *Lestes dryas* Kirby, 1890, *Lestes macrostigma* (Eversmann, 1836), *Coenagrion puella* (Linnaeus, 1758), *Coenagrion pulchellum* (Vander Linden, 1825), *Coenagrion scitulum* (Rambur, 1842), *Enallagma cyathigerum* (Charpentier, 1840), *Ischnura pumilio* (Charpentier, 1825), *Aeshna affinis* Vander Linden, 1820, *Aeshna isoceles* (Müller, 1767), *Anax imperator* Leach, 1815, *Lindenia tetraphylla* (Vander Linden, 1825), *Libellula depressa* Linnaeus, 1758, *Libellula fulva* Müller, 1764, *Orthetrum brunneum* (Fonscolombe, 1837) and *Sympetrum meridionale* (Selys, 1841) are the species determined to be new records for the Meriç Delta wetland. While the Odonata fauna of the Meriç Delta was represented so far by 14 species, this number increased to 31 with the addition of 17 new species during this study. The diversity of the Odonata species recorded in the delta and their conservation categories according to the Red List criteria of the World Conservation Union (IUCN) are also considered.

Key words: Odonata, wetland, diversity, fauna, Meriç Delta, Edirne.

INTRODUCTION

The Meriç Delta is an important wetland and lies along the westernmost land border between Greece and the European part of Turkey (Dochy, 2008). The delta formed by the Meriç River (*Evros* in Greek and *Maritza* in Bulgarian), which originates from the Rila Mountain of Bulgaria and flows northwards, then eastwards and southward to the Aegean Sea, is shared by two countries: Turkey and Greece. The total surface area of the delta is about 188 km², and 150 km² of it lies in Greek land. The 100 km² of the Evros Delta (the Greek part) in Greece is protected under the Ramsar Convention as a wetland of international importance (Kibaroglu *et al.*, 2005). The Turkish part of the delta, named the Meriç Delta, is located inside the borders of the Enez and İpsala municipalities in the Edirne province in Turkish Thrace. The delta includes Lake Gala National Park, which was declared in 2005, and is characterized by different wetland systems, i.e., lagoons, lakes, seasonal swamps, large marshes, floodplains, paddy fields and the Meriç River system, all of which have different features (Kantarci, 1989; Karauz Er, 2006).

Although none of the studies performed so far in the Meriç Delta paid special attention to the local Odonata fauna, 14 Odonata species were already known before our work based on few samplings mostly done in Lake Gala and its surroundings, and Enez was given as the locality for some of these species (Havza, 1987; Hacet and Aktaş, 2004). The diversity and abundance of water sources with different features in the delta make it possible for more representatives of the order to be present. In addition, when Odonata species recorded from both the Greek part of the delta and from the nearby regions within Turkish Thrace are also considered (Yazıcıoğlu, 1982; Havza, 1987; Hacet and Aktaş, 2004, Lopau, 2010), it is also possible to expect more species to be present in the Meriç Delta.

This study was performed to determine the current composition and status of the Odonata fauna in the Turkish part of the Meriç Delta wetland. The results of the study will provide the most recent data to fill the gaps concerning the fauna in the region. Odonates are considered to be an important bioindicator group (Bulánková, 1997; Bhandari *et al.*, 2016), and the data obtained in the present study can be used in the evaluation of the delta wetlands from many points of view. In addition, species listed under a threatened conservation status in the Mediterranean and European Odonata Red Lists by the IUCN were recorded during this study (Boudot *et al.*, 2009; Riservato *et al.*, 2009; Kalkman *et al.*, 2010). This will contribute to update and improve their conservation status.

MATERIALS AND METHODS

The Meriç Delta wetland and a buffer zone in its vicinity, all corresponding to 27.490 ha, were designated as wetland protection area in 2008 (Köse, 2015). This protected area includes important water surfaces (river, lakes, and lagoons), which were sampled in this study. The study material was collected from locations selected in lagoons and lakes, as well as along the Meriç River in the Meriç Delta wetland (Fig. 1). Samplings

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were carried out from May to October in 2014-2015, which corresponds to the flight period of odonates in the region.

Observations and collections were made every month in all localities as planned (Fig. 1), except for some locations in Meriç River and Gala Lake when it was impossible to enter to the sampling locations for sampling. The periodic sampling allowed for i) the determination which species were adapted to different water types in the delta, ii) the observation any possible changes in the species' compositions in the sampling locations from May to October and iii) the sampling the species during different periods (Table 1).

Odonate records in Hacet and Aktaş (2004) were given with a single locality information, Enez, although the samplings were performed in various parts, i.e. Lake Gala and the bank of Meriç River, inside municipality borders of Enez. However, detailed locality information of these records have been formerly given in the Doctoral Dissertation (Hacet, 2000) which Hacet and Aktaş (2004) based their work on. So, for water bodies studied in the present paper, the distribution data in Hacet (2000) were also given in Table 1.

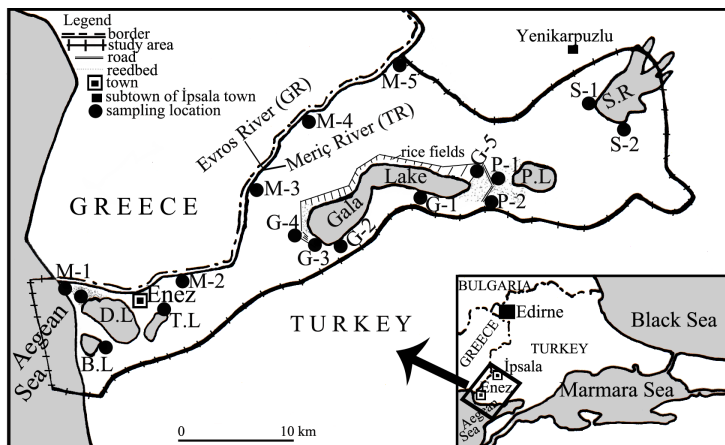


Fig. 1. Sampling locations in the Meriç Delta Wetland. (D.L: Dalyan Lagoon; B.L: Bücürmene Lagoon; T.L: Taşaltı Lagoon; P.L: Lake Pamuklu; S.R: Siğircı Reservoir).

The Shannon-Wiener index was used to determine the species diversity of the sampled localities (Krebs, 1999). The Bray-Curtis similarity index was used to compare the similarities of the wetlands in terms of species compositions (Krebs, 1999). The results were supported by a correspondence analysis index (Krebs, 1999).

Waters sampled in the Meriç Delta

The Meriç Delta is an 'A Class' wetland area according to international standards (Yaşar, 2010). The ecological equilibrium of the delta needs special attention to allow many animals and plants to survive in the delta, in particular, the birds that use the delta as wintering and breeding areas.

Water sources in the delta can be classified in two groups: freshwater and brackish water. The inland freshwater ecosystems are Lake Gala, Lake Pamuklu, the Siğircı

Reservoir as well as the Meriç River. Brackish water ecosystems are located on the Aegean Sea coast and are represented by the Dalyan, Taşaltı and Bücürmene lagoons (Kantarci, 1989).

Table 1. The list of Odonata species recorded in Meriç Delta Wetland up to present, and recording months.

Species names	water bodies studied in the Meriç Delta							E.T	months of record					
	TL	BL	DL	MR	GL	PL	SR		M	J	J	A	S	O
Family: Calopterygidae														
<i>Calopteryx splendens</i> (Harris, 1780) *			+	+(3,4,5)	+				+	+	+			
Family: Lestidae														
<i>Lestes barbarus</i> (Fabricius, 1798)			+, b					c		b, c	+			
<i>Lestes dryas</i> Kirby, 1890 *			+							+				
<i>Lestes macrostigma</i> (Eversmann, 1836) *	+		+						+	+				
Family: Coenagrionidae														
<i>Coenagrion puella</i> (Linnaeus, 1758) *					+	+				+				
<i>Coenagrion pulchellum</i> (Vander Linden, 1825) *					+	+	+			+				
<i>Coenagrion scitulum</i> (Rambur, 1842) *			+								+			
<i>Enallagma cyathigerum</i> (Charpentier, 1840) *			+		+	+					+			+
<i>Erytroma viridulum</i> (Charpentier, 1840)			+	+(1,3)	+,a,b	+		c	+	+a,b,c	+	+		
<i>Ischnura elegans</i> (Vander Linden, 1820)	+	+	+	+(1,2,3,4,5)	+	+	+	b,c	+,b,c	+,b,c	+	+, b	+	+
<i>Ischnura pumilio</i> (Charpentier, 1825) *			+				+					+		
Family: Platynemididae														
<i>Platynemis pennipes</i> (Pallas, 1771)			+	+(2,3,4,5)b	b			c	+,b,c	+,b,c	+			
Family: Aeshnidae														
<i>Aeshna affinis</i> Vander Linden, 1820 *	+	+	+						+	+	+			
<i>Aeshna isocetes</i> (Müller, 1767) *	+		+	+(2)	+	+	+		+	+	+			
<i>Aeshna mixta</i> Latreille, 1805	+	+	+, b	+(3,4), b	+	+	+	c	+	+, b,c	+		+	+
<i>Anax ephippiger</i> (Burmeister, 1839)			+					b,c				+,b,c		
<i>Anax imperator</i> Leach, 1815 *	+		+		+				+	+		+		
<i>Anax parthenope</i> (Selys, 1839)		+	+		+,b,a	+		b,c	+	+a,b,c	+	+	b,c	
Family: Gomphidae														
<i>Lindenia tetraphylla</i> (Vander Linden, 1825) *					+					+				
Family: Corduliidae														
<i>Somatochlora meridionalis</i> Nielsen, 1935								b,c					b,c	

Abbreviations: (T.L) Taşaltı Lagoon; (B.L) Bücürmene Lagoon; (D.L) Dalyan Lagoon; (M.R) Meriç River; (G.L) Lake Gala; (P.L) Lake Pamuklu; (S.R) Sığircı Reservoir; (E.T) Enez Town. Markings: (+) present data; (1,2,3,4,5) locations in the Meriç River (1: brackish water; 2-5: fresh waters); (a) Havza, 1987; (b) Hacet, 2000; (c) records given from Enez town as location by Hacet and Aktaş (2004); (*) new record for the delta; (**) new record for Turkish Thrace Region.

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Table 1. Continued.

Species names	water bodies studied in the Meriç Delta							E.T	months of record					
	TL	BL	DL	MR	GL	FL	SR		M	J	J	A	S	O
Family: Libellulidae														
<i>Crocothemis erythraea</i> (Brullé, 1832)	+, a	+, a	+, a, b	+(12345)b	+, b	+	+	c	+bc	+,b,c	+	+,a	+	
<i>Libellula depressa</i> Linnaeus, 1758 *	+				+				+		+			
<i>Libellula fulva</i> Müller, 1764 *					+	+			+	+	+			
<i>Libellula quadrimaculata</i> Linnaeus, 1758 **				+(2)					+					
<i>Orthetrum albistylum</i> (Selys, 1848)	+	+	+	+(345)	+	+	+	b,c	+	+,b,c	+	+	+	
<i>Orthetrum brunneum</i> (Fonscolombe, 1837) *	+	+	+	+(2,3)			+		+	+	+	+	+	
<i>Orthetrum cancellatum</i> (Linnaeus, 1758)	+	+	+	+(2345)b	+, b	+	+	c	+	+,b,c	+	+		
<i>Orthetrum coerulescens</i> (Fabricius, 1798)	+		+		a	a	+, a		+	+,a	+	+, a		
<i>Sympetrum fonscolombii</i> (Selys, 1840)	+, a	+, a	+, a	+(12345)b	+	+, a	+, a	b,c	+, b,c	+,b,c	+	+, a	+	+
<i>Sympetrum meridionale</i> (Selys, 1841)*	+	+	+	+(1,3,4)	+	+			+	+		+	+	+
<i>Sympetrum striolatum</i> (Charpentier, 1840)	+, a		+						+	+		+, a		
Number of species in the water bodies	15	10	24	13	20	15	11	12						

Macrophytes determined in the freshwater ecosystems are reeds (*Phragmites australis*), bulrushes (*Typhpha latifolia*), club-rushes (*Schoenoplectus lacustris*), water lilies (*Nymphaea alba*), pondweeds (*Potamogeton fluitans* and *Potamogeton pectinatus*), hornwort (*Ceratophyllum demersum*), water plantain (*Alisma plantago-aquatica*) and filamentous green algae. White poplar (*Populus alba*) and willows (*Salix* sp.) are common along the shores of the Meriç River (Kantarci, 1989).

Taşaltı (Harmanlı) Lagoon (T.L.): Edirne- Enez, sampling location: 40°43'11"N, 26°05'47"E, sea level.

This lagoon covers an area of 70 ha and is characterized by brackish water (Yaşar, 2010). The total area of the lagoon widens with the effects of rainfall in winter, but it narrows during the summer, and the lagoon transforms into a swampy area with the withdrawal of water. The lake shows a shallow lagoon feature (Balci Akova, 2008). While there are paddyfields in its north border, the others are occupied by marshes.

Bücürmene (Işık or Üzmene) Lagoon (B.L.): Edirne- Enez, sampling location: 40°42'17"N, 26°04'02"E, sea level.

This lagoon is located on the Aegean Sea coast and occupies an area of 76 ha, but the flooded surface may change seasonally. The water of the lake is brackish. The north and east parts of the lagoon are surrounded by a high vegetation of reeds, shrubs and club-rushes, while the other shores are mostly composed of sandy areas (Balci Akova, 2008; Çamur-Elipék and Kırız, 2010).

Dalyan Lagoon (Tekke or Peso) (D.L.): Edirne-Enez, sampling location: 40°43'39"N, 26°02'41"E, sea level.

This lagoon is a brackish water ecosystem covering an area of 3.4 km². The area of the lagoon changes in summer and winter according to the amount of water carried by the Meriç River. The coastal part of the lagoon as well as its other sides are covered with sand (Balci Akova, 2008).

There are a few small water deposits with reeds around them and both swampy and sandy areas covered by shrubs and short reeds in the part of lagoon near the Meriç River.

The Meriç River (M.R): Edirne- Enez, sampling locations: M-1, brackish water, M-(2-5), fresh waters. M-1: 40°43'46"N, 26°02'20"E, sea level; M-2: 40°44'11"N, 26°06'03"E, 9 m; M-3: 40°46'16"N, 26°07'41"E, 7 m; M-4: 40°48'11"N, 26°09'14"E, 6 m; Edirne- İpsala, sampling location: M-5: 40°50'20"N, 26°12'53"E, 8 m.

The river forms the border between Greece and Turkey. The sampling locations in the study area are located within both the Enez and İpsala municipalities.

The amount of water carried by the Meriç River is quite important, and in the rainy seasons, it sometimes leads to overflowing events around it. Wide reeds margins are typical in natural vegetation and willows are present in coastal areas. The river is connected to Lakes Pamuklu and Gala and to Dalyan Lagoon (Balci Akova, 2008; Yaşar, 2010).

The Sığircı Reservoir (S.R.): Edirne- İpsala, sampling locations: S-1: 40°49'10"N, 26°18'29"E, 15 m; S-2: 40°48'54"N, 26°19'30"E, 8 m.

Sığircı Dam Lake, covering an area of 1.8 km², is located in the south of the İpsala town in the Edirne province. It is a shallow lake and was built between 1989 and 1994. The water level of the pond falls in the summer. Some of its coastal regions have reeds, and some parts turn into marshes (Anonymous, 2017).

Pamuklu (P.L) and Gala Lakes (G.L): Pamuklu Lake: Edirne- İpsala, sampling locations: P-1: 40°46'46"N, 26°14'26"E, 7 m; P-2: 40°46'07"N, 26°13'59"E, 4 m.

Gala Lake: Edirne- Enez, sampling locations: G-1: 40°46'18"N, 26°13'10"E, 6 m; G-2: 40°44'59"N, 26°10'55"E, 14 m; G-3: 40°45'15"N, 26°10'05"E, 7 m; G-4: 40°45'30"N, 26°09'35"E, 5 m; G-5: 40°47'08"N, 26°14'02"E, 10 m.

Lake Gala is an alluvial lake formed by the Meriç River and is separated into two parts as the Great Gala and Small Gala Lakes during the summer (Çamur-Elipek and Kırğız, 2010). The alluvium brought by the river occasionally closes the river mouth of the lake and causes the lands around the lake to be submerged, thereby merging the lake with Lake Pamuklu (Balci Akova, 2008). Lakes Gala and Pamuklu (2.369 ha) were declared to be a nature conservation area in 1991. The border of the nature protection area was later expanded (6.087 ha), and it was declared a national park in 2005 (Köse, 2015).

The Small Gala and Pamuklu Lakes are wetlands that consist of bulrushes and reeds and contain, from time to time, water lilies, pondweeds and filamentous green algae. Some parts of the lakes turn into marshes with the withdrawal of water during

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summer months. Great Gala Lake, which is mostly bordered by a forest ecosystem, is also an important wetland where bulrushes are common and is partly covered with water lilies and has shrubs.

RESULTS AND DISCUSSION

Thirty Odonata species (12 Zygoptera and 18 Anisoptera) were recorded in the study area (Table 1).

Libellula quadrimaculata Linnaeus, 1758 is reported as a new species both for the Meriç Delta and the Thrace region (see the updated species distribution in Boudot and Kalkman (2015). Its nearest known locality is in north-eastern Greece, close to the Bulgarian border) (Lopau, 2010). In addition, *Calopteryx splendens*, *Lestes dryas*, *L. macrostigma*, *Coenagrion puella*, *C. pulchellum*, *C. scitulum*, *Enallagma cyathigerum*, *Ischnura pumilio*, *Aeshna affinis*, *A. isoteles*, *Anax imperator*, *Lindenia tetraphylla*, *Libellula depressa*, *L. fulva*, *Orthetrum brunneum* and *Sympetrum meridionale* are new species for the Meriç Delta.

Although the Odonata fauna of the Meriç Delta were represented by 14 species, these 17 new species increased the total number of Odonata species known from the delta to 31.

Conclusions on some species recorded in the study area

Libellula quadrimaculata has a Holarctic distribution and is widely distributed in Eurasia and North America (Askew, 2004; Boudot and Kalkman, 2015). The species had not been recorded so far in Turkish Thrace Region. While shallow ponds and lake edges with emergent vegetation have been given as the habitats where *L. quadrimaculata* generally breeds, it has also been reported that the species is common in the pools situated in mossy and open areas in the north of Europe (Askew, 2004). *Libellula quadrimaculata* was recorded from only one location, a bushy area in at the border of a swampy area near the edge of the Meriç River about 7 or 8 km inwards from the shore of the Aegean Sea. Since this species could not be found in any of our other sampling sites, neither along the river nor in other water bodies in the delta, we conclude that it is not likely for *L. quadrimaculata*, known as a vagrant and migrant species (Askew, 2004), to be native in our study area, so that this record most likely belongs to a vagrant individual. *Libellula quadrimaculata* is known from Greece (Lopau, 2010) and might occur in the Evros Delta, which is the part of the Meriç Delta in Greece. The record from the Meriç Delta probably represents an individual originating from a Greek population. When both the dispersal capacity of this species and its adaptation to various water types are considered, the presence of suitable habitats in the delta indicates that *L. quadrimaculata* might well settle in this area in the future.

Lestes dryas and *Lestes macrostigma* are species rarely recorded in the Thrace region. The main suitable habitats for the latter species are in brackish wetlands with sea club-rush (*Bolboschoenus maritimus*), common club-rush (*Scoenoplectus lacustris*) and/or sea rush (*Juncus maritimus*) in abandoned salt pans, salt marshes and dune and steppe lakes with salinity up to ca 20‰ (Boudot and Kalkman, 2015).

In this study, *L. macrostigma* was found on the banks of two lagoon lakes (i.e., Taşaltı and Dalyan). The habitats preferred by *L. dryas* were stagnant and well-vegetated waters, shallow ponds and swamps (Askew, 2004; Kalkman, 2006). This species was recorded at the edge of a swamp area at the border of the Dalyan Lagoon near the Meriç River. While the presence of *L. dryas* in the Meriç Delta is represented by two specimens caught in this lagoon lake, *L. macrostigma* was found in Dalyan Lagoon in higher numbers than in Taşaltı Lagoon. *L. macrostigma* is assessed as vulnerable (VU) in the European IUCN Red List (Kalkman *et al.*, 2010). Therefore, the presence of this threatened species in the delta makes the Meriç Delta an important area in terms of Odonates.

Coenagrion pulchellum, *C. scitulum* and *E. cyathigerum* are rarely seen in the Thrace region and the known records of these species have been gathered in the north of the region (Kalkman and Van Pelt, 2006; Hacet, 2017). These three species are known from locations in Greece close to the Meriç delta (Lopau, 2010). The present records of *C. pulchellum*, *C. scitulum* and *E. cyathigerum* in the delta extended their distributional range to include the southern parts of the Thrace region. However, their densities in the delta was low. *C. pulchellum* was evaluated as Near Threatened (NT) in the Red List of the Mediterranean Region Basin (Riservato *et al.*, 2009). The Meriç Delta is therefore an important location for these species in Turkish Thrace.

Lindenia tetraphylla, also assessed as VU according to the IUCN European Odonata Red List (Kalkman *et al.*, 2010), is a new species for the delta. This nomadic species tends to migrate (Schneider, 1981) and has a distributional range extending from Central Asia to the West Mediterranean Basin through the Middle East. Permanent populations in the Mediterranean basin are known from the Adriatic coast of the Balkans and Turkey (Dijkstra and Lewington, 2006; Boudot and Kalkman, 2015). A number of new localities became recently available within the known species range (Gastarov and Beshkov, 2010; Kulijer *et al.*, 2012; Brochard and van der Ploeg, 2013; De Knijf *et al.*, 2013; Stille *et al.*, 2014; Boudot and Kalkman, 2015). The known distribution of the species from Turkey are mainly localized to the south of Anatolia, but the species has also been recorded in the Thrace region and Gökçeada Island in the north of the Aegean Sea (Hacet and Aktaş, 2006; Kalkman and Van Pelt, 2006; Hacet, 2017). The finding of *L. tetraphylla* in the Meriç Delta is based only on a dead male found around Gala Lake, and no other specimens could be found during the subsequent samplings in the area. *Lindenia tetraphylla* is prompt to vagrancy and shows often a migratory behaviour. Therefore, some recent records in Europe may represent temporary populations (Boudot *et al.*, 2009). It is possible that the single specimen found in our study area was either a vagrant or a migrant coming from Greece, where the indigenous populations of the species occur.

Lindenia tetraphylla is known from lakes and rivers with hydrophytes and helophytes (Schorr *et al.*, 1998; Boudot, 2014). Recent data have shown that man-made dam lakes may also constitute breeding places (Kalkman and Van Pelt, 2006; Hardersen and Leo, 2011; Brochard and van der Ploeg, 2013; Boudot, 2014; Hamzaoui *et al.*, 2015). *L. tetraphylla* is a widespread eremic species occurring in North African and

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Asian deserts (Suhling *et al.*, 2003; Hamzaoui *et al.*, 2015). A species that adapts to desert conditions will undoubtedly be more successful in spreading than a species that is specific to certain habitats. The water resources in the delta have features that can support permanent populations of *L. tetraphylla*, and, therefore, it may be expected that this species will be able to establish itself here in the future, although it is not a native population today.

Abundance and biodiversity of the species in the Meriç Delta

According to the value of the Shannon-Wiener index, the Dalyan Lagoon shows the highest species diversity ($H'=1.38$), followed by the Lake Gala ($H'=1.2$), the Taşaltı Lagoon, the Pamuklu Lake, the Meriç River ($H'=1.1$) and the Bücürmene Lagoon and the Sığircı Reservoir ($H'=1.0$) (Fig. 2).

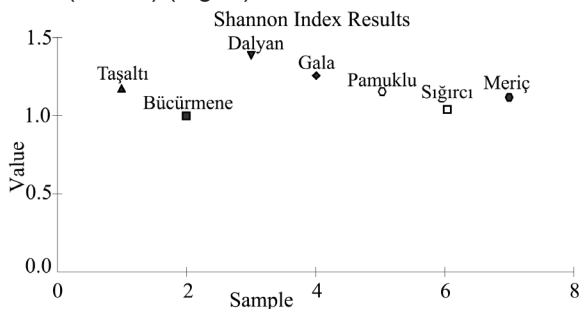


Fig. 2. The results of Shannon diversity index of Odonata in the sampling localities.

When the sampled localities were evaluated in terms of the number of species, the Dalyan Lagoon was found to harbour the highest number of species. The sampling point selected in the Dalyan Lagoon is close to the Meriç River. This edge of the Dalyan Lagoon includes marshy areas covered by reeds and also a sandy area covered by short reeds and shrubs throughout the sea border. Furthermore, this location has small pools and a channel bordered by short herbaceous plants, which enters inward from the Meriç River. This location, characterized by brackish water, is close to the Meriç River, which creates a suitable habitat both for different species occurring in either river or lagoon waters and for species showing tolerance to a broad spectrum of aquatic habitats. This should explain why it contains more species than the other sampled localities.

According to the results of the Bray-Curtis similarity index, Lakes Gala and Pamuklu, both with freshwaters, were found to be the most similar in the wetlands with 87 % similarities. The second leading similarity values were obtained for the Taşaltı and Bücürmene Lagoons (72 % similarity) and the Taşaltı and Dalyan Lagoons (71 %) (Fig. 3).

When the species recorded within the delta were evaluated according to their distributions in lagoons and fresh waters, 25 species were found to exist in lagoons and 23 species in fresh waters (Table 1). The numbers in lagoons and fresh waters are close to each other. The reason for this seems to be that *I. elegans*, *I. pumilio*, *E. viridulum*, *L. barbarus*, *L. macrostigma*, *A. mixta*, *A. ephippiger*, *O. cancellatum*, *S.*

fonscolombii, *S. striolatum* and *C. erythraea* are tolerant to both fresh and brackish waters and that the present water bodies in the delta are close and linked to each other, allowing species to disperse from their native habitats to another one. The Meriç River is linked to Lakes Gala and Pamuklu (Balci Akova, 2008). Excess water from Gala Lake is discharged to the Taşaltı Lagoon by the Taşyarma Canal, and this lagoon is also linked to the Dalyan Lagoon (Yaşar, 2010).

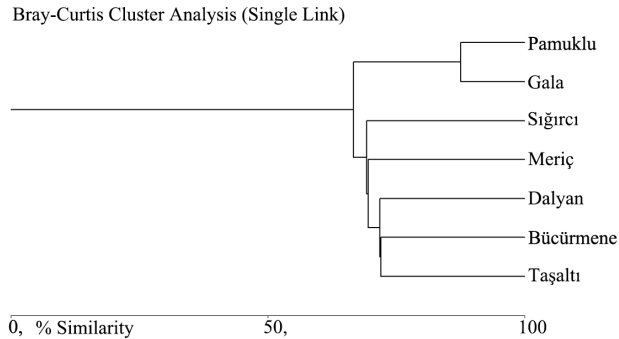


Fig. 3. Dendrogram of similarity of species composition for Odonata in the Meriç Delta Wetland.

The Bray-Curtis results were supported by correspondence analysis. Accordingly, the sampled localities were grouped according to the species sampled within them (Fig. 4).

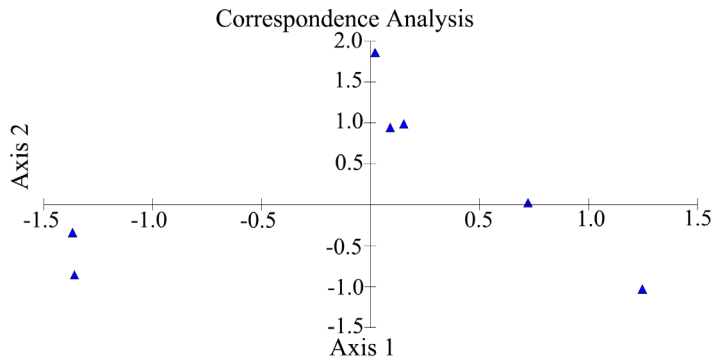


Fig. 4. Corresponding analysis results of species composition for Odonata in the sampling localities.

The most common species in the 7 different wetlands sampled are *I. elegans*, *A. mixta*, *C. erythraea*, *O. albistylum*, *O. cancellatum* and *S. fonscolombii*, while *A. isoceles* and *S. meridionale* were common in 6 wetlands, and *O. brunneum* and *O. coerulescens* were common in 5 wetlands (Table 1). The common occurrence of these species in different water types, i.e., lagoons with brackish to salty water and river and lakes with fresh water, can be explained by their low specialisation in terms of habitat preferences (i.e., ubiquist species).

Lestes barbarus, *L. dryas*, *C. puella*, *C. pulchellum*, *C. scitulum*, *E. cyathigerum* and *I. pumilio* were observed in low numbers in the delta. Their sampling dates showed

Diversity and Distribution of Odonates of the Meriç Delta

that they have earlier flight periods than other species (Table 1), which accounts for their lower frequency in our records. *Ischnura elegans*, *A. mixta*, *A. parthenope*, *C. erythraea*, *O. albistylum*, *O. brunneum*, *S. fonscolombii* and *S. meridionale* were observed in large numbers and showed longer flight periods (Table 1).

The number of species recorded from the Meriç Delta represents approximately half of the Odonata fauna from the Thrace region. Anisoptera and Zygoptera were found to be represented in the study area by about 61 % and 38 % respectively. The dominant families in the region were Libellulidae (36 %) and Coenagrionidae (23 %) (Fig. 5). The results of this study showed that the Meriç Delta constitutes an important living area for ubiquitous Odonata. The delta is also important especially for species listed in the threatened and near threatened categories in the European and Mediterranean Odonata Red lists.

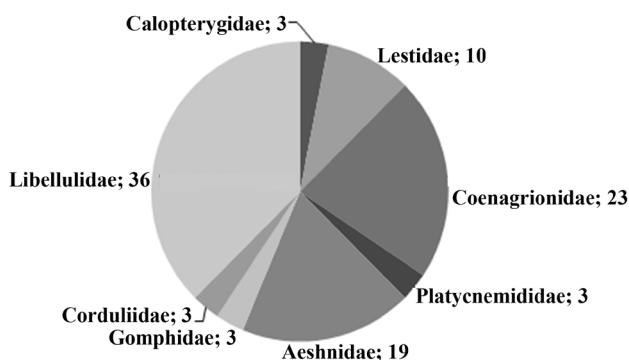


Fig. 5. Family distribution of Odonata in the Meriç Delta Wetland.

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***Ceutholopha isidis* (Zeller, 1867), a New Phycitinae Record from Turkey (Lepidoptera: Pyraloidea)**

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ABSTRACT

Ceutholopha isidis (Zeller, 1867) is a new genus and species record for the Pyraloidea fauna of Turkey. Distribution, synonyms and host plants of the species are summarized. The adult male and the male genitalia are figured.

Key words: *Ceutholopha isidis*, Phycitinae, fauna, new record, Şanlıurfa, Turkey.

INTRODUCTION

Phycitinae is one of the most species-rich groups of snout moths (Pyraloidea), with currently over 3.100 species in more than 650 genera (Nuss *et al.*, 2003-2018 (The Pyraloid Planet Vol.11)). It is known with 303 species in Turkey (Koçak, 2014; Akın, 2016; Slamka and Plant, 2016; Kemal and Koçak, 2016; 2017a,b,c,d; Akın and Seven, 2017). Except for the northern and southern extremes, the group has a very wide spread on the earth (Solis and Mitter, 1992). Roesler (1973) subdivided Phycitinae into 4 tribus: Cryptoblabini, Phycitini, Cabniini and Anerastiini with Phycitini divided into two subtribus, Phycitina and Acrobasiina-a system that is largely still in use today (Nuss *et al.*, 2003-2018). All tribes except Cabniini comprise quadrifid and trifold groups, having vein m_3 reduced or veins m_2 and m_3 fused in the hindwing.

Ceutholopha isidis (Zeller, 1867) was described from Manfalut, Egypt, its synonyms from Algeria and Tunisia. With this study, it is aimed to contribute to the Pyraloidea fauna of Turkey.

MATERIAL AND METHODS

The specimens were collected in Ceylanpınar (Şanlıurfa province) using a 160 W mercury vapor lamp at a white sheet and a generator as an energy source. The male genital was prepared following Robinson (1976).

RESULTS

Ceutholopha isidis (Zeller, 1867), Fig. 1A

Nephopteryx [sic] (*Ceutholopha*) *isidis* Zeller, 1867: 464-465, pl. 24 fig. 6.

=*Phycita gilvibasella* Ragonot, 1888: 12. (Leraut, 2014)

=*Heterographis aequalisella* D. Lucas, 1948: 89. (Roesler, 1973)

=*Syria parallelolineata* D. Lucas, 1950: 142. (Roesler, 1973)

Material examined: Turkey, Şanlıurfa Prov., Ceylanpınar: 2 ♂♂, Tigem, Akrepli management, 29.08.2008; 5 ♂♂, 3 ♀♀ TİGEM, 1 km west from Urfa Gate, 23.07.2008 (G. P. 258 K. A. (Figs. 1 B-D); 2 ♂♂, TİGEM, Gökçayır way 13 km, near Atatürk forest, 04.08.2008. Leg. K. Akın.

Host plants: *Acacia tortilis* (Forssk.) Hayne, *A. farnesiana* (L.) Willd., *A. nilotica* (L.) Delile (Fabaceae) and *Rhus oxyacantha* Aubrév. (Anacardiaceae) (Leraut, 2014; De Prins and De Prins, 2018).

Acacia farnesiana (called “tatlı akasya” in Turkish) is used as park and garden tree while *Rhus* genus is known with only one species, *R. coriaria* L. (called “sumak” in Turkish), in Turkey (Güner *et al.*, 2012).

Distribution: In Europe, *C. isidis* is recorded from Great Britain, France (Corsica) and Malta (Leraut, 2014), Portugal (Corley *et al.*, 2014), Spain (Revilla and Gaston, 2014) and Cyprus (Rebel, 1939). In the Palearctic east of Europe, the species is known to occur in Iran (Amsel, 1959), United Arab Emirates (De Prins and De Prins, 2018),

Ceutholopha isidis (Zeller, 1867), a New Phycitinae Record from Turkey

Pakistan (Amsel, 1968) and Afghanistan (Amsel, 1970). On the African continent, *C. isidis* is recorded in the Morocco, Tunisia, Sub-Saharan Africa (Leraut, 2014), Algeria (Ragonot, 1888), Egypt (Zeller, 1867), Sudan (Amsel, 1965) and South Africa (Balinsky, 1991). Revilla and Gaston (2014) furthermore mention India, Sri Lanka and Kenya as distribution records.

As result of this study, *C. isidis* (Zeller, 1867) is recorded as new genus and new species for the Pyraloidea fauna of Turkey. The specimens were collected in the Ceylanpınar district (Şanlıurfa) in the southeast of Turkey near the Turkish-Syrian border, making it likely that this species is also present in Syria.

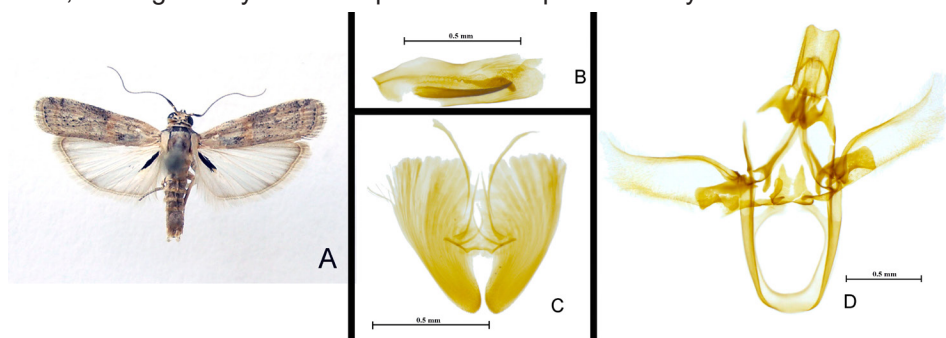


Fig.1. *Ceutholopha isidis* (Zeller, 1867), (A) Adult male, (B) Phallus, (C) Coremata, (D) Male genitalia armature.

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New Records of *Metreletus balcanicus* (Ulmer, 1920) (Ephemeroptera: Ameletidae) from Republic of Macedonia

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ABSTRACT

Within the present paper we elaborate the first country records for the larval and subimaginal stadium of *Metreletus balcanicus* (Ulmer, 1920) and the second locality for the presence of male imago from the territory of R. Macedonia. Furthermore, illustrated description of larval and adult characteristics, with an overview of species zoogeography and ecology is given.

Key words: Mayfly, larva, subimago, ecoregions.

INTRODUCTION

Metreletus balcanicus is a rare European mayfly species, described as pontomediterranean faunistic element by Haybach (1998: 161, nec Bauernfeind and Soldán, 2012). Its southernmost distribution on the Balkan Peninsula reaches Southern Bulgaria (Russev and Vidinova, 1994; Presolska, 2014; Chertoprud and Palatov, 2017), Macedonia (Bauernfeind and Soldán, 2012) and European part of Turkey (Kazancı, 2001; Kazancı and Türkmen 2012; Salur *et al.*, 2016). According to the Fauna Europaea database, the species is present in: Bulgaria, Czech Republic, Germany, Luxemburg, French Mainland, Hungary, Poland and European part of Turkey (de Jong *et al.*, 2014). As *M. balcanicus* was not observed in Flanders (Belgium) since 1990 (Lock and Goethals, 2011), nowadays the species is confirmed from the Kiev region in Ukraine (Martynov, 2016).

Concerning the taxonomy of *M. balcanicus* important contributions were done by Ujhelyi (1960) and Puthz (1977), who synonymized *Ameletus hessei* Fizaine, 1931, *Metretopus goetghebueri* Lestage, 1938 and *Metreletus hungaricus* Ujhelyi, 1960 with *Metreletus balcanicus* (Ulmer, 1920). Later, Jażdżewska and Wojcieszek (1997) overviewed in detail the distribution, taxonomy and ecology as well as the ecological niche of this species. Studemann *et al.* (1988) provided a redescription of *M. balcanicus*. Presolska (2014) mentioned it as “rare” mayfly, known only from several localities in Bulgaria, but populations usually rich in individuals. Martynov (2016) presented it also as very rare species in Ukraine, with only one country record since its first finding in 2011.

The aim of the paper is to present new country records of *M. balcanicus* from R. Macedonia.

MATERIAL AND METHODS

The material (4 larvae and 1♂ subimago) was collected from a small river above the lake of Suvodol (periodically drying up stream, affluent of the river Crna Reka; N 41.081075°; E 21.556038°). The specimens were collected at the end of April 2015. Larvae were picked up by hand net, while the adult was collected with an entomological net. Collected specimens were preserved in 80% ethanol. The species identification was done according to Studemann *et al.* (1992) and Bauernfeind and Humpesch (2001). The drawings were prepared on a microscope Olympus BX41.

RESULTS AND DISCUSSION

Until now the only known locality for this species in R Macedonia was reported in Bauernfeind and Soldán (2012)-1♂ specimen. This material was collected by Wolfram Graf, near village Dolno Divjaci (on 07.07.2010) at elevation of 851m (Bauernfeind, pers. comm.). According to Illies (1978) this locality belongs to the zoogeographic region 6, Hellenic Western Balkan.

The main characters distinguishing the larvae of the species from that of the related ones are elongated, plate-like tracheal gills, submarginally with a fine sclerotized line

New Records of Metreletus balcanicus from Republic of Macedonia

along lateral borders (Fig. 1a) and the apically widened maxilla with pectinate bristles (Fig. 1b) (Bauernfeind and Soldán, 2012).

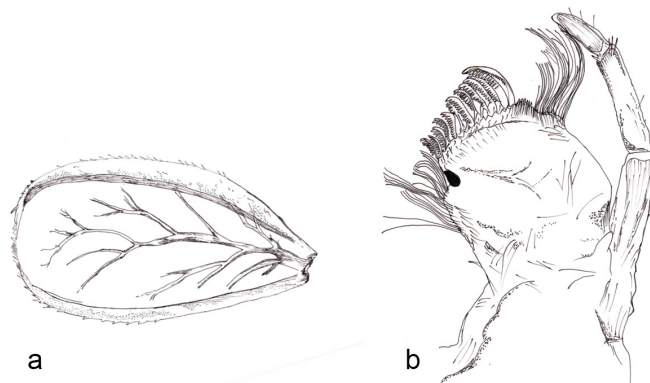


Fig.1: Gill (a) (40x) and Maxilla, ventral view (b) (100x), the larval stadium of *M. balcanicus* (drawings by B. Rimcheska).

In male imago and subimago the posterior border of the forceps base has a deep median incision (Fig. 2a). The penis lobes are typically tubular and strongly sclerotized with acutely pointed longitudinal fold projection (Fig. 2b).

The stream bed from the sampled site is 0.3-0.5 m wide. The substrate was dominated by sand, gravel and small to large sized boulders. The streambank and riparian vegetation were represent with a small amount of decaying trees and vegetation surrounding the locality. In the literature it is familiar that *M. balcanicus* prefers slow flowing streams with muddy and clay bottom, covered with aquatic vegetation and often coexisting with *Siphonurus* species (Russev and Vidinova, 1994). Furthermore, in periodically drying up streams, *M. balcanicus* life cycle is characterized by adaptation to the peculiar environmental condition, when the imagines emerge in spring before drying up of the stream bed; hatched eggs survives the dry period and in autumn the nymphs of next generation appear (Jażdżewska and Wojcieszek, 1997).

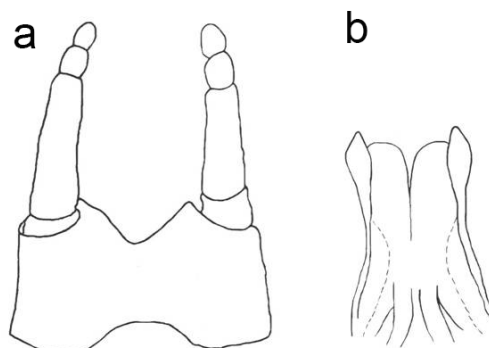


Fig. 2. Forceps, ventral view (a) (40x) and penis lobes, dorsal view (b) (40x), subimago of *M. balcanicus* (drawings by B. Rimcheska).

The species has one generation per year (Soldán, 1978; Jażdżewska and Wojcieszek, 1997; Martynov, 2016). Two types of monovoltine life cycles of *M. balcanicus* are known for Central Europe (Czech Republic and Poland): overwintering only in the egg stadium and overwintering simultaneously both the egg and larval stadia (Martynov, 2016). In Poland, the mature nymphs and imagines have been collected during May and early June (Jażdżewska and Wojcieszek, 1997). In Ukraine, larvae begin to hatch in March, where the middle and elder larvae are recorded in the middle of April, with overwintering of the egg stadium (Martynov, 2016). We recorded the existence of the elder larval stage and subimago at the same period-the end of April.

From a faunistic point of view, *M. balcanicus* represents a rare species for the R. Macedonia, wherein, up to date there are no records from the 7th Ecoregion (Eastern Balkan). This statement would imply that some population of the species should exist in the eastern part of the country, having in mind the records from the 7th Ecoregion from South-Western Bulgaria (Russev and Vidinova, 1994; Presolska, 2014; Chertoprud and Palatov, 2017). Generally this is the second report not just from the territory of R. Macedonia, but from the 6th Ecoregion as well.

In summary, more detailed studies of the sampled site and its surroundings are needed in order to estimate the overall tendency of species abundance. Thus, we could measure the impact of the anthropogenic influence (when high degradation of the river bed is achieved) at this locality, especially during dry summer period. Furthermore, we select some sites with the potential preferred habitat of the species from the nearby border area with neighboring Bulgaria for potential confirmation of our assumption that *M. balcanicus* exist and in the Eastern part of the country.

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Material examined: Ankara, Altındağ, Çubuk Dam Lake, 900 m, 29.06.1998, 1 ♂; Kalecik, 600 m, 24. 07. 2001, 2 ♀♀, Kalecik, 800 m, 25. 07. 2001, 3 ♀♀

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