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Host Range Expansion by the Invasive Herbivore *Corythucha marmorata* (Uhler, 1878) is not Caused by Better Quality of New Hosts

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ABSTRACT

Phytophagous insects may become serious pests of crops when introduced into a new place. Better nutritional quality and lower toxicity of new host plants and escape from natural enemies can enhance survival. The chrysanthemum lace bug, *Corythucha marmorata* (Hemiptera: Tingidae), is native to North America, where it exploits mainly goldenrod and its relatives (Asteraceae). It was accidentally introduced into Japan by about 2000. Since then, many reports of injury to sweet potato (Convolvulaceae) and eggplant (Solanaceae) by this species have been published. Here, we tested larval performance on goldenrod, sweet potato, eggplant, and three other known or potential host plants to investigate why the lace bug began to exploit the new host plants. Survival to adult stage was nil on eggplant, extremely low on blue daze (Convolvulaceae) and crown daisy (Asteraceae), moderate on sweet potato, and ca. 80% on goldenrod and sunflower. Developmental time was shorter and adults grew larger on goldenrod and sunflower than on the other plants. These results show that plant nutritional or toxicological qualities are not major factors that facilitate host range expansion of *C. marmorata* in Japan. Possible factors are discussed.

Key words: Tingidae, Asteraceae, Convolvulaceae, specialist herbivore, larval performance, development, survival, biological invasion

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INTRODUCTION

The invasion of insect pests can cause ecological and economic problems (Sydnor, Bumgardner, & Todd, 2007; Basavaraju, Chakravarthy, Doddabasappa, Nagachaitanya, & Yathish, 2010; Gandhi & Herms, 2010). Phytophagous insects may become serious pests of crops even if they are not problematic in their original range. Explanations include escape from natural enemies and from plant resistance. Host shift or host range expansion in new areas also may make them pests (e.g., Bowers, Stamp, & Collinge, 1992; Louda, Kendall, Connor, & Simberloff, 1998; Fukano & Doi, 2013; Davis & Cipollini, 2014).

Most phytophagous insects have limited host range (Bernays & Graham, 1988; Schoonhoven, Van Loon, & Dicke, 2005). Traditionally, insect species that exploit only one plant species, or several species within a genus are called monophagous, while species that exploit several plant species among different genera within a family are called oligophagous, and species that exploit plant species of more than one families are called polyphagous (Bernays & Chapman, 1994), although the trait is not stable (Bernays & Graham, 1988). Host shift and host range expansion by various insects are frequently reported (Tuda et al, 2009; Kohyama, Matsumoto, & Katakura, 2012). Insects introduced for biological control have become pests of non-target native plants (Howarth, 1991; Pemberton, 2000), in most cases adopting new host plants closely related to their original hosts within the same family (Wheeler, 1987; Louda et al.,1997; Pemberton, 2000).

The chrysanthemum lace bug, *Corythucha marmorata* (Hemiptera: Tingidae), is native to North America and is a specialist (mono- or oligophagous species) of asteraceous plants, such as goldenrod (*Solidago altissima*) and sunflower (*Helianthus annuus*). It is the most abundant herbivore on *Solidago* species and several other asteraceous plants, such as *Ambrosia*, *Chrysanthemum*, and *Xanthium* (Wheeler, 1987; Fontes, Habeck, & Slansky Jr., 1994; Kato & Ohbayashi, 2009). It causes chlorosis typical of injury by lace bugs (Root, 1996; Cappuccino, 2000), which suggests that it feeds on leaf cellular materials by sucking (Ishihara & Kawai, 1981). Injury occasionally leads to plant death (Miyatake, 2005).

The North American *S. altissima* was introduced into Japan around 1900 as an ornamental plant (Nakagawa & Enomoto, 1975). It escaped from gardens, spread naturally after World War II (Nakagawa & Enomoto, 1975) and is now a common weed throughout Japan.

In 2000, *C. marmorata* was first reported in Nishinomiya City, Hyogo Prefecture, western Japan, and its geographic range has since expanded rapidly (Tomokuni, 2002; Kato & Ohbayashi, 2009; Hoshino, 2011). Although there is only one report that it utilizes plants other than asteraceous plants in its native range (Wheeler, 1987), in Japan it utilizes non-asteraceous plants such as sweet potato (*Ipomoea batatas*, Convolvulaceae; e.g., Shiga Pest Control Center, 2005; Aichi Prefecture, 2005; Gifu Plant Protection Center, 2005), blue daze (*Evolvulus pilosus*, Convolvulaceae; e.g., Nara Plant Protection Center, 2005; Okayama Plant Protection Center, 2005; Kagawa

Plant Protection Office, 2005), and eggplant (*Solanum melongena*, Solanaceae; e.g., Gifu Plant Protection Center, 2005; Kagawa Plant Protection Office, 2005; Kochi Prefecture Crop Pest Control Center, 2005) as food and oviposition substrate. It can be inferred that *C. marmorata* has expanded its host range to novel plants. This is unusual in that these new hosts belong to different families from that of the original host species.

The central question in insect-plant relationships is what is the key factor which determines the host range of an herbivore. *Corythucha marmorata* may give a clue. The most plausible scenario is that *C. marmorata* enjoys higher larval performance on these new host plants. Here, to examine this hypothesis, we evaluated the quality of novel and original host plants as food for *C. marmorata* in terms of larval performance. In addition to the five species named above, we tested crown daisy (*Glebionis coronaria*, Asteraceae), which is an important vegetable crop in Japan.

MATERIALS AND METHODS

Materials

We collected *C. marmorata* individuals from wild goldenrod growing on the campus of Mie University (Tsu City, Mie Prefecture, Japan) from April 2012 to November 2014. To conduct the experiments with insects as close to wild as possible, we used only descendants two generations after these collected individuals, and therefore made several collections.

We tested goldenrod, eggplant (*cv.* Senryo No. 2), sweet potato (*cv.* Beniazuma), blue daze, sunflower, and crown daisy as food for the lace bug. The goldenrod leaves were collected on campus. The other plants were bought as seeds or seedlings at a DIY store in Tsu City. The plants were grown in pots filled with a ca. 2:1 mixture of *akadama* (granular loam) and leaf litter.

Methods

Adults collected in the field were allowed to reproduce in the laboratory. Larvae were reared to adults in an environmental chamber (25 °C, 16L:8D) on leaves of goldenrod. About six adults (both male and female) were kept in a plastic cup (A-PET, 200 ml, ø 101 mm × 44 mm) with two leaves of goldenrod in the same environmental chamber as before. Each leaf stalk was covered with a piece of sterile cotton soaked with tap water. Any leaf on which eggs were laid was replaced with a new leaf, and was transferred to another plastic cup. Each hatched larva was put individually into a plastic cup and reared on a leaf of one of the six plants in the chamber. All leaves were kept fresh with wet cotton on the stalk, and were checked daily and replaced as necessary. When an individual died, the instar was recorded. As do other lace bugs, this species passes through five larval stages until eclosion. Sex, head width, and body length of adults were recorded. Head width and body length were measured in ImageJ software in photographs taken under a digital microscope (Keyence VH-5000 equipped with a VH-Z25 zoom lens) connected to a PC.

All statistical analyses were performed in R v. 3.5.0 software (R Core Team, 2018). Pairs of survival rate curves were compared by log-rank test. The results were corrected by Bonferroni's method for multiple comparisons. To examine whether developmental time, head width, or body length differed among host plants, we fitted generalized linear models (GLMs) with those as the response variables and plant species as the explanatory variable. Multiple comparisons were conducted using the Tukey–Kramer method. All GLMs had a Gaussian distribution (We show only the result of multiple comparison tests).

RESULTS

Larvae reared on goldenrod and sunflower attained significantly higher survival rates than those reared on the other plants (Log Rank Test with Bonferroni correction, $P < 0.01$; Fig. 1). Larvae reared on sweet potato attained a significantly higher survival rate than those reared on blue daze, eggplant, and crown daisy (Log Rank Test with Bonferroni correction, $P < 0.01$). No larvae grew to adult stage on eggplant, and only 2 adults emerged on crown daisy. Larval mortality was extremely high on eggplant (100%), blue daze (89.6%), and crown daisy (96.7%), especially during the first instar (Fig. 1). Larval mortality was higher during the first instar than during the later stages on sweet potato.

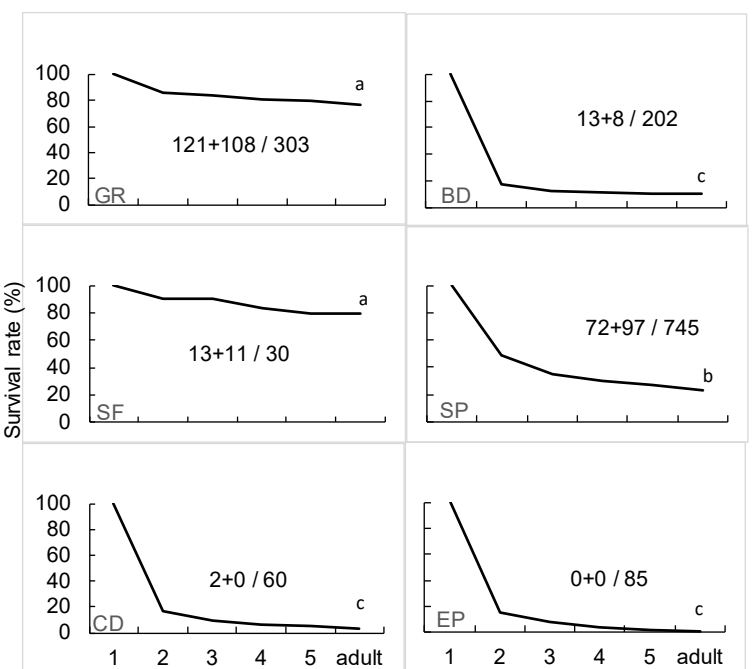

Fig. 1. Larval survival rate by stage on goldenrod (GR), sunflower (SF), crown daisy (CD), blue daze (BD), sweet potato (SP), and eggplant (EP). Survival curves labelled on the right with the same letter are not significantly different (log-rank test with Bonferroni's correction, $P < 0.01$). Equations show

number of emerged females + males over initial number of first instar larvae.

Larvae reared on goldenrod and sunflower had significantly shorter developmental times (both female and male) than those reared on the other plants (Tukey-Kramer multiple comparison test, $P < 0.05$; Fig. 2). There were no significant differences in developmental time among the novel hosts.

Both females and males reared on goldenrod and sunflower had significantly larger head width and body length than those reared on blue daze and sweet potato (Tukey-Kramer multiple comparison test, $P < 0.05$; Figs. 3, 4). Adults reared on sweet potato had no significant differences in head width or body length from those reared on blue daze. Females tended to be larger than males.

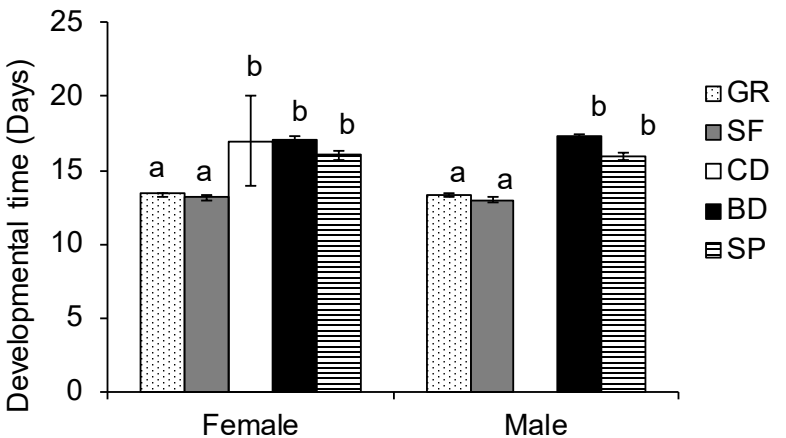

Fig. 2. Developmental time (days, mean ± SE) of females and males. Bars with the same letter are not significantly different within each sex. See Fig. 1 for plant name codes.

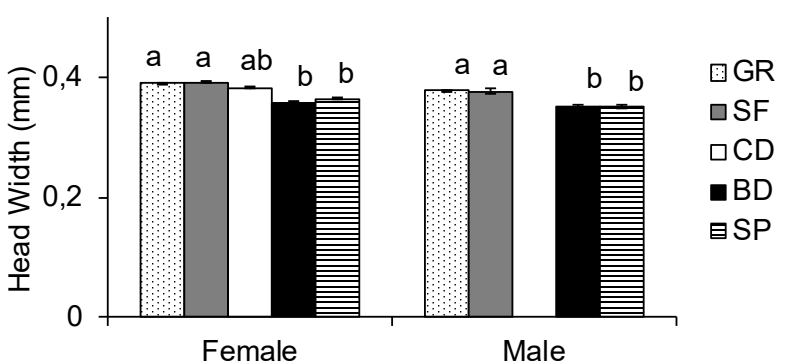

Fig. 3. Head width (mm, mean ± SE) of female and male adults. Bars with the same letter are not significantly different within each sex. See Fig. 1 for plant name codes.

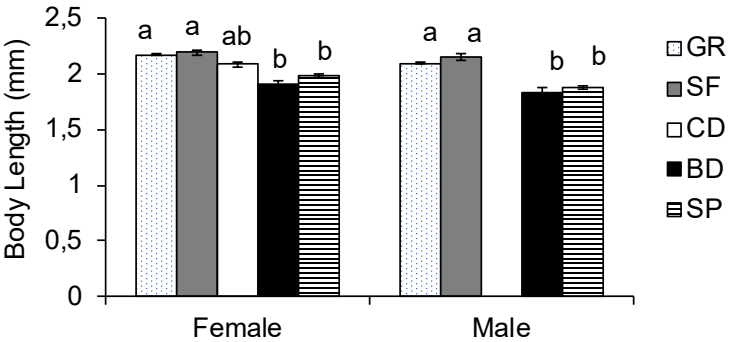

Fig. 4. Body length (mm, mean ± SE) of female and male adults. Bars with the same letter are not significantly different within each sex. See Fig. 1 for plant name codes.

DISCUSSION

As previously reported (e.g., Nara Plant Protection Center, 2005, Chiba Prefecture Agriculture and Forestry Research Center, 2011), *C. marmorata* fed on and grew to adult on sweet potato and blue daze (Convolvulaceae), but not as well as on goldenrod and sunflower. Furthermore, they did not grow to adult on eggplant, even though many records report injury to eggplant by *C. marmorata* (e.g., Gifu Plant Protection Center, 2005; Kagawa Plant Protection Office, 2005; Kochi Prefecture Crop Pest Control Center, 2005). Thus, what has driven the host range expansion is not better larval performance on (intact leaves of) the new hosts. Therefore, our hypothesis that *C. marmorata* expanded its host range to convolvulaceous plants because the plant quality is higher than the original host is not accepted.

On the two convolvulaceous plants, most larvae died during early development, especially in the first instar. However, most of those that passed through this stage survived. Therefore, most of the larvae might not have recognized these plants as food and starved to death. On the other hand, most of the individuals that recognized a plant as food were able to feed and develop on it, though some might be killed by poor nutrition. Herbivorous insects distinguish their hosts mainly by chemical compounds (Ehrlich & Raven, 1964; Futuyama & Agrawal, 2009). The azalea lace bug *Stephanitis pyrioides* (Hemiptera: Tingidae) distinguishes azalea varieties by leaf surface lipids (Balsdon, Espelie, & Braman, 1995). The Convolvulaceae are only distantly related to the Asteraceae (Angiosperm Phylogeny Group, 2009), and so their chemical components would be different. However, as some individuals fed on them, some of the chemicals could be similar to those of the original hosts. Such similarity might accelerate host range expansion to different families from the original hosts (Tuda et al, 2014).

Some previous studies have reported low larval performance in some phytophagous insects on novel host plants due to physiological non-adaptation (Bowers et al, 1992;

Keeler & Chew, 2008; Dai, Lu, Zhang, & Ding, 2014), whereas others suggested that pre-adaptation can contribute to similar performance as on the original hosts (Fox & Caldwell, 1994; Leclaire & Brandl, 1994; Newman, Borman, & Castro, 1997). Here, we found higher survival rate, shorter developmental time, and larger head width and body length on goldenrod and sunflower than on the novel plants. Even though the adults reared on novel hosts recognized the plant as food, the shorter developmental time and larger sizes show that the original hosts are more suitable than the other four species for the development of *C. marmorata*. Thus, *C. marmorata* has not yet adapted to the novel hosts.

Many studies, including ours, show that the suitability of novel plants for development may be not critical in host range expansion (e.g., Dai et al, 2014). One factors that is more important for range expansion would be high oviposition preference for novel plants. Adult females of some species lay eggs even on plants on which their offspring perform poorly (Gratton & Welter, 1998; Thompson, 1988b), as *C. marmorata* does. Yet even if the offspring performance is low, if some grow to the adult stage, those female adults may prefer their natal host plant (Hopkins' host selection principle). Therefore, such apparent mistakes in oviposition can be the first step toward a host shift (Thompson, 1988a; Thompson & Pellmyr, 1991; Davis & Cipollini, 2014). Even if most of the offspring on a novel plant die, even limited success may make the mother's oviposition on it adaptive.

Despite the many reports of injury by *C. marmorata* on eggplant, larvae did not complete their development to adult stage. This suggests that female adults cannot assess the unsuitability of eggplant for the larvae correctly in the field, and the injury found was mostly made by adults because the larvae would die soon after egg hatch. If this was the case, addition of eggplant to the host plants of *C. marmorata* would not occur in near future.

The sunflower and crown daisy belong to the same family as the goldenrod, Asteraceae. On sunflower, the survival rate was mostly the same as that on goldenrod. On the other hand, only two male adults were obtained from 60 larvae on crown daisy. As Asteraceae is a large family with large phylogenetic diversity (Mandel et al, 2019), it is not surprising that only a part of this family is suitable as the host of *C. marmorata*.

We used leaves with no or very low damage of sap feeding by *C. marmorata* for the experiment. This might have led our conclusion differently from what is occurring in nature, as injury level become high in summer and autumn, when the population density become high (Kato & Ohbayashi, 2009). When relative quality of the goldenrod become lower than the other plants, females may choose novel plants such as sweet potato. Investigation on larval performance on damaged leaves would be required.

Although *C. marmorata* is considered a specialist herbivore of several asteraceous plants, it has expanded its host range to distantly related plants in its introduced range. Many insects have been introduced as biological control agents all over the world (Howarth, 1991; Pemberton, 2000). Our results suggest that invasive species can have an unpredictable impact on native environments. When non-native organisms

are introduced as biological control agents, circumspect research about the agent ecology is necessary before introduction.

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Research Article
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Proof on the Divergence Times of Two Sympatric Species, *Rhynchophorus ferrugineus* and *R. vulneratus* (Coleoptera: Curculionidae) by Molecular Clock Analysis

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ABSTRACT

Molecular clock analysis on the oil palm weevil has separated the Red Palm Weevil, *Rhynchophorus ferrugineus* and Asiatic palm weevil, *Rhynchophorus vulneratus* as two sympatric species. Calibration was performed using the fossil of *R. cruentatus* which had evolved approximately 1 mya using sequences data of combined *COI* and *Cytb*. Divergence time indicated that *R. cruentatus*, the outgroup species evolved around 80.598±10 mya and finally speciated to form *R. ferrugineus* (≈1.926±10 mya) and *R. vulneratus* (≈4.857±10 mya). Additionally, the construction of Neighbour Joining (NJ) and Maximum Parsimony (MP) trees showed distinct separation using *COI, Cytb* and combination of *COI* and *Cytb*. These findings were highly supported by genetic distance analysis. Although a limited number of individuals from the small geographical area was used in this study, the genetic molecular clock analysis of mitochondrial data was able to effectively differentiate both species. These results reveal the first analysis to use the molecular clock to confirm the separation of these two sympatric species.

Key words: Red Palm Weevil, fossil, time divergence, molecular clock, evolution, mitochondria, *COI* and *Cytb.*

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INTRODUCTION

Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier, 1970) is categorised under the family Curculionidae of the weevil species and poses a serious threat to the trees of Arecaceae family (Molet, Roda, & Jackson, 2011). The invasion of the RPW significantly reduces the yield of coconut trees in the west coast of Peninsular Malaysia (Idris et al, 2014), and other palm trees e.g. dates and coconut around the world (Mazza et al, 2014). This species has complicated biology, for instance, the pest inhabits inside the tree trunk (Wattanapongsiri, 1966), and the larval and adult stages feed on different resources that renders developing control strategies difficult.

The adult of RPW displays a high degree of color polymorphism, especially for the two species namely *R. ferrugineus* and *R. vulneratus*. This color polymorphism has been discussed and studied by the taxonomists and other researchers. Hallet et al (1993) have studied the aggregation pheromones *R. ferrugineus* and *R. vulneratus* and stated that there was no significant difference between the two species in pheromone production, according to the chemical composition. According to Hallet, Crespi, & Borden (2004), the two colour-morphs in the weevil species, which is having orange or black marking, and black or red stripe are recognized as a single species, *R. ferrugineus*. Hallet et al (2004) have synonymise both species based on the morphological characters, molecular-genetic and breeding data. All that findings have claimed synonymous in both species, consequently invited a great debate among the researchers to prove that both species are cryptic species due to sympatric speciation.

The *R. ferrugineus* and probably its synonymized species, *R. vulneratus* are native from South-East Asia. They have expanded their distributional ranges, which indicates a geographic overlap in both species (*R. ferrugineus* and *R. vulneratus*) (Giblin-Davis, Faleiro, Jacas, Peña, & Vidyasagar, 2013). Rugman-Jones et al (2013) noted that distribution in the *R. ferrugineus* was distributed worldwide and had been found native to the continental southeast Asia (northern and western parts), Sri Lanka and Philippines, while *R. vulneratus* was distributed more to the southern part across Indonesia and had invaded California, U.S.A. It was also suggested that a few cryptic species of the *Rhyncophorus* might exist within the studied populations of both species. Thus, it is permissible to state that both species gathered in this study were species are native to Malaysia and other countries in the native range.

Both species have spread to other countries and regions through the import and export of dates and coconut plants within and outside the country (El-Mergawy et al, 2011). The study by Rugman-Jones et al (2013) has proven that *R. ferrugineus* and *R. vulneratus* are two different species using molecular-genetic of the *COI* mitochondrial data. The phylogenetic tree from the study has also been generated in three distinct lineages of *Rhynchophorus*, which are *R. ferrugineus, R. vulneratus* and *R. bilineatus*. The need for thorough research on these two sympatric species was raised and highlighted due to their high degree of polymorphism in coloration (Hallett et al 2004). Due to all the above reasons, both species were claimed as sympatric species, whereby either *R. vulneratus* or *R. ferrugineus* had evolved earlier, but both had finally been found inhabiting the same locality.

Therefore, this study aimed to investigate the difference between the Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier) and Asiatic Palm Weevil, *R. vulneratus* (Panzer) (Coleoptera: Curculionidae) using molecular data (*COI* and *Cytb*) by implementing molecular clock analysis to estimate the divergence time of each species. With that, the species separation can be seen clearly.

MATERIAL AND METHODS

Insect sampling and morphological identification

The sampling of palm weevils was conducted between April to June 2013. A total of eight palm weevils were collected from Kuala Selangor, Selangor (*R. vulneratus*) and Kuala Terengganu, Terengganu (*R. ferrugineus*), located at the center and east-coast part of Peninsular Malaysia (Fig. 1). The specimens collected were preserved in 70% alcohol and brought to the Entomology Laboratory in Universiti Kebangsaan Malaysia (UKM) for morphological identification prior to the molecular work. The morphological identification was based on taxonomical keys by Wattanapongsiri (1966) using the StemiD4 stereo microscope.

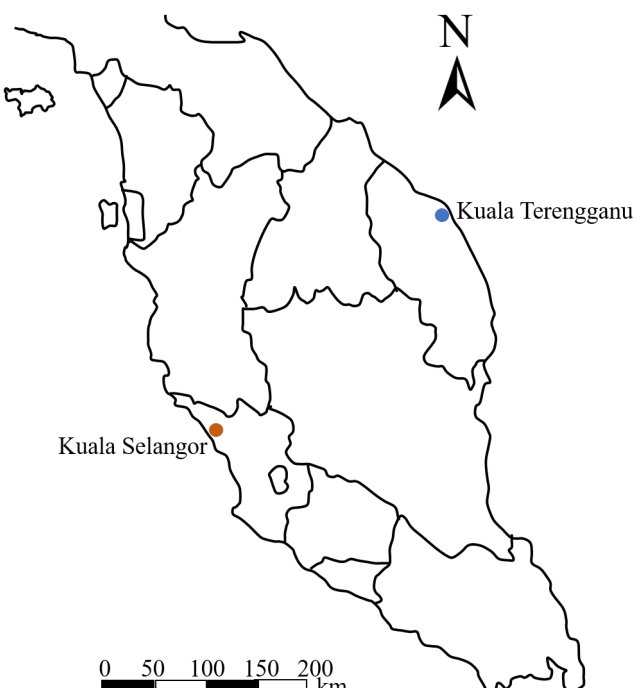


Fig. 1. Map of samples location collected in this study.

DNA extraction and PCR amplification

Tissue samples were taken from the posterior part of the metasomal tergite of the black or red stripe weevil (*R. vulneratus*), and the orange or black marking weevil (*R. ferrugineus*) (Rugman-Jones et al, 2013). DNA extraction of the tissue samples was performed using DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA).

The extracted samples were then subjected to PCR amplification. Optimization of PCR amplification for cytochrome oxidase subunit I (*COI*) and cytochrome B (*Cytb*) region were done for each of the individuals. A total of 25 μl PCR mixture consisted of 0.5 μl of 0.2mM dNTPs, 10 pmol of each primer, 1.25U of Taq polymerase, and 1 μl of 15mM $MgCl_2$ from Vivantis were prepared. PCR was performed using MyGene MG96G Thermalcycler or Thermocycler Perkin Elmer 240 under different conditions for each primer combination, starting with initial denaturation for 3 minutes at 94°C, followed by 39 cycles; denaturation for 1 min at 92°C, annealing for 1 min at 47°C, an extension for 1 min at 72°C and final extension for 5 mins at 72°C (Mohammed, Aman-Zuki, Yusof, S., Md-Zain, & Yaakop 2017; Halim et al, 2018; Aman-Zuki, Mohammed, Md Zain, & Yaakop 2019). Two sets of universal primers used were *COI* [Ron (5' GGA TCA CCT CAT ATA GCA TTC CC 3') (Forward); Nancy (5' CCC GGT AAA AAT TAA AAT ATA AAC TTC 3') (Reverse)] (Simon et al, 1994; Monteiro & Pierce, 2001) and *Cytb* [(CB-J-10933 5' TCT TTT TGA GGA GCW ACW GTW ATT AC 3'; CB-N-11367 5' AAT TGA ACG TAA AAT WGT RTA AGC AA 3'] (Smith, Kambhampati, Völkl, & Mackauer, 1999; Smith & Kambhampati, 1999) to yield 600 bp and 560 bp fragments.

Sequencing and phylogenetic analyses

PCR products for each species were then sent to First Base Sdn. Bhd., Selangor, Malaysia for sequencing. The sequences were then edited using Sequencher 4.8 and aligned using MacClade 4.08. Before the phylogenetic analyses, the genetic distance between the species was obtained for both markers. For phylogenetic analyses (Table 1), the Neighbor Joining (NJ) tree was constructed using Kimura-2 parameter and the bootstrap was analysed (Kimura & Ohta, 1972). The maximum parsimony (MP) tree(s) were generated using PAUP* 4.0- test version 4.0d63 (Swofford, 1998) to get the most parsimonious tree(s). A heuristic parsimony search (Hillis, Moritz, & Mable, 1996) was performed using 100 replicates of random addition sequences, including the TBR (tree bisection reconnection) option for branch swapping. Each base was treated as an unordered character with equal weight, with gaps treated as missing data. Statistical support was obtained by bootstrap analysis with 100 replications (Felsenstein, 1985).

For Bayesian analysis (BI), nucleotide substitution model was selected using jModelTest 2.1.4 (Ronquist et al, 2012). Bayesian Inference trees for *COI*, *Cytb* and combined *COI* and *Cytb* were generated using MrBayes 3.1.2 software with Markov Chain Monte Carlo algorithm (Huelsenbeck, Larget, & Alfaro 2004). The length of the generation chain was analyzed until the value of split frequency is lower than 0.01. The burnin of the final tree was set up at 25% (Drummond, Ho, Phillips, & Rambaut 2006).

Table 1. List of samples used in the molecular and phylogenetic analyses.

Code sample	Species	Locality	Accession No. *COI*	Accession No. *Cytb*
K01	*Rhynchophorus vulneratus*	Malaysia: Selangor, Kuala Selangor	MG051024	MG051032
K02	*Rhynchophorus vulneratus*	Malaysia: Selangor, Kuala Selangor	MG051025	MG051033
K04	*Rhynchophorus vulneratus*	Malaysia: Selangor, Kuala Selangor	MG051026	MG051034
K05	*Rhynchophorus ferrugineus*	Malaysia: Terengganu: Kuala Terengganu	MG051027	MG051035
K06	*Rhynchophorus ferrugineus*	Malaysia: Terengganu: Kuala Terengganu	MG051028	MG051036
K07	*Rhynchophorus ferrugineus*	Malaysia: Terengganu: Kuala Terengganu	MG051029	MG051037
K08	*Rhynchophorus ferrugineus*	Malaysia: Terengganu: Kuala Terengganu	MG051030	MG051038
K10	*Rhynchophorus ferrugineus*	Malaysia: Terengganu: Kuala Terengganu	MG051031	MG051039
GBMIN32436	*Rhynchophorus ferrugineus*	Japan	GU581524	-
GBMIN32437	*Rhynchophorus ferrugineus*	Japan	GU581522	-
GBMIN32267	*Rhynchophorus ferrugineus*	Japan	GU581521	-
GBMIN32268	*Rhynchophorus ferrugineus*	Japan	GU581519	-
GBMIN32269	*Rhynchophorus ferrugineus*	Japan	GU581518	-
GBMIN32439	*Rhynchophorus ferrugineus*	Japan	GU581517	-
	Rhynchophorus palmarum	-	GU581629	GU581656
	Rhynchophorus cruentatus	-	AY131113	

Molecular clock analysis

The analysis to measure and estimate the time divergence of the RPW species was done using a combination sequence data of *COI* and *Cytb*. Two compatible softwares used for the time divergence analysis were BEAUti and BEAST version v1.10.4 (Suchard et al, 2018). The nucleotide substitution model was chosen using jModelTest (Ronquist et al, 2012) prior to analysis. The chosen model was HKY+G. The clock partitioning was set for ingroup and outgroup taxa with *R. cruentatus* (AY131113) as outgroup and set with tip date from time fossil for Palmetto Weevil, *Rhynchophorus cruentatus* Fabricius (Insecta: Coleoptera: Curculionidae) which is 1 million years ago (Weissling & Giblin-Davis, 1997). Lognormal relaxed clock was chosen for clocks model referring to Heath, Huelsenbeck, & Stadler (2014) to remove the assumption of a strict molecular clock in the uncertain or unresolved topology of phylogenetic tree. The Speciation: Birth-Death Process was chosen for the tree before estimation of the speciation with the rate of birth (branching speciation) and death (extinction) incorporated in the lineage following (Stadler, Gavryushkina, Warnock, Drummond, & Heath, 2018) and (Herbst, 1795). The time fossil for *Rhynchophorus* used was 48.6-40.4 ± 10 mya as reported from (Rambaut & Drummond, 2015). The length of the chain for this analysis was fixed at 10,000,000 generations of Markov Chain Monte Carlo with tree sampling at every 1000 generations. The burnin of the final tree was set up at 25% from total tree using TreeAnnotator 1.7.5 (Drummond & Rambaut, 2007). Molecular clock tree was visualized using FigTree 1.4.2 (Posada, 2008).

RESULTS

Morphological identification

The weevil samples were identified based on the color morph according to Wattanapongsiri (1966) description, in which orange with black marking was characterized for the *R. ferrugineus*, while black with red stripe was for the *R. vulneratus*. Three individuals collected from Kuala Selangor were identified as *R. vulneratus* (K1-K2, K4), while other specimens collected from Kuala Terengganu (K5-K8, K10) were identified as *R. ferrugineus.*

Phylogeny tree reconstruction and genetic distances

All individuals were successfully extracted and amplified using both markers, viz. *COI* and *Cytb* in the length of 600 and 560 bp. Phylogeny analyses have confirmed that both spotted and striped weevils were located in different clades for each molecular data in NJ, MP and BI analyses (Figs. 2a-c, 3a-c and 4a-c). In the phylogeny results, *Rhynchophorus vulneratus and R. ferrugineus* are located in different clades and supported with 100% bootstrap values in both analyses. The specimens from Japan were added in the *COI* datasets and yet still showed the separation of the outgroup and ingroups (*R. cruestatus* and *R. palmarum*) at different lineages and highly supported with 100% bootstrap value. The genetic data differences showed that individuals of the *R. ferrugineus* from Malaysia and Japan were 0.047 (Table 2).

Table 2 and 3 show the genetic distance for individuals in *R. vulneratus* and *R. ferrugineus* using *COI* and *Cytb* data. Genetic distance for both *COI* and *Cytb* markers showed that individuals among the *R. vulneratus* had high divergences compared to *R. ferrugineus*. In *COI*, genetic distances among the *R. vulneratus* were 0.010-0.029, while among *R. ferrugineus* the values were 0.000-0.047. In *Cytb*, genetic distances among *R. vulneratus* were 0.016-0.019, while among *R. ferrugineus* the values were 0.000-0.05.

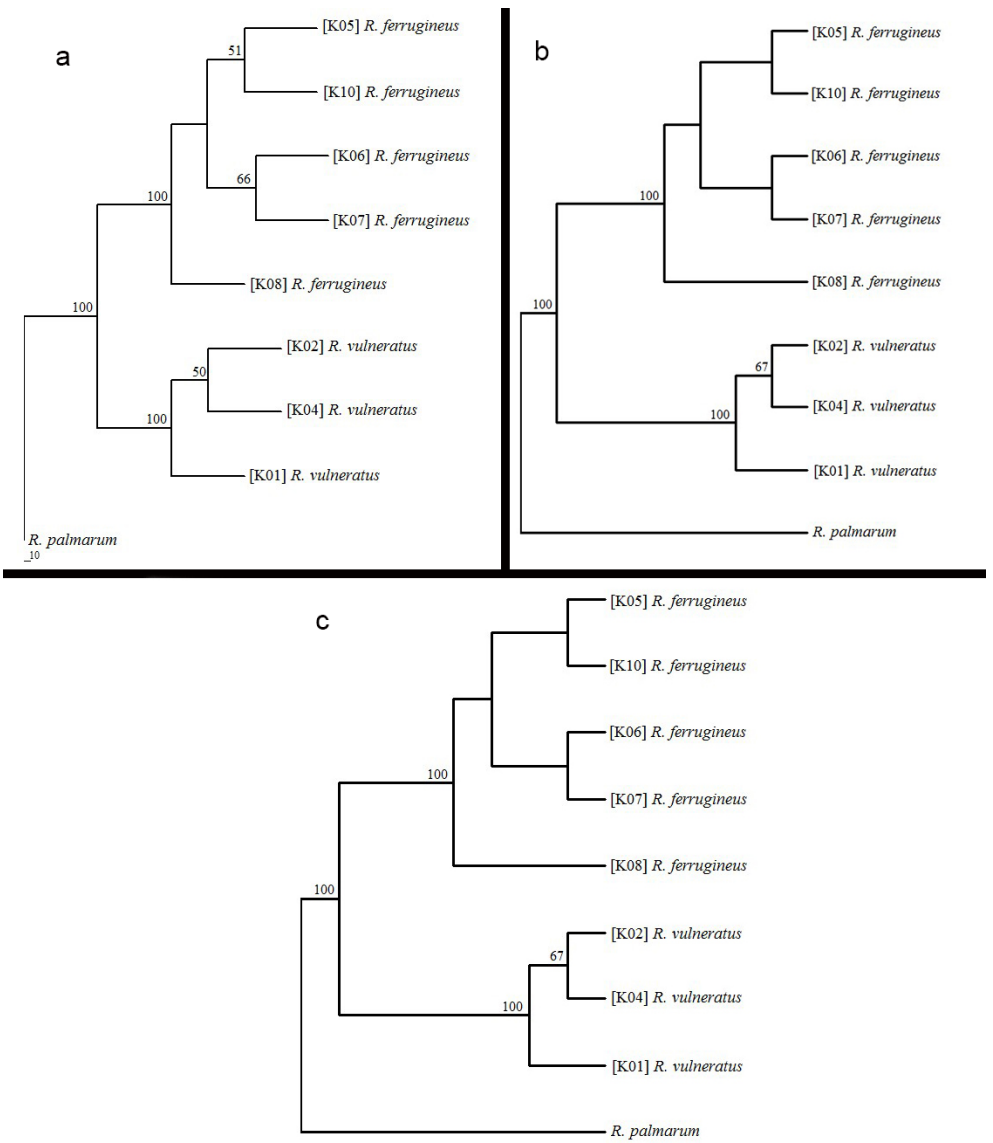
Estimating divergence time

The result of molecular clock analysis (Fig. 5) based on the combination of *COI* and *Cytb* (1367 bp) molecular data showed different divergence times in both sympatric species (*R. vulneratus* and *R. ferrugineus*) and the outgroup species, *R. cruentatus* Fabricius (Coleoptera: Curculionidae). The divergence times showed that *R. cruentatus* had diverged earlier compared to *R. vulneratus* and *R. ferrugineus* since around 80.598±10 mya followed by *R. vulneratus* at ≈4.857±10 mya and the latest was *R. ferrugineus* at ≈1.926±10 mya.

DISCUSSION

In this paper, the Malaysian samples were taken as a model species to measure the time divergence of *Rhynchophorus ferrugineus* and *R. vulneratus* from the Oriental region. Although a small-scale area (Peninsular Malaysia only) was considered in

this study, it was very significant in influencing the speciation process. In this study, *R. palmarum* was selected as the outgroup for comparing the two cryptic species. According to Löhr, Vásquez-Ordóñez, & Lopez-Lavalle (2015), the cryptic species (*R. ferrugineus* and *R. vulneratus*) was distinctly separated from the *R. palmarum* using *COI* sequences data with the support of phylogenetic analysis in species clustering.



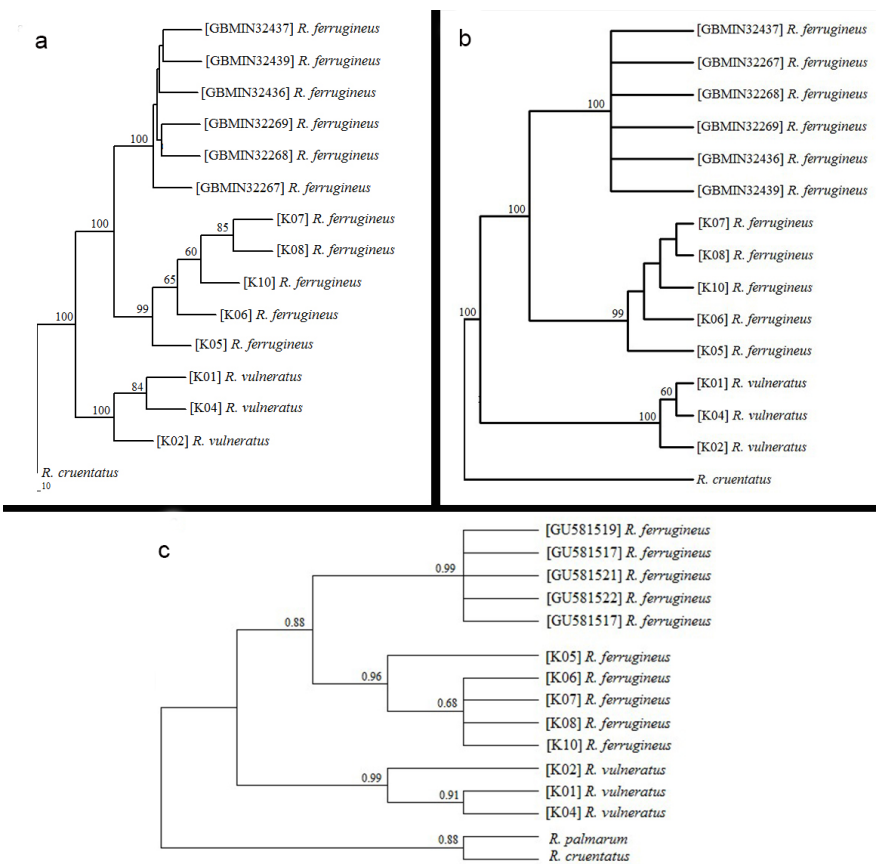
Figs. 2a-c. Phylogenetic tree of the *Rhynchophorus ferrugineus* and *R. vulneratus* based on NJ (a), MP (b) and BI (c) analyses using *Cytb* data sequences.

Table 2. Genetic distance of species, *R. vulneratus*, *R. ferrugineus* and *R. palmarum* using *COI* data.

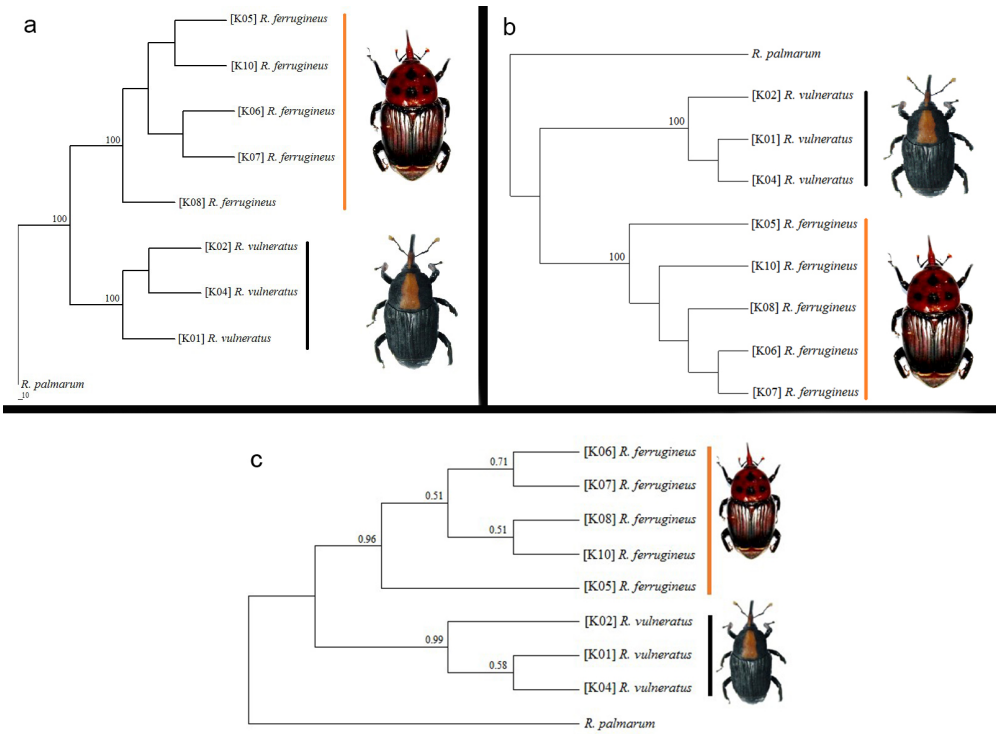
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
(1) [K01] *R. vulneratus*															
(2) [K02] *R. vulneratus*	0.029														
(3) [K04] *R. vulneratus*	0.010	0.024													
(4) [K05] *R. ferrugineus*	0.141	0.139	0.136												
(5) [K06] *R. ferrugineus*	0.141	0.139	0.136	0.000											
(6) [K07] *R. ferrugineus*	0.141	0.139	0.136	0.000	0.000										
(7) [K08] *R. ferrugineus*	0.141	0.139	0.136	0.000	0.000	0.000									
(8) [K10] *R. ferrugineus*	0.141	0.139	0.136	0.000	0.000	0.000	0.000								
(9) [GBMIN32436] *R. ferrugineus*	0.150	0.156	0.150	0.047	0.047	0.047	0.047	0.047							
(10) [GBMIN32437] *R. ferrugineus*	0.150	0.156	0.150	0.047	0.047	0.047	0.047	0.047	0.000						
(11) [GBMIN32267] *R. ferrugineus*	0.150	0.156	0.150	0.047	0.047	0.047	0.047	0.047	0.000	0.000					
(12) [GBMIN32268] *R. ferrugineus*	0.150	0.156	0.150	0.047	0.047	0.047	0.047	0.047	0.000	0.000	0.000				
(13) [GBMIN32269] *R. ferrugineus*	0.150	0.156	0.150	0.047	0.047	0.047	0.047	0.047	0.000	0.000	0.000	0.000			
(14) [GBMIN32439] *R. ferrugineus*	0.150	0.156	0.150	0.047	0.047	0.047	0.047	0.047	0.000	0.000	0.000	0.000	0.000		
(15) *R. palmarum*	0.222	0.216	0.216	0.182	0.182	0.182	0.182	0.182	0.198	0.198	0.198	0.198	0.198	0.198	

Table 3. Genetic distance of species, *R. vulneratus*, *R. ferrugineus* and *R. palmarum* using *Cytb* data.

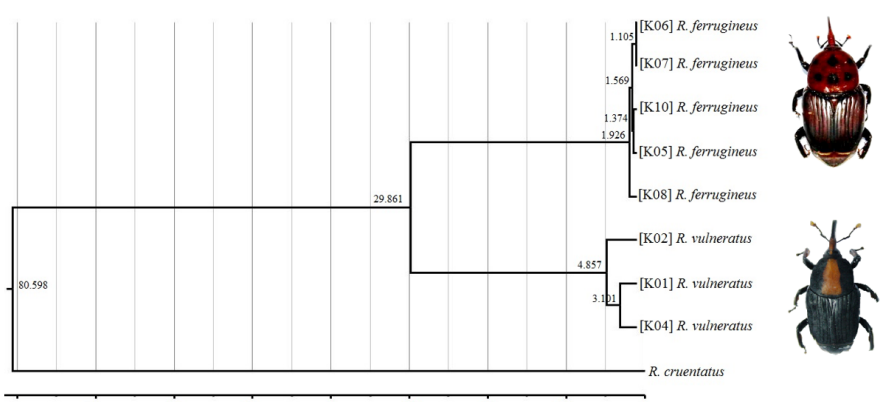
	K01	K02	K04	K05	K06	K07	K08	K10	*R. palmarum*
[K01] *R. vulneratus*	-								
[K02] *R. vulneratus*	0.019								
[K04] *R. vulneratus*	0.019	0.016							
[K05] *R. ferrugineus*	0.146	0.154	0.149						
[K06] *R. ferrugineus*	0.143	0.157	0.153	0.003					
[K07] *R. ferrugineus*	0.143	0.157	0.153	0.003	0.000				
[K08] *R. ferrugineus*	0.150	0.157	0.153	0.003	0.005	0.005			
[K10] *R. ferrugineus*	0.150	0.157	0.153	0.003	0.005	0.005	0.005		
R. palmarum	0.282	0.292	0.282	0.294	0.290	0.290	0.299	0.300	-



Figs. 3a-c. Phylogenetic tree of the *Rhynchophorus ferrugineus* and *R. vulneratus* based on NJ (a), MP (b) and BI (c) analyses using *COI* sequences data.



Figs. 4a-c. Phylogenetic tree of the *Rhynchophorus ferrugineus* and *R. vulneratus* based on NJ (a), MP (b) and BI (c) analyses using combination of *Cytb+COI* sequences data.



Figs. 5. Molecular clock tree of *Rhynchophorus ferrugineus* and *R. vulneratus* using combination of *Cytb* + *COI* sequences data.

Even though the body colour and spots could not clearly differentiate the two studied species, *R. ferrugineus* and *R. vulneratus,* the *COI* marker on the other hand,

has shown a species separation. Although a small number of weevil specimens was examined in this study as compared to that of (Rugman-Jones et al 2013), a study of the species time divergence to clarifying the *R. ferrugineus* and *R. vulneratus* as a sympatric species would be the most effective method and a significant item to be measured and investigated, despite the fact the factors for the speciation of the species are still very much in doubt. In addition, only a small scale of samples need to be utilized to elucidate a complex morphology or cryptic species. Likewise, there had also been similar studies on the different genetic data among cryptic species of gecko (Oliver, Adams, & Doughty, 2010), and we believed that this could reveal a similar pattern in insect species.

Previous studies on *R. ferrugineus* and *R. vulneratus* only focused on either morphological (Wattanapongsiri, 1966; Sazali, Hazmi, Abang, & Jemain, 2018) or molecular (Rugman-Jones et al, 2013) aspects but none reported on the incorporation of both aspects and species, except by Hallet et al (2004). As a result, various conclusions regarding the species status of *R. ferrugineus* and *R. vulneratus* were presented. According to Wattanapongsiri (1966), the identification of *R. ferrugineus* and *R. vulneratus* was mainly based on the colour morph, the shape of the pronotum as the second morphological characters while Rugman-Jones et al (2013) debunked that the protonum shape was one of the distinguished characters in two species. A study by Sazali et al (2018) reported that the morphological differences between *R. ferrugineus* and *R. vulneratus* were significant in terms of the size of the species. *Rhynchophorus vulneratus* is slightly larger compared to *R. ferrugineus* with the length of pronotum for *R. vulneratus* is measuring 12.87 mm, while for *R. ferrugineus* it is 11.51 mm, and the mean width of elytra is 13.60 mm, it is 11.44 mm for *R. vulneratus* and *R. ferrugineus*, respectively.

Results from the molecular studies indicated that *R. ferrugineus* and *R. vulneratus* differed from each other and this was supported by the morphological features depicted by Wattanapongsiri (1966). According to Hallett et al (2004), the crossbred of *R. ferrugineus* and *R. vulneratus* have successfully produced a fertile F1. However, the survival of the hybrid was not further discussed in the paper. Additionally, they provided a weak single proof of DNA similarity in which only 201 bp of mitochondrial DNA sequence in the *COI* gene were identical between the two species. In addition, a study by Sukirno et al (2018) had confirmed that the undescribed color polymorphism existed in Indonesia *R. vulneratus* samples, in which rusty red polymorphisms had been wrongly identified as *R. ferrugineus*. The low interspecific variability was also detected in *COI* sequences in the Indonesia and Saudi Arabia samples, despite *COI* having proven to be a great marker for species separation. Sukirno, Tufail, Rasool, & Aldawoo (2020) also had stated that pronotal markings and *COI* sequences were able to separate the *R. ferrugineus* under three haplotypes number. Moreover, in this study, the *COI* marker has proven effective in species separation, especially on the Malaysian samples, but using different primers that have not been used earlier by Sukirno et al (2018) and Sukirno, Tufail, Rasool, Husain, & Aldawood (2020b).

Referring to Filchak, Roethele, & Feder (2000), speciation may occur due to changes in hosts and does not only depend upon geographical isolation integrating sterility and life development. We assumed that the changing of the pronotum coloration in both *Rhynchophorus* species from orange or black marking (*R. ferrugineus*) to black or red stripe (*R. vulneratus*) or vice versa indicated major changes in the evolution and speciation to finally become two distinct species. A study by Kelley, Fitzpatrick, & Merilaita (2013) also reported that the coloration and spots of coral reef fishes were investigated, and they found that the presence of spots and stripes on the fish body were correlated with several parameters. Due to this, fish biology seems to be developing as a whole in parallel with the fish's evolution, for example in body length, feeding behavior and habitat preferences.

To date, no clear studies have been performed on biological and geographical factors that separate the weevil species from Malaysia and other areas in their native range. Furthermore, the divergence time for both species took place much earlier at ≈29.861±10 mya. The molecular clock analysis also revealed that *R. vulneratus* formed as a new species earlier at ≈4.857±10 mya as compared to *R. ferrugineus* at ≈1.926±10 mya. These findings provide a baseline data of molecular clock study in *Rhynchophorus* spp. regardless of the sample size.

In terms of evolutionary processes, we hypothesize that the changes in coloration from a fully black weevil (*R. palmarum*) to black or red stripe (*R. vulneratus*) is a characteristic that commonly changes through evolution. For example, a similar change took place in the *Timema* species of walking sticks in their dorsal stripes through their interactions with the host plants (Sandoval & Crespi, 2008; Farkas et al, 2013). However, this process required a very long time due to geographical isolation in parallel with a gradual process of speciation that led to reproductive isolation (Mayr, 1942). In this situation, we believed that *R. palmarum*, which is native to South America and countries nearby, took approximately 500,000 years to become *R. vulneratus* is native to Southeast Asia and Indo-Malaya.

Furthermore, both sympatric species started diverging into different subpopulations from 29.861 mya and started to speciate after a relatively short time, approximately 3 mya. This may have been influenced by changing the host plant from the coconut tree to the date tree. We believed that the process started in an area where both coconut and date trees grew together. For this reason, competition occurred that correlated with the availability of the plant host itself as a food source. A population of *R. vulneratus* changed its host plant from coconut to date trees and had speciated to form *R. ferrugineus* within a short time. Similar events have happened in species not limited to insects as seen in studies by Bush et al (1989), Bush & Butlin, (2004), but also in other kinds of animal species, for example in the coral-dwelling fish genus *Gobiodon* (Munday, Van Herwerden, & Dudgeon, 2004).

The results of the molecular clock analysis of this study supported that *R. ferrugineus* and *R. vulneratus* were two valid species with *R. vulneratus* being formed as a new species earlier than *R. ferrugineus*. The time of separation for *R. ferrugineus* and *R. vulneratus* is generally accepted as that for the separation of *R. ferrugineus*,

which for *R. bilineatus*, *R. phoenicis*, *R. cruentatus* and *R. palmarum* was from 2.3 to 3.7 million years ago. Furthermore, a study by Hallett et al (2004) stated that *R. ferrugineus* and *R. vulneratus* had separated from *R. bilineatus* about 5 million years ago.

In this study, the genetic distance among members of *R. vulneratus* was higher than that of *R. ferrugineus* for both markers, indicating a high divergence in *R. vulneratus* species (Figs. 2-4, Table 2-3). However, the value of the *COI* obtained from the genetic distance analysis was lower (0.000-0.047) compared to the values obtained by El-Mergawy et al (2011) in *R. ferrugineus* at a range of 0.058–0.095. Even though the results showed slight difference in the genetic distance value however, there are a few assumptions that can be made regarding this situation. Firstly, there may be cryptic species within *R. vulneratus* that have created a higher genetic distance value (Rugman-Jones et al, 2013). Secondly, the occurrence of sympatry within the *R. vulneratus* species may create higher values for genetic distance. Sympatry within species has been reported in Curculionidae, for example in the *Laparocerus* weevil (Faria et al, 2016) and *Mecinus* weevil (ToŠevski et al, 2011). Furthermore, high genetic diversity is a characteristic exhibited in the native range of the species. For example, the high genetic diversity in *R. ferrugineus* from Pakistan indicates that this species is native to Pakistan (Yasin, Rugman-Jones, Wakil, & Stouthamer, 2016).Molecular clock studies provide important information regarding the species being studied. In our study, molecular clock analysis was able to prove the status of *R. ferrugineus* and *R. vulneratus* as two valid species. Likewise, a study by (Zhang et al, 2005) provided proof of the existence of a *Coptalabrus* species (Carabidae) using the divergence time derived from a phylogeographic study of this species in South Korea. This demonstrates that information regarding divergence time is important for phylogeographic study. This is because phylogeographic study involves the evolutionary history, population genetics, geography and divergence time of the species (Edwards & Beerli, 2000).

CONCLUSION

Molecular clock analysis using the combined data of *COI* and *Cytb* has firmly separated the sympatric species, *R. ferrugineus* and *R. vulneratus.* Based on the divergence time, we have discovered that the species speciate to form *R. ferrugineus* from ≈1.926±10 mya and *R. vulneratus* ≈4.857±10 mya. The separation was also supported by phylogenetic analyses of NJ, MP and BI, as well as proven by the data of the distance analysis. The information gathered by this study will be able to provide information for future regional studies related to the RPW.

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First Insights into the Springtime Butterfly (Rhopalocera) Fauna of Podgorica (Montenegro, Balkan Peninsula)

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ABSTRACT

The work shows the results of research on butterfly species richness, which took place in Podgorica (Montenegro) between April and June of 2017. The material was gathered on 14 sites located within the city borders. Observations confirmed the presence of 48 species of butterflies representing 5 families: Hesperidae (5 species), Papilionidae (3 species), Pieridae (9 species), Lycaenidae (13 species) and Nymphalidae (18 species). The most common species were I*philcides podalirius*, *Papilio machaon*, *Colias croceus*, *Coenonympha pamphilus*, *Polyommatus icarus* and *Aricia agestis*. Results are discussed on a background of two species lists from other urban areas of Balkan Penisula (Zagreb and Patras) as well as a diversity of the butterfly fauna of Montenegro. It is the first analysis of the butterfly fauna of Podgorica city.

Key words: Lepidoptera, urban fauna, urban entomology, species richness, butterfly survey.

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INTRODUCTION

Urbanisation is amongst the most important reasons of biodiversity loss. Constant expansion of urban infrastructure onto natural ecosystems makes it one of the key problems of today's environmental protection (McKinney, 2002; McKinney, 2006; Clark, Reed, & Chew, 2007). Impermeable urban spaces reduce the amount of water reaching the soil, affecting the composition and distribution of plant communities. Local fauna is dependent on a mosaic fragmented landscapes created by small patches of vegetation and spaces covered with buildings, streets and pavements (McKinney, 2002; Alberti, 2005). Cities host a very specific set of species. Plant and animal communities are often simplified and dominated by synanthropic organisms, show lower diversity than natural areas and also are vulnerable to disturbances or influence of invasive species (Rebele, 1994; Alberti, 2005). Urban fauna is also characterized by high similarity, even between very distant cities (McKinney, 2006). Therefore, comprehensive biodiversity inventories are an indispensable element of current studies. Recognition of faunal communities might be a good base for further ecological studies as well as research focused on long term changes in species composition and distribution patterns (Pollard, 1977; Blair, 1999; McKinney 2007, Rochat, Manel, Deschamps-Cottin, Widmer & Joost, 2017; Lang, Dixon, Klaver,Thompson & Widrlechner, 2019; Aguilera, Ekroos, Persson, Petersson & Öckinger, 2019).

Butterflies are considered good indicators of changes associated with urbanization gradients and are frequently studied in cities all over the world (Blair & Launer, 1997; Thomas, 2005; Bergerot, Fontaine, Julliard, & Baguette, 2011; Konvicka & Kadlec, 2011; Dallimer et al, 2012; Koren, Zadravec, Ńtih, & Hlavati, 2013; Matsumoto, 2015; Ramírez-Restrepo & Macgregor-Fors, 2017; Rochat et al, 2017; Sobczyk, Pabis, Wieczorek, & Salamacha, 2017; Luppi, Dondina, Orioli, & Bani, 2018; Lang et al, 2019; Tzortzakaki, Kati, Panitsa, Tzanatos, & Giokas, 2019). At the same time our current knowledge on the butterfly fauna of larger cities on the Balkan Peninsula is poor. This area is situated in the Mediterranean sea basin - one of the 25 global biodiversity hotspots (Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). Moreover the Balkan is an area with the highest level of endemism in Europe (Krystufek & Reed, 2004), what makes it more than appropriate to observe human influence on biodiversity.

The urbanisation level of Monetenegro is above 60%, which means that more than half of population lives in the cities. Podgorica is inhabited by about 30% of citizens of Montenegro and is the largest city in this country. The latest checklist of Montenegrian butterflies comprises 192 species (Franeta, 2018). Butterflies of Montenegro have mostly been investigated in larger natural ecosystems or protected areas like the Durmitor National Park, while the rest of the country is still poorly described in terms of butterfly diversity (Nicholl, 1899; Nicholl, 1902; Gibbs, 1913; Rebel, 1913; Sijarić 1984; Sijarić, Lorković, Carnelutti, & Jakšić, 1984; Koçak, 1989; Jakšić & Ristić, 1999; Radović et al, 2008; Švara, Zakńek, & Verovnik, 2015; Sobczyk & Gligorović, 2016). None of the studies was focused strictly on the urban areas (Franeta, 2018), although the region neighbouring to Podgorica was recently studied by Švara et al (2015) as well as Sobczyk & Gligorović (2016).

The aim of this study was to analyse the species richness of butterflies in Podgorica. It is the first study of the butterfly fauna of this city.

MATERIAL AND METHODS

Study area

Podgorica is the capital of Montenegro and is situated on the Zeta river plateau and surrounded by Kučke and Piperske mountain chains (Stešević, Caković, & Jovanović, 2014). The city consists of three basic districts: Novi Grad, Nova Varos, and Stara Varos, which are naturally separated by two rivers - Morača and Ribicnia (Stešević et al, 2014; Vujadinović, 2016). Podgorica is a developing city, where plenty of green space can be found, especially in the newest district Novi Grad, where green belts are distributed along most of the streets (Vujadinović, 2016). It is also worth to point out a special value of migration corridors for fauna along river banks as well as the presence of interesting xerothermic sites located on hills like Gorica or Malo Brdo. Podgorica is not a metropolitan type of city, thus it is difficult to draw strict borders delimiting urbanization zones. The most densely inhabited space can be found in the Stara Varos and the adjacent part of Nova Varos, while the rest of the residental areas are characterised by more dispersed buildings. The area surrounding the residential areas is used for industry and agriculture.

Data were collected on 14 sites (Fig. 1). Sites were chosen to represent different types of habitats. Investigated areas can be described as a mosaic of ruderal vegetation, parks, forests, meadows, hills, bushes, wastelands, crop fields and pastures. Specific characteristics of each site are given in Table 1.

Field studies

Data about the butterfly fauna of Podgorica were collected between April 15th and 30th of June 2017. Sites 1 - 8 were investigated 8 to 12 times during the whole observation period. The Mareza (site 9) was visited five times and sites 10-14 were visited only once (Table 1). Observations were qualitative and were carried out between 9 am - 6 pm under appropriate weather conditions: no rain, no strong wind, preferable sunny or mostly sunny days (Van Swaay, Brereton, Kirkland, & Warren, 2012). Time spent on particular site depended on its size. Four size classes were distinguished: small (up to 2 ha), medium (2-6 ha) and large (above 10 ha) sites. Butterflies were identified alive and photographed either, in natural conditions or after capture with an entomological net.

Data analysis

Analysis of ecological attributes of all recorded species was done based on the literature data (Sielezniev & Dziekańska, 2010; Tolman & Lewington, 1997) according to the method proposed by Shreeve, Dennis, Roy, & Moss (2001). Bray-Curtis similarity index was used to analyse ecological similarity of species.

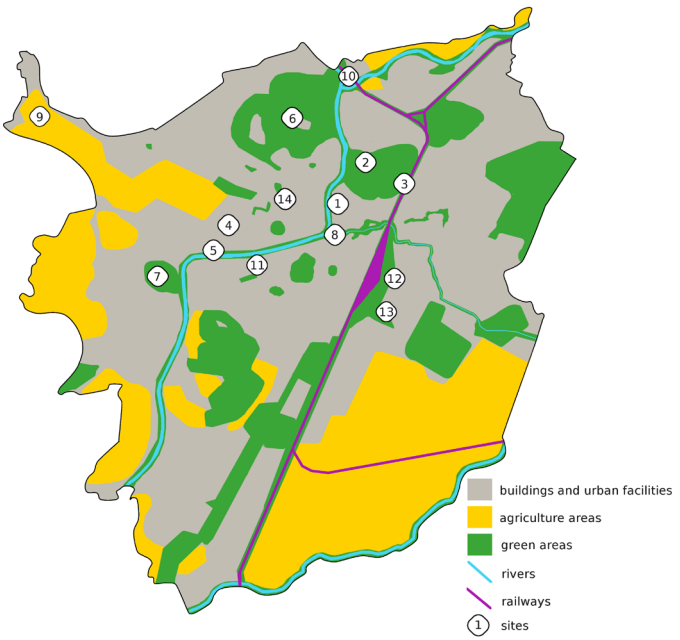

Fig. 1. Map of Podgorica with sites distribution on simplified image of landscape usage according to Stešević et al (2014).

Table 1. Location and description of observation sites.

No.	Observation site	Co-ordinates	Approximate site area [ha]	Size class	Number of visits	Habitat types
1	Milenium Bridge	42.446228, 19.260141	1,9	Small	10	Ruderal, Wasteland, Bushes
2	Park Šuma Gorica N-W	42.450784, 19.264975	3,9	Medium	11	Park, Meadow, Forest, Hill, Bushes
3	Park Šuma Gorica S-E	42.447554, 19.278419	4,5	Medium	9	Meadow, Hill, Bushes
4	Wasteland next to University of Montenegro	42.441740, 19.239559	3,0	Medium	12	Ruderal, Wasteland, Bushes
5	Wasteland next to Delta city mall	42.434825, 19.236777	0,5	Small	8	Wasteland, Meadow, Bushes
6	Malo Brdo	42.457750, 19.252788	8,7	Large	10	Hill, Meadow, Bushes, Pasture
7	Gorica Hill	42.428867, 19.221493	6,6	Large	9	Hill, Meadow, Bushes, Pasture
8	Old Bridge on Ribicnia River	42.439336, 19.258913	0,8	small	10	Ruderal, Park
9	Mareza	42.460069, 19.189503	39,2	Vast	6	Meadow, Pasture, Bushes, Agricultural landscape
10	Zeta and Moraca connection point	42.466689, 19.264685	4,9	Medium	2	Meadow, Bushes
11	Park Šuma Ljubović	42.431158, 19.254193	3,6	Medium	1	Park, Hill, Forest
12	Wasteland next to railway station	42.432230, 19.271820	11,2	Vast	1	Ruderal, Wasteland, Meadow
13	Stari Aeodrom district next to Tuški put	42.423857, 19.269952	2,5	Medium	1	Wasteland, Forest
14	Momišići	42.447793, 19.255759	3,1	Medium	1	Hill, Ruderal

The matrix for similarity comparison consisted of the following attributes: wing span (small: up to 3 cm; medium: 3 - 4 cm; big: above 4 cm), host plants used by caterpillars (polyphagous, oligophagous, monophagous), type of host plant used by caterpillars (grasses, herbs, shrubs, trees), dispersal potential (good disperser, poor disperser), myrmecophily (myrmecophilous, non myrmecophilous), habitat preference (rocky, ruderal, dry, humid, open, forest), overwintering stadium (egg, caterpillar, pupa, imago). Hierarchical agglomerative clustering and group average grouping method was used (Shreeve et al, 2001; Clarke, Gorley, Somerfield, & Warwick, 2014).

The analysis was performed in Primer 5.0 (Clarke et al., 2014).

RESULTS

Altogether 48 species of butterflies were observed from five different families: Nymphalidae (18 species), Lycaenidae (13 species), Pieridae (9 species), Hesperiidae (5 species), Papilionidae (3 species) (Table 2). The most common species were: *Iphilcides podalirius*, *Papilio machaon*, *Colias croceus*, *Coenonympha pamphilus*, *Polyommatus icarus* and *Aricia agestis*. They were recorded on all or almost all sites.

Seven species (*Zerynthia polyxena*, *Cupido decolorata*, *Lysandra bellargus*, *Plebejus argus*, *Cyaniris semiargus*, *Hipparchia volgensis* and *Euphydryas aurinia*) were found on only one of the investigated sites.

The highest total number of species was found on hill Malo Brdo (30 species), and the lowest number of species was recorded next to the Old Bridge on Ribicnia River (14 species). On average 7 species were observed during a single visit, the lowest number being 4 species per visit (Old Bridge on Ribicnia River, Wasteland next to Delta city mall) and the highest 10 species per visit (Malo Brdo, Mareza). Distribution of each species is given in Table 2.

All butterflies found in Podgorica have been assigned to Least Concern (LC) category on the Red List of Mediterranean Butterfly Species (Numa et al, 2016).

The Bray-Curtis similarity analysis distinguished five ecological groups of species (Fig. 2). For 1 - 4 groups, the similarity is 50% or higher and for group 5 is 38%. Group 1 includes mostly migratory (good dispersers) oligophagus species feeding on grasses. Group 2 consists of mostly large body size butterflies feeding on herbs or grasses. Group 3 is mainly comprised of small myrmecophilous species. Butterfly species in group 4 have an average body size, feed on herbs and prefer dry and open habitats. Group 5 consists of polyphagous species which are good dispersers. The full ecological characteristics of particular clusters are described in Table 3.

DISCUSSION

This study was a first attempt to describe the butterfly fauna of Podgorica by using monitoring scheme. The butterfly fauna of Podgorica is rich in species. Despite the relatively short study period (from April to the end of June) about 25 % of all Montenegrian butterflies were found in the city (Franeta, 2018). Some of the species

like *Celastrina argiolus* and *Antocharis cardamines* might not have been recorded only due to their early spring activity. Two species of *Hipparchia* were observed, but only one (*Hipparchia fagi*) was captured. The other species was only observed from a distance because of its flickering flight. It has been assumed to be *Hipparchia volgensis* because its sister species *Hipparchia semele* has not been recorded in Montenegro (Franeta, 2018). Previous studies have not provided records from urban parts of Podgorica, but do give information from the river valleys not far away from the city (Švara et al, 2015; Sobczyk & Gligorović, 2016). Švara et al. (2015) studied tree sites located in the river valley of Cijevna within 8 - 16 km distance to of the city center, from where 36 species of butterflies were recorded (Table 4). Twenty one of those species were found also in presented study (Table 4). Sobczyk & Gligorović (2016) also studied areas located in close proximity to Podgorica. Two sites from their study were situated very close to the sites presented in this study. Cypress forest site was set about 5 km from a centre of Nova Varos next to sites 12 and site 13. For Cypress forest site Sobczyk & Gligorović (2016) have noted 4 species: *Aricia agestis*, *Polyommatus icarus*, *Coenonympha pamphilus* and *Vanessa cardui*, while during presented observations on sites 12 and 13 - seven species were recorded (Table 2).

Table 2. List of species with occurence on investigated sites.

Family	No.	Species	Present on sites:
Hesperiidae	1	*Carcharodus alceae* (Esper, [1780])	1, 4, 6
	2	*Ochlodes sylvanus* (Esper, 1777)	3, 6
	3	*Spialia orbifer* (Hübner, [1823])	4, 5, 7
	4	*Thymelicus acteon* (Rottemburg, 1775)	3, 6, 7
	5	*Thymelicus silvestris* (Poda, 1761)	2, 6, 7
Papilionidae	6	*Iphilcides podalirius* (Linnaeus, 1758)	1, 2, 3, 4, 5, 6, 7, 8, 9
	7	*Papilio machaon* Linnaeus, 1758	1, 2, 3, 4, 5, 6, 7, (8), 9, 11, 14
	8	*Zerynthia polyxena* ([Denis & Schiffermüller], 1775)	9
Pieridae	9	*Colias croceus* (Fourcroy, 1785)	1, 2, 3, 4, 5, 6, 7, (8), 9, 10
	10	*Euchloe ausonia* (Hübner, [1804])	1, 2, 3, 4, 6, 7, 10, 12
	11	*Gonopteryx rhamni* (Linnaeus, 1758)	3, 6, 9
	12	*Leptidea sp.*	3, 5, 6, 9
	13	*Pieris brassicae* (Linnaeus, 1758)	6, 7, 8
	14	*Pieris ergane* (Geyer, [1828])	2, 3, 6, 7
	15	*Pieris napi* (Linnaeus, 1758)	1, 8, 9
	16	*Pieris rapae* (Linnaeus, 1758)	1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 13, 14
	17	*Pontia edusa* (Fabricius, 1777)	3, 4
Lycaenidae	18	*Aricia agestis* ([Denis & Schiffermüller], 1775)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14
	19	*Celastrina argiolus* (Linnaeus, 1758)	3, 7
	20	*Cupido argiades* (Pallas, 1771)	1, 2, 5, 9
	21	*Cupido decolorata* (Staudinger, 1886)	1
	22	*Glaucopsyche alexis* (Poda, 1761)	1, 2, 3, 4, 5, 9, 10, 11, 12, 13
	23	*Lycaena phlaeas* (Linnaeus, 1761)	1, 2, 3, (8)
	24	*Lysandra bellargus* (Rottemburg, 1775)	2
	25	*Plebejus argus* (Linnaeus, 1758)	9
	26	*Polyommatus icarus* (Rottemburg, 1775)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14
	27	*Cyaniris semiargus* (Rottemburg, 1775)	5
	28	*Polyommatus thersites* (Cantener, 1835)	2, 6, 9
	29	*Pseudophilotes vicrama* (Moore, 1865)	2, 3, 6, 13
	30	*Satyrium spini* ([Denis & Schiffermüller], 1775)	3, 5, 6, 7

Family	No.	Species	Present on sites:
Nymphalidae	31	*Coenonympha pamphilus* (Linnaeus, 1758)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13
	32	*Euphydryas aurinia* (Rottemburg, 1775)	3
	33	*Hipparchia fagi* (Scopoli, 1763)	2, 3
	34	*Hipparchia volgensis* (Mazochin-Porshnjakov, 1952)	6
	35	*Aglais io* (Linnaeus, 1758)	2, 9
	36	*Issoria lathonia* (Linnaeus, 1758)	2, 4, 6
	37	*Lasiommata maera* (Linnaeus, 1758)	1, 2, 6, 7, 8
	38	*Lasiommata megera* (Linnaeus, 1767)	1, 2, 3, 6, 7
	39	*Limenitis reducta* Staudinger, 1901	3, 6, 14
	40	*Libythea celtis* (Laicharting, 1782)	8, 9
	41	*Maniola jurtina* (Linnaeus, 1758)	1, 2, 3, 5, 6, 7, 9
	42	*Melanargia larissa* (Geyer, 1828)	2, 3, 6, 7
	43	*Melithaea didyma* (Esper, 1778)	1, 3, 4, 5, 6, 7, 9
	44	*Melithaea phoebe* ([Denis & Schiffermüller], 1775)	2, 3, 4, 6, 7, 9
	45	*Pararge aegeria* (Linnaeus, 1758)	1, 8, 14
	46	*Polygonia egea* (Cramer, 1775)	1, 6
	47	*Vanessa atalanta* (Linnaeus, 1758)	2, 6, (8)
	48	*Vanessa cardui* (Linnaeus, 1758)	2, 3, 4, 6, 7

Table 3. Species composition and characteristic features of particulary ecological groups obtained with Bray-Curtis similarity analysis.

<table>
<tr><th>No. of similarity group</th><th>Species composition</th><th colspan="2">Characteristic features for group</th></tr>
<tr><td>1</td><td>Colias croceus
Lasiommata megera
Thymelicus acteon
Maniola jurtina
Vanessa atalanta
Polygonia egea
Hipparchia volgensis
Melanargia larissa
Iphilcides podalirius
Papilio machaon</td><td colspan="2">Mostly large body size, Herbal or grass host plant for caterpillar</td></tr>
<tr><td>2</td><td>Cupido decolorata
Cupido argiades
Polyommatus icarus
Pseudophilotes vicrama
Lysandra bellargus
Polyommates thersites
Coenonympha pamphilus
Carcharodus alaceae
Lycaena phlaeas
Cyaniris semiargus Glaucopsyche alexis
Spialia orbifer</td><td colspan="2">Small body size,
Myrmecophilus (in case of Lycenidae)
Poor dispersers
Dry, open and rocky habitats
Overwintering as caterpillars</td></tr>
<tr><td>3</td><td>Leptidea sp.
Pieris ergane
Euchloe ausonia
Pontia edusa
Pieris napi
Pieris rapae
Melithaea phoebe
Euphydryas aurinia
Melithaea didyma
Aricia agestis
Issoria lathonia</td><td colspan="2">Average body size
Herbal host plant for caterpillar
Dry, open and ruderal habitats</td></tr>
<tr><td rowspan="2">4</td><td>Ochlodes sylvanus
Thymelicus silvestris
Hipparchia fagi
Lasiommata maera</td><td rowspan="2">Oligophagus, mostly good dispersers,
grass host plant for caterpillar,
overwintering as caterpillars</td><td>Open habitats</td></tr>
<tr><td>Limenitis reducta
Pararge aegeria</td><td>Moist habitat</td></tr>
<tr><td rowspan="2">5</td><td>Plebejus argus
Celastrina argiolus
Satyrium spini</td><td rowspan="2">Good dispersers
Polyphagus caterpillar
Open and woody habitats</td><td>Small, myrmecophilus</td></tr>
<tr><td>Zerynthia polyxena
Gonopteryx rhamni
Pieris brassicae
Aglais io
Vanessa cardui</td><td>Large</td></tr>
</table>

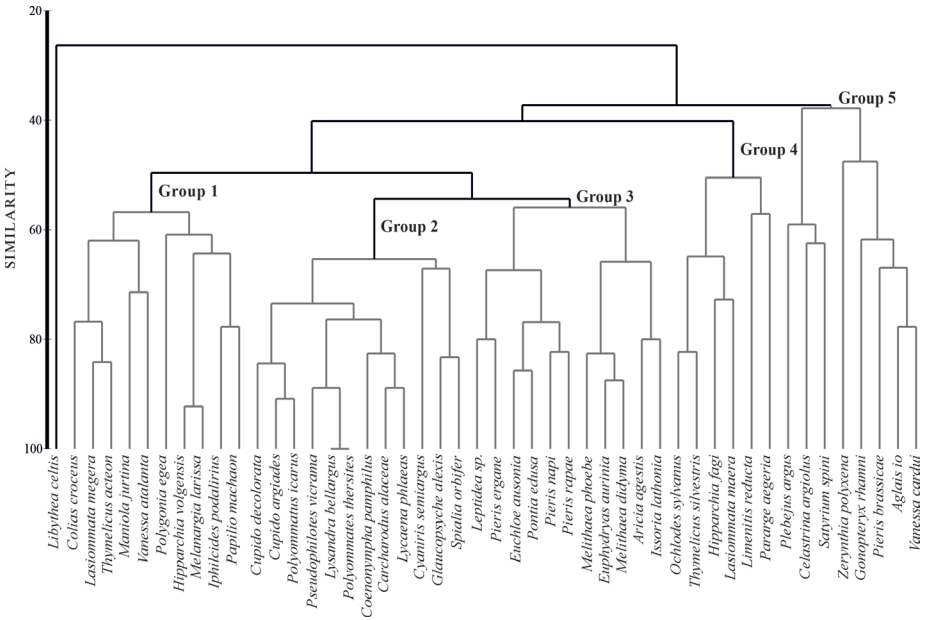

Fig. 2 Dendrogram

The site Mareza was established by Sobczyk & Glogorovic (2016) in 6 km distance from Novi Grad and can be described as contiguous (and possibly overlapping at some point) to site 9 of the present study (Mareza). Sobczyk & Gligorović (2016) listed 10 species on this site and only *Nymphalis polychloros* was not recorded in Podgorica during present observations. Complete list of species from Zeta-Skadar Plain provided by Sobczyk & Gligorović (2016) consists of 76 species and almost half of them (37 species) was confirmed in Podgorica urban area (Table 4). A comparison of the species composition between Podgorica and areas located outside the city (Švara et al, 2015; Sobczyk & Gligorović, 2016) demonstrate that those areas can be treated as a potential species pools for urban populations.

There is a lack of studies of the butterfly fauna from other cities located on the Balkan Peninsula. Koren et al (2013) studied the butterfly fauna of a small village located in the vicinity of Zagreb. Zagreb is situated on higher altitude (122 m a.s.l) than Podgorica (44 m a.s.l.) and despite covering only a half of area of Podgorica, it has four times more inhabitants. Studies lasted two full seasons, which resulted in a list of 88 species (Koren et al, 2013) including 37 species that were recorded also in Podgorica (Table 5). *Glaucopsyche alexis* was described as rare in Vugrovec, but in Podgorica, according to the definition given by Koren et al (2013) this species would be treated as uncommon - it is present on a few sites, but there were no more than 15 specimens observed. *Pseudophilotes vicrama* was given the status uncommon in Vugrovec and such a status could be also applied to this species in Podgorica. In 2019 a study of butterfly fauna of Patras (coastal Greece) was also carried out (Tzortzakaki et al, 2019). Patras is located in about 500 km distance from Podgorica.

The list of species observed in this city gives a good reference for comparison with the butterfly fauna of Podgorica, especially since both studies were carried out at a similar time of the year, from April to June (2015 - Patras, 2017 - Podgorica). Forty one species of butterflies were noted in Patras (Tzortzakaki et al, 2019), including 29 species common for both cities (Table 5). A comparison of the butterfly fauna of Zagreb, Patras and Podgorica demonstrates that there is a group of species that are most probably typical for various urban areas on the Balkan Peninsula. For these three particular urban areas, there were 25 common species (Table 5). Most of them are ubquitous and/or large size species with high dispersal potential.

An analysis using Bray-Curtis similarity indices showed that most of the species living in Podgorica prefer dry and open types of habitats (meadows and low shrubs). Their caterpillars were mostly oligophagous, i.e feeding on host plants from mainly one plant family and the species were very common in urbanised areas (Table 3). Many butterfly species were related to Fabaceae - plants that are common in the whole Podgorica (Stešević et al, 2014), about 30% of species which caterpillars feeding on herbs prefer this plant family and, additionaly Fabaceae melliferous flowers are also interesting for a lot of imagines of other species. Other habitat features that can also be assumed to be attractive elements of an investigated landscape and which were included in the analyses are: exposed rocky fields, woodland and ruderal areas. Sites characterised by the highest number of species, like Malo Brdo (30 species), Gorica (22 species), and two sites in Park Suma Gorica (NW part - 26 species, SE part - 28 species) fit mentioned patterns by combining almost all preferable habitat types. Additionally, all of the mentioned sites were located on hills, which could be an extra factor for a constant presence of species with hilltopping behaviour like Papilionidae (Pe'er, Saltz, Thulke, & Motro, 2004). Interestingly, a relatively high number of species (20 species) was recorded in the Milenium Bridge site, close to the city center. The high number of species here might be associated to a green corridor alongside the Morača river.

Some hints about distribution patterns of species group distinguished by Bray-Curtis analysis might only be indicated in case of group 1 and group 2 (Table 3). Group 1 is represented mostly by species appearing respectively on sites 6, 7, 2, 3 - already mentioned as hills with the highest number of species listed (Table 3, Fig. 1). Species from Group 2 are mostly found on sites 1, 2 and 5 (Table 3, Fig. 1), what can be associated with the available host plants along with the host ants and open space of the sites. Species composition of the other three groups includes butterflies that do not show a preference to particular sites.

Table 4. Comparison of species list from this study and from Zeta-Skadar Plain (Sobczyk & Gligorović, 2016) and from nearby sites in Cijevna valley (Švara et al. 2015). Species common to diiferent sites are underlined. Species recorded only during present study are marked in bold.

Family	Present Podgorica study	Zeta-Skadar Plain (Sobczyk & Gligorović, 2016)	Sites in Cijevna valley in close proximity to Podgorica (Švara et al. 2015)
Hesperiidae	*Carcharodus alceae* (Esper, [1780]) ***Ochlodes sylvanus* (Esper, 1777)** *Spialia orbifer* (Hübner, [1823]) *Thymelicus acteon* (Rottemburg, 1775) ***Thymelicus silvestris* (Poda, 1761)**	*Carcharodus alceae* (Esp er, 1780) *Erynnis tages* (Linnaeus , 1758) *Gegenes pumilio* (Hoffmannsegg, 1804) *Pyrgus serratule* (Rambur, 1839) *Pyrgus sidae* (Esp er, 1784) *Spialia orbifer* (Hübner, 1823)	*Erynnis tages* (Linnaeus, 1758) *Spialia orbifer* (Hubner, 1823) *Thymelicus acteon* (Rottemburg, 1775)
Papilionidae	*Iphilcides podalirius* (Linnaeus, 1758) *Papilio machaon* Linnaeus, 1758 *Zerynthia polyxena* ([Denis & Schiffermüller], 1775)	*Iphiclides podalirius* (Linnaeus , 1758) *Papilio machaon* Linnaeus , 1758 *Zerynthia polyxena* (Denis & Schiffermüller, 1775)	*Iphiclides podalirius* (Linnaeus, 1758) *Papillio machaon* Linnaeus, 1758 *Zerynthia polyxena* (Dennis & Schiffermuller, 1775)
Pieridae	*Colias crocea* (Fourcroy, 1785) *Euchloe ausonia* (Hübner, [1804]) *Gonopteryx rhamni* (Linnaeus, 1758) *Leptidea sp.* *Pieris brassicae* (Linnaeus, 1758) *Pieris ergane* (Geyer, [1828]) *Pieris napi* (Linnaeus, 1758) *Pieris rapae* (Linnaeus, 1758) ***Pontia edusa* (Fabricius, 1777)**	*Anthocharis cardamines* (Linnaeus , 1758) *Aporia crataegi* (Linnaeus , 1758) *Colias crocea* (Fourcroy, 1785) *Colias erate* (Esp er, 1805) *Euchloe ausonia* (Hübner, 1804) *Gonepteryx farinosa* (Zeller, 1847) *Gonepteryx rhamni* (Linnaeus , 1758) *Leptidea duponcheli* (Staudinger, 1871) *Leptidea sinapis* (Linnaeus , 1758) *Pieris brassicae* (Linnaeus , 1758) *Pieris mannii* (Mayer, 1851) *Pieris napi* (Linnaeus , 1758) *Pieris rapae* (Linnaeus , 1758) *Pontia chloridice* (Hübner, 1813)	*Antocharis cardamines* (Linnaeus, 1758) *Colias crocea* (Geoffroy, 1785) *Gonepteryx rhamni* (Linnaeus, 1758) *Pieris ergane* (Geyer, 1828) *Pieris mannii* (Mayer, 1851) *Leptidaea sinapis/juvernica*
Lycenidae	*Aricia agestis* ([Denis & Schiffermüller], 1775) *Celastrina argiolus* (Linnaeus, 1758) *Cupido argiades* (Pallas, 1771) ***Cupido decolorata* (Staudinger, 1886)** ***Cyaniris semiargus* (Rottemburg, 1775)** *Glaucopsyche alexis* (Poda, 1761)	*Aricia agestis* (Denis & Schiffermüller, 1775) *Callophrys rubi* (Linnaeus , 1758) *Celastrina argiolus* (Linnaeus , 1758) *Cupido argiades* (Pall as, 1771) *Glaucopsyche alexis* (Poda, 1775) *Iolana iolas* (Ochsenheimer, 1816) *Lycaena dispar* (Haworth, 1802) *Lycaena ottomanus* (Linnaeus , 1758) *Lycaena phlaeas* (Linnaeus , 1761) *Phengaris arion* (Linnaeus , 1758) *Plebejus argus* (Linnaeus , 1758) *Plebejus argyrognomon* (Bergsträss er, 1779) *Plebejus idas* (Linnaeus , 1761)	*Aricia agestis* (Dennis & Schiffermuller, 1775) *Callophrys rubi* (Linnaeus, 1758) *Cupido minimus* (Fuessly, 1775) *Glaucopsyche alexis* (Poda, 1761) *Iolana iolas* (Ochsenheimer, 1816) *Lycaena ottomana* (Lefebvre, 1830)

Table 4. Continued.

Family	Present Podgorica study	Zeta-Skadar Plain (Sobczyk & Gligorović, 2016)	Sites in Cijevna valley in close proximity to Podgorica (Švara et al. 2015)
Lycenidae	*Lycaena phlaeas* (Linnaeus, 1761) ***Lysandra bellargus* (Rottemburg, 1775)** *Plebejus argus* (Linnaeus, 1758) *Polyommatus icarus* (Rottemburg, 1775) *Polyommatus thersites* (Cantener, 1835) *Pseudophilotes vicrama* (Moore, 1865) *Satyrium spini* ([Denis & Schiffermüller], 1775)	*Polyommatus amandus* (Schneider, 1792) *Polyommatus coridon* (Poda, 1761) *Polyommatus escheri* (Hübner, 1823) *Polyommatus icarus* (Rottemburg, 1775) *Polyommatus thersites* (Cantener, 1835) *Pseudophilotes vicrama* (Moore, 1865) *Satyrium acaciae* (Fabricius , 1787) *Satyrium ilicis* (Esp er, 1779) *Satyrium pruni* (Linnaeus , 1758) *Satyrium spini* (Denis & Schiffermüller, 1775) *Scolitantides orion* (Pall as ,1771) *Tarucus balkanicus* (Freyer, 1844)	*Lycaena phleas* (Linnaeus, 1761) *Polyommatus icarus* (Rottemburg, 1775) *Scolitantides orion* (Pallas, 1771) *Tarucus balkanicus* (Freyer, 1844)
Nymphalidae	*Aglais io* (Linnaeus, 1758) *Coenonympha pamphilus* (Linnaeus, 1758) *Euphydryas aurinia* (Rottemburg, 1775) *Hipparchia fagi* (Scopoli, 1763) ***Hipparchia volgensis* (Mazochin-Porshnjakov, 1952)** *Issoria lathonia* (Linnaeus, 1758) ***Lasiommata maera* (Linnaeus, 1758)** *Lasiommata megera* (Linnaeus, 1767) *Libythea celtis* (Laicharting, 1782) *Limenitis reducta* Staudinger, 1901 *Maniola jurtina* (Linnaeus, 1758) *Melanargia larissa* (Geyer, 1828) *Melithaea didyma* (Esper, 1778) *Melithaea phoebe* ([Denis & Schiffermüller], 1775) *Pararge aegeria* (Linnaeus, 1758) *Polygonia egea* (Cramer, 1775) *Vanessa atalanta* (Linnaeus, 1758) *Vanessa cardui* (Linnaeus, 1758)	*Aglais io* (Linnaeus ,1758) *Aglais urticae* (Linnaeus , 1758) Argynnis adippe (Denis & Schiffermüller, 1775) Argynnis paphia (Linnaeus , 1758) *Brenthis hecate* (Denis & Schiffermüller, 1775) *Brintesia circe* (Fabricius , 1775) *Coenonympha pamphilus* (Linnaeus , 1758) *Erebia ligea* (Linnaeus , 1758) *Euphydryas aurinia* (Rottembu rg, 1775) *Euphydryas maturna* (Linnaeus , 1758) *Hipparchia fagi* (Scopoli, 1763) *Issoria lathonia* (Linnaeus , 1758) *Lasiommata megera* (Linnaeus , 1764) *Limenitis reducta* (Staudinger, 1901) *Maniola jurtina* (Linnaeus , 1758) *Melanargia galathea* (Linnaeus , 1758) *Melitaea cinxia* (Linnaeus , 1758) *Melitaea didyma* (Esp er, 1779) *Melitaea phoebe* (Denis & Schiffermüller, 1775) *Melitaea trivia* (Denis & Schiffermüller, 1775) *Nymphalis antiopa* (Linnaeus , 1758) *Nymphalis polychloros* (Linnaeus , 1758) *Pararge aegeria* (Linnaeus , 1758) *Polygonia c-album* (Linnaeus , 1758) *Polygonia egea* (Cramer, 1775) *Vanessa atalanta* (Linnaeus , 1758) *Vanessa cardui* (Linnaeus , 1758) *Libythea celtis* (Laicharting, 1782)	*Argynnis niobe* (Linnaeus, 1758) *Argynnis paphia* (Linnaeus, 1758) *Brintesia circe* (Fabricius, 1775) *Coenonympha arcania* (Linnaeus, 1761) *Coenonympha pamphilus* (Linnaeus, 1758) *Euphydryas aurinia* (Rottemburg, 1775) *Hypparchia syriaca* (Staudinger, 1871) *Issoria lathonia* (Linnaeus, 1758) *Lasiommata megera* (Linnaeus, 1767) *Libythea celtis* (Laicharting, 1782) *Limenitis reducta* (Staudinger, 1901) *Melanargia larissa* (Geyer, 1828) *Melitaea didyma* (Esper, 1778) *Nymphalis antiopa* (Linnaeus, 1758) *Satyrus ferula* (Fabricius, 1793) *Vanessa atalanta* (Linnaeus, 1758)

Table 5. Comparison of species lists from Podgorica and other cities on the Balkan Peninsula.

Common species for Podgorica, Zagreb and Patras (Koren et al., 2013; Tzortzakaki et al., 2019)	Common species just for Podgorica and Zagreb (Koren et al., 2013)	Common species just for Podgorica and Patras (Tzortzakaki et al., 2019)
Aricia agestis *Carcharodus alaceae* *Celastrina argiolus* *Coenonympha pamphilus* *Colias crocea* *Glaucopsyche alexis* *Gonopteryx rhamni* *Iphilcides podalirius* *Lasiommata megera* *Leptidea sp.* *Limenitis reducta* *Lycaena phlaeas* *Maniola jurtina* *Melithaea didyma* *Ochlodes sylvanus* *Papilio machaon* *Pararge aegeria* *Pieris brassicae* *Pieris rapae* *Polyommatus icarus* *Polyommatus thersites* *Pseudophilotes vicrama* *Thymelicus silvestris* *Vanessa atalanta* *Vanessa cardui*	*Cupido argiades* *Cupido decoloratus* *Cyaniris semiargus* *Glaucopsyche alexis* *Hipparchia fagi* *Aglais io* *Issoria lathonia* *Lasiommata maera* *Melitaea phoebe* *Pieris napi* *Plebejus argus*	*Euchloe ausonia* *Pontia edusa* *Thymelicus acteon* *Zerynthia polyxena*

CONCLUSIONS

Podgorica is very interesting for butterfly monitoring by being a developing city that does not yet have a metropolitan character, and where landscape planning could benefit from information obtained from an indicator group like butterflies. The list of 48 recorded species of butterflies is a preliminary list as observations were only carried out in the spring.

For a better understanding of the urban butterfly fauna additional observations from a wider range of sites and over a wider time span is needed. Additional sites might include areas like urban lawns, smaller parks and some ruderal sites. Species distribution results obtained in the present study demonstrate the importance of hills and ruderal sites, especially those connected to Morača river valley. Those facts should be taken into consideration for example during further urbanisation planning of Malo Brdo or Gorica as well as for developing business centres in Novi Grad.

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In Vitro Bioassay of *Purpureocillium lilacinum* and *Bacillus thuringiensis* for Control of *Meloidogyne incognita* on Black Pepper (*Piper nigrum* L.) in Sarawak, Malaysia, Northern Borneo

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ABSTRACT

This study aimed to evaluate strains of the entomopathogenic fungus *Purpureocillium lilacinum* and the bacterium *Bacillus thuringiensis* from Bintulu, in north-eastern Sarawak, Malaysia for their *in vitro* nematicidal properties against different developmental stages of *Meloidogyne incognita*. Ten indigenous novel strains of *P. lilacinum*, a commercial strain of the fungus (*P. lilacinum* M), and a strain of *B. thuringiensis* carrying Cry6 and Cry14 gene sequences were screened for parasitism against *M. incognita* females and eggs, egg hatching inhabitation and 2nd stage of active juveniles (J2) mortality. Our study demonstrates that *P. lilacinum* A and *P. lilacinum* B were the most effective biocontrol agent against *M.incognita* females, eggs and J2 due to highest infection of females, eggs and significant decrease in egg hatching events. *P. lilacinum* A, *P. lilacinum* B, and *P. lilacinum* M (positive control) demonstrated highly significant infection (>90%, P≤0.01) on *M.incognita* female nematodes. The present study revealed that spore suspension (10^5 spore/mL) of *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M resulted in 78.8%, 66.0% and 73.4% parasitism on eggs, respectively. *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M with low mortality of 6.0%, 5.5% and 5.7%, respectively, showed significant (P<0.05) differences in mortality effect on J2 of *M.incognita* as compared with control treatment (2.3%). Hatching of *M. incognita* eggs incubated in spore suspension of *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M for seven days were reduced by 89% when corrected for control mortality (26%).

Key words: Laboratory bioassay, *Meloidogyne incognita*, *Purpureocillium lilacinum, Bacillus thuringiensis*, biocontrol, root-knot nematodes

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INTRODUCTION

Black pepper (*Piper nigrum* L.) (Piperales: Piperaceae), the king of spices, is an important cash crops supporting the livelihood of about 67,000 rural dwellers in upland areas of Sarawak, Malaysia in northern Borneo. Such areas are loosely defined as generally remote, interior, hilly to mountainous landscapes and tablelands at moderate to high elevations where dryland farming is dominant (Li, 1999). Sarawak has the right latitude, sufficient level of rainfall, and suitable soil profile and topography for planting quality pepper. The black pepper industry is the largest agricultural export commodity of Sarawak, with production area of approximately 16,021 hectares producing 34,294 tonnes of peppercorn (MPIC, 2013). Annual export income was valued at RM 1.95 billion or USD 0.48 billion in 2019. *Piper nigrum* is attacked by several pests and diseases (Kueh,1986), the most economically important of which in Sarawak are root-knot nematodes (RKN), *Meloidogyne* spp. (Heteroderidae), that threaten the viability and sustainability of the black pepper industry (Kueh & Teo, 1978; 1990; Leong, 1986; Kueh & Sim, 1992; Ramana & Eapen, 2000; Eng, 2001).

In the absence of effective control of RKN, badly infested vines are stunted with some yellowing and gall formation on the roots. Vines subsequently become unproductive and are abandoned, resulting in substantial economic losses to pepper farmers. Currently, no RKN resistant black pepper cultivar is available in Sarawak (Eng, 2001) and nematicides are usually expensive and may raise problems of environmental pollution and/or of accumulation of toxic residues in edible plant products. They also require frequent application to be effective against nematodes (Gowen,1997). On the other hand, microbial control agents (MCAs) are gaining popularity in integrated nematode management programs due to promising results of their use and relative safety with respect to synthetic nematicides (Mukhtar & Pervaz, 2003; Dong & Zhang, 2006).

Purpureocillium lilacinum ((Thom) Luangsaard et al (2011)) (*Ophiocordycipitaceae*), a saprophytic soil fungus known as a natural facultative egg parasite of root-knot and cyst nematodes (Kannan & Veeravel, 2012) has gained attention of the researchers over the past decade due to its efficacy as a parasite for suppressing populations of phytophagous nematodes (Jatala, 1986; Dube & Smart, 1987; Freitas, Ferraz, & Muchovej, 1995; Khan et al, 2006a; Kiewnick & Sikora, 2006; Oclarit & Cumagun, 2009; Brand, Soccol, Sabu, & Roussos, 2010; Kannan & Veeravel, 2012; Timper, 2014). It was reported with high frequency of occurrence in the tropics and subtropics (Morgan-Jones, White, & Rodriguez-Kabana, 1984; Akyazi & Dickson, 2014) and can be found in most of agricultural soils (Brand et al., 2010). Eng (2001) reported that 82.9% of 43 surveyed farms in Sarawak contained *P. lilacinum* despite intensive application of nematicides in the farms. Cabanillas et al. (1989) observed maximum growth of *P. lilacinum* at temperature ranged from 24ºC-30ºC. They reported that *P. lilacinum* was able to grow on a wide range of common organic substrates and remain competitive with other microbes in the soil. It also tolerated broad range of soil pH and was able to grow well at 15°-30°C. Since *P. lilacinum* has high adaptability in its life strategy, it is competitive in a broad spectrum of range adaptability.

Bacillus thuringiensis (Bt) Berliner (*Bacillaceae*), a well-known entomopathogenic bacterium used for the control of insects for more than four decades, (Brar, Verma, Tyagi, Valéro, & Surampalli, 2006) has also drawn intensive studies on its nematicidal effects against economically important phytophagous nematodes (Devidas & Rehberger, 1992; Siddiqui & Mahmood, 1994; Carneiro, Souza, & Belarmino, 1998; Mozgovaya, Byzov, Ryabchenko, Romanenko, & Zvyagintsev, 2002; El-Nagdi & Youssef, 2004; Mohammed et al, 2008; Khan et al, 2010). Carneiro et al (1998) claimed that *B. thuringiensis* was efficacious in killing freshly hatched 2nd stage juveniles (J2) of sugarcane eelworm, *Meloidogyne javanica* (Treub,1885) Chitwood, 1949. Mozgovaya et al (2002) reported 80% mortality of nematodes after *in vitro* treatment with *B. thuringiensis*. El-Nagdi & Youssef (2004) found that soaking faba beans with *B. thuringiensis* reduced the population density of *M. incognita* and increased the plant growth. According to Osman, Ameen, Mohamed, El-Mohamedy, & Elkelany (2018), *B. thuringiensis* applied alone improved the growth parameters of eggplant and reduced nematode development. Mohammed et al (2008) reported that the spore/crystal proteins of *B. thuringiensis* showed high nematicidal activity against *M. incognita* (Kofoid and White) Chitwood. However, no current report was found on the positive effect of *B. thuringiensis* in controlling RKN development in black pepper. Therefore, studies were conducted to investigate the feasibility for use of *P. lilacinum* and *B. thuringiensis* as alternatives to the chemical nematicides currently used to control RKN in black pepper. The objective of this study was to evaluate the local virulent strains of *P. lilacinum* and *B. thuringiensis* for their *in vitro* nematicidal properties against different developmental stages of *M. incognita* in the laboratory bioassay.

MATERIAL AND METHODS

Establishment of pure *Meloidogyne incognita* (MI) culture

A pure population of *M. incognita* was established on a local variety of tomato (*Solanum lycopersicum* L. (Solanales: Solanaceae) from a single egg mass of *M. incognita*, and maintained on tomato plants raised in pots filled with sterilized pot mixture (2: 1: 1 sand, loam, cocoa peat, respectively).

Isolation, screening, and identification of *Purpureocillium lilacinum* (PL)

Seven indigenous strains of *P. lilacinum*, designed as *P. lilacinum* A, *P. lilacinum* EJ1, *P. lilacinum* EJ2, *P. lilacinum* EK1, *P. lilacinum* EK2, *P. lilacinum* EK3, and *P. lilacinum* EK4 were isolated from egg masses and females of *M.incognita* while three indigenous strains, designed as *P. lilacinum* 1A, *P. lilacinum* B and *P. lilacinum* SA were obtained from soil. *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M were further confirmed at molecular level with primer pairs: ITS1-ITS4 (Inglis & Tigano, 2006) and EF4-EF3 (Glass & Donaldson, 1995) before being assessed for their *in vitro* antagonism on egg masses, eggs, egg hatch and 2nd stage of juveniles (J2) of *M. incognita*. The sequences for ITSI, ITS4 and EF3, EF4 genes were listed as follows:

ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3'),

ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and

EF3 (5′-TCCTCTAAATGACCAAGTTTG-3′),

EF4 (5′-GGAAGGG[G/A]TGTATTTATTAG-3′).

Identification of *P. lilacinum* was based on classical (morphological and morphometric properties) and molecular methods (Luangsa-Ard et al, 2011). *P. lilacinum* M, a commercial strain (SUBOTANI, Malaysia) served as positive control in all tests. Roots and rhizosphere soils around black pepper roots were collected from several areas in two black pepper farms, one at Bintulu (3.2167°N, 113.0989°E) in north-eastern Sarawak and the other at Kuching (1.4497°N, 110.1339°E) in the south-eastern Sarawak, where RKN disease is prevalent. Root pieces were washed in gentle running tap water for 5 min. Females and egg masses were extracted from roots using method described by Sun, Gao, Shi, Li, & Liu (2006) before transferred to PDA+ [Potato Dextrose Agar (Merck, Darmstadt, Germany) amended with 0.01%(w/w) chloramphenicol (Sigma, USA) and 3% (w/w) sodium chloride] plate. To isolate from soil: Serial dilution and a standard pour plate technique were used (Thomas, Sekhar, Upreti, Mujawar, & Pasha, 2015). Dilution at 10^{-1}, 10^{-2} and 10^{-3}, were subsequently spread onto PDA+ plates and incubated at room temperature (28° ± 1°C) for 7 days.

Determination of the effect of *P. lilacinum* on female nematodes of *M. incognita*

P. lilacinum A, *P. lilacinum* EJ1, *P. lilacinum* EJ2, *P. lilacinum* EK1, *P. lilacinum* EK2, *P. lilacinum* EK3, *P. lilacinum* EK4, *P. lilacinum* 1A, *P. lilacinum* B, *P. lilacinum* SA and *P. lilacinum* M were preliminarily screened for their efficacies in infecting *M.incognita* female nematodes. Five female nematodes of approximately equal size (from pure culture) were surface-sterilized with 1.0% sodium hypochlorite for 5 minutes after extracted from tomato roots. These were later collected on a 200-µm sieve and were rinsed 3 times with chlorine-free sterile tap water (Sun et al*.,* 2006) before placing at the edge of 5-day-old fungal colony grown on water agar 2.0 % (w/w). Plates were arranged according to a Simple Randomized Design (SRD), run in four replicates and incubated at room temperature (28°± 2 °C). After 4 days, females were observed under stereo microscope (45x) to detect emerging mycelium from the body surface as sign of colonization.

P. lilacinum* (PL) infection rate on females and egg masses of *M.incognita

Infection rate on *M.incognita* females by selected *P. lilacinum* was conducted with ten females per plate and four replications per fungus treatment. Sign of colonization was observed daily until the 6th day. Similarly, the procedure was repeated for egg masses.

Infection on eggs of *M. incognita*

M.incognita egg suspensions were prepared as described by Nitao, Meyer, & Chitwood (1999). Fifty (50) µL freshly prepared egg suspensions (approximately 300 fresh eggs) was pipetted separately into sterile McCartney bottles and to which

1 mL of fungus spore suspension (10^5 spores/mL) prepared in sterile distilled water was added. Sterile water was added for control treatment. The entire procedure was conducted in aseptic condition. Bottles were sealed with Parafilm (Sigma, USA), arranged in a CRD and placed in the dark at room temperature (28° ± 2°C) with four replications per fungus treatment. Four days later, drops of egg suspension from each replicate (approximately 100 eggs) were pipetted onto glass slides, stained with lactophenol cotton blue and examined under a compound microscope (×400) for signs of parasitism.

Impacts of spore suspension of *P. lilacinum* on egg hatch and 2nd stage juveniles (J2) of *M. incognita*

Procedures described for egg parasitism test were repeated with fresh material but the incubation period was extended to seven days. Egg hatch rate and juvenile stage 2 (J2) mortality were determined by counting all eggs, J2 and dead J2 in a counting disc under a stereo-microscope, which were later calculated to determine the percentage of egg hatch and percentage of J2 mortality according to the following formula:

Egg hatch percentage = 100 x J2/(eggs + J2)

J2 mortality percentage = 100 × dead J2/total J2,

A piece of eyelash attached to a tooth- pick was used to probe the tail of J2. They were considered dead if they became rigid and did not react when probed by the eyelash.

Isolation of *Bacillus thuringiensis* (Bt)

Soil samples were randomly collected from several locations of a cattle farm at the University Putra Malaysia (UPM) Bintulu Sarawak Campus (3.2058°N, 113.0999°E). Sodium acetate- (0.25 M) -selection heat-pasteurization, and 50% ethanol treatment methods were used for *B. thuringiensis* isolation as described by Xavier, Reena Josephine, & Sreeramanan (2007). *B. thuringiensis* colonies were selected based on *B. thuringiensis* colony morphology, with the appearance of a fried egg on the plates (Barathi, Sangeetha, Karthick, Govindaraju, & Indra Arulselvi, 2012). *B. thuringiensis* was again inoculated in nutrient broth medium for sporulation. The culture was further examined and confirmed under phase contrast microscope.

The production of parasporal crystal (a solid crystalline protein which is produced next to the endospore during spore formation of *B. thuringiensis*) in *B. thuringiensis* culture was detected by adopting crystal protein staining method of Sharif & Alaeddinoğlu (1998). Smear of 2-day-old culture of *B. thuringiensis* prepared on a glass slide was dipped in a Coomassie brilliant blue solution (0.25% Coomassie brilliant blue, 50% ethanol and 7% acetic acid) for 3 minutes, then washed with tap water, dried and observed under a light compound microscope at 1000x magnificent without cover and oil immersion.

Detection of Cry Gene with Molecular Method and forward

DNA of an overnight *B. thuringiensis* culture was extracted according to DNA extraction procedure for *P. lilacinum* except incubation of pellet in 180 µL of TE buffer at 100°C for 10 minutes was omitted. Forward and backward primers of Cry6 and Cry14 (Salehi Jouzani et al, 2008) were performed, replacing ITS and EF primers, in order to detect nematode-specific Cry active genes, Cry6 and Cry14. The sequences for Cry6 and Cry14 genes were as follows: Cry6+F (5'-TGG CGT AGA GGC TGT TCA AGT A-3'), Cry6-R (5'-TGT CGA GTT CAT CAT TAG CAG TGT-3') and Cry14+F (5'-ATA ATG CGC GAC CTA CTG TTG T-3'), Cry14-F (5'-TGC CGT TAT CGC CGT TAT T-3').

Bioassay on the toxicity of *B. thuringiensis* parasporal crystal on 2nd stage juveniles (J2) of *M. incognita*

A bioassay method developed by Zi-Quan et al (2008) was modified and used for testing the parasporal crystal protein of *B. thuringiensis* against *M. incognita*. *B. thuringiensis* isolate that carry Cry active genes (Cry6 and Cry14) was selected for this study and named as *B. thuringiensis* Bt 614. The presence of Cry6 and Cry14 genes in *B. thuringiensis* served as an indication for the production of parasporal crystal 6 and crystal 14, which can be toxic and show activity against root-knot nematodes. The selected *B. thuringiensis* 614 initially was grown in nutrient broth on a rotary shaker (180 rotations per minute) for 2 days at 32°C in order to reach stationary phase with cell density of (1-5) x 10^9 CFU/mL (Mozgovoya et al, 2002). *B. thuringiensis* broth culture was subjected to centrifugation at 12,000 g for 20 minutes for precipitation of cells, spores and crystals (Carneiro et al, 1998). The precipitates were washed two times in chilled distilled water by centrifugation before re-suspended in sterile distilled water to the initial volume.

A suspension (1.85 x 10^4 CFUs) of *B. thuringiensis* spore, crystals and cells were two-fold serially diluted to achieve concentrations of 50%, 25%, 12.5% and 6.25%. Two mL of each concentration was added to 3 mm diameter glass dish. Two mL of J2 *M. incognita* suspension (average 62 *M. incognita* juveniles/mL) was later added into each dish. Glass dish containing 2 mL of sterile tap water and 2 mL of J2 suspension served as control. There were four replications for each treatment, run in a CRD. After 24, 48 and 77 hours of incubation periods, each dish was stirred for 30 seconds to mix the content before placed under a dissecting microscope to determine the number of paralyzed J2 from a total count of 50 nematodes. Mean percentage of paralyzed J2 was estimated.

Statistical analysis

Means of data in percentage subjected to arc-sine transformation, were analysed according to standard procedure for analysis of variance (ANOVA). Differences between means were compared using SAS version 9.0 for significance according to Duncan multiple range test ($P<0.01$ and $P<0.05$). Untransformed arithmetic means are reported.

RESULTS AND DISCUSSION

Determination of the impact of *P. lilacinum* on female nematodes of *M. incognita*

Fig. 1 showed that infection of *M. incognita* females by all *P. lilacinum* isolates differed according to the isolate, with isolate *P. lilacinum* Ek2, *P. lilacinum* Ek4 and *P. lilacinum* EJ2 causing lower infection levels and *P. lilacinum* 1A, *P. lilacinum* EK1, *P. lilacinum* EK3 in moderate infection levels while *P. lilacinum* A, *P. lilacinum* M, *P. lilacinum* B, *P. lilacinum* EJ1 and *P. lilacinum* SA resulting in highly significant (P<0.0001) infection levels (>80%). Similar results were reported by Eapen, Beena, & Ramana (2005) on the significant infection of *P. lilacinum* isolate on *M. incognita* females. A stereo microscope (40×) observation demonstrated radiating mycelia from the female's body (Fig. 2a and b) unrestricted to vulva, anus or broken opening, which corroborates that appressoria are not involved in the penetrating process due to the lack of a tough cross-linked chitin layer on the female body as described in early studies (Morgan-Jones et al,1984; Holland, Williams, & Khan, 1999; Khan et al,2006b). However, according to Jatala (1986), Peruvian isolate of *P. lilacinum* infected the female of *M. javanica* (Treub) only by body opening. Khan et al (2006b) suggested that this penetrating process may be attributed to different profile enzymes of each strain that help in the direct cuticle penetration.

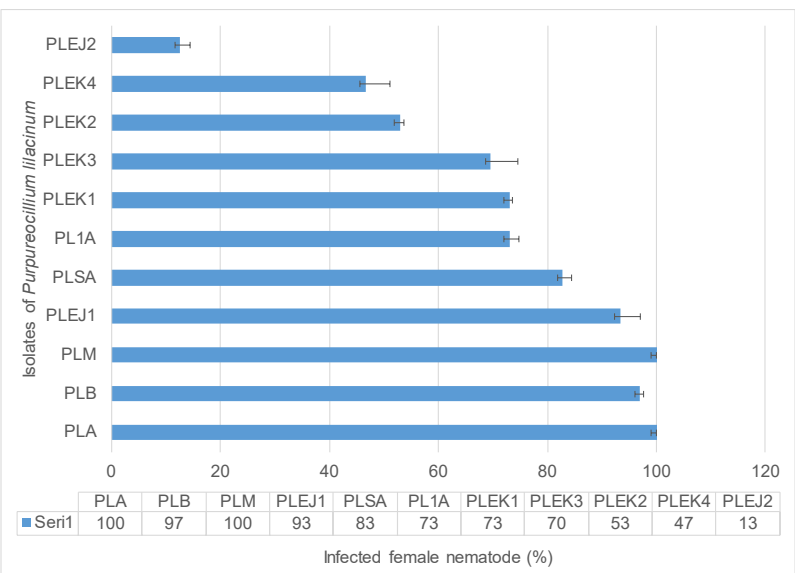

Fig. 1 Percentage of female Meloidogyne incognita infection by *Purpureocillium lilacinum*, determined by observing emerging hyphae from the body surface after 4 days of incubation under a stereo microscope at 40×. Value represents mean (%) of five replications. Means followed by the same letter are not significantly different according to Duncan Multiple Range Test at P < 0.01. Vertical bars indicate standard error of the means.

P. lilacinum infection rate on females and egg masses of *M. incognita*

Fig. 2a demonstrated higher rates of infection on *M. incognita* females by isolates *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M. Both *P. lilacinum* A and *P. lilacinum* M on the 1st day reached 50% infection rate followed by *P. lilacinum* B with a rate of 10%. Nevertheless, all isolates eventually achieved 100% infection on the 6th day. It was observed that from the 3rd day onwards, there was no significant increase in percentage of infection on female nematodes by the three above isolates.

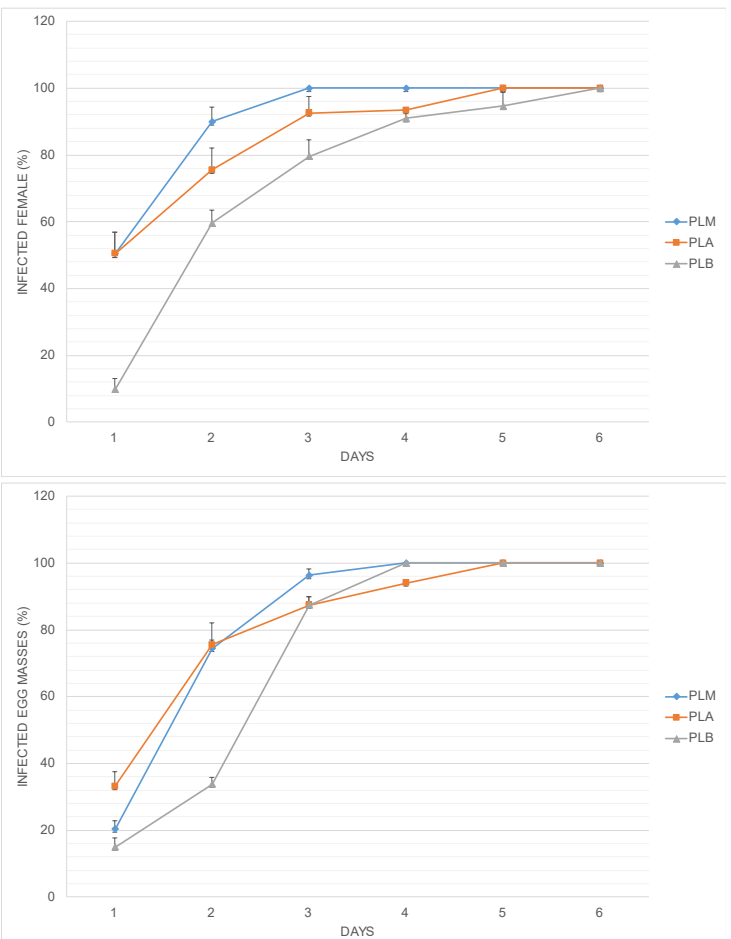

Fig. 2 Infection rate of *Purpureocillium lilacinum* on *Meloidogyne incognita* (a) infected female nematodes and (b) infected egg masses under in vitro condition. Sign of infection by observing the emerging hyphae from the surface of specimens (female or egg mass). Each value represents the mean [a % of infected female; b % of infected egg masses] of 5 replications (n=5). Means followed by the same letter for each respective day are not significantly different according to Duncan Multiple Range Test at $P < 0.05$. Vertical bars indicate standard error of the means.

The infection of egg masses by *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M was confirmed by observing the emerging mycelium from the surface of egg masses under a stereomicroscope 40× (Figs. 3a, 3b and 3c). On the first day, the rates of infection of egg masses ranging from 15 to 38% were recorded by *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M and then it increased to 90-95% on the third day and eventually achieved complete infection on the fifth day (Fig. 2b). There was no significant increase in percentage of infection of egg mass among *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M from the 3rd day onwards. This was due to the presence of antimicrobial compound (as suggested by Orion, Kritzman, Meyer, Erbe, & Chitwood, 2001) in gelatinous matrix (GM) of an egg mass can protect eggs from microbial infection (Kannan & Veeravel, 2012). An effective parasite generally should be able to utilize GM as source of nutrient and then reproduce in it (Sharon et al, 2007). Since the mycelium was detected on the surface of GM in the present study, suggesting that *P. lilacinum* A and *P. lilacinum* B possess resistance towards antimicrobial compound present in the GM. This observation is in agreement with the studies on *Meloidogyne* spp. by Zaki & Batti (1991) and Eapen et al (2005).

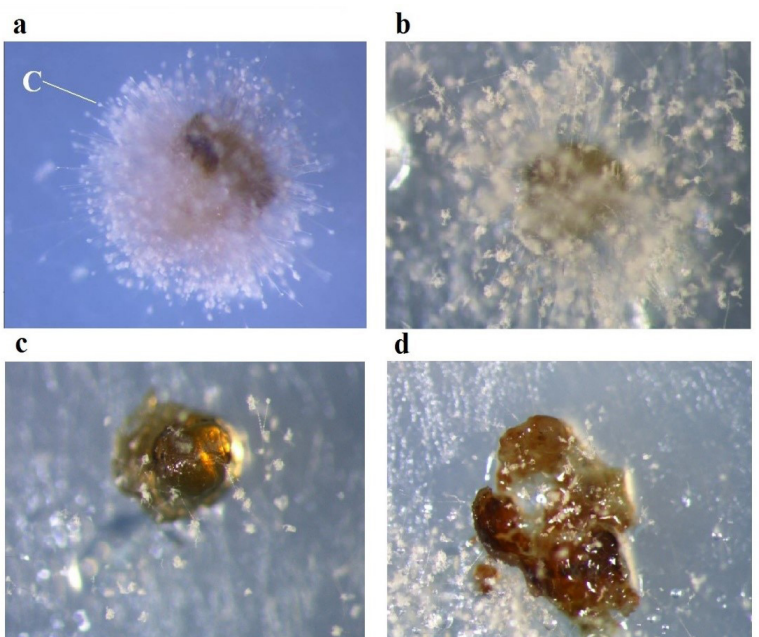

Fig. 3. Infection on *Meloidogyne incognita* female by PLM (a) & PLA (b); hundreds of conidiosphores (C) radiating from the body surface. c and d Infection on egg mass by PLA; growing mycelium was detected on the surface of egg mass (c and d). Sign of infection was detected by observing the emerging hyphae from the surface of specimens (female or egg mass) under a stereo microscope at 40×.

P. lilacinum parasitism on eggs of *M. incognita*

The results demonstrated significant (P<0.05) parasitic effect on *M. incognita* eggs with 78.5%, 73.4% and 66.0% parasitism for *P. lilacinum* A, *P. lilacinum* M and *P. lilacinum* B as compared with the control. However, it was noted that they did not differ significantly in percentage parasitism among themselves (Table 1). This result confirmed the observation made by Sun et al (2006) who reported a high *in vitro* parasitism rate of *P. lilacinum* strain YES-X-2-14 on *M. hapla* Chitwood eggs. In addition, another thirty *P. lilacinum* isolates of Sun et al (2006) were reported to parasitized 100% of *M. hapla* eggs. Al Kader (2008) stated that 77% infection of *M. incognita* eggs after 4 days of incubation.

Table 1. Impact of PL spore suspension (10^5 spores/ml) on parasitism of Meloidogyne incognita eggs, egg hatch inhibition and J2 mortality.

Isolate	Parasitized egg (%)	Hatch Inhibited egg (%)	J2 Mortality (%)
PLA	78.5 ± 2.4 [a]	88.3 ± 1.4 [a]	6.0% ± 1.8 [a]
PLB	66.0 ± 5.2 [b]	88.2 ± 1.7 [a]	5.5% ± 0.8 [a]
PLM	73.4 ± 0.6 [ab]	89.4 ± 1.0 [a]	5.7% ± 1.0 [a]
Control	0.0 ± 0 [c]	25.9 ± 3.12 [b]	2.3% ± 0.8 [b]

Each value (%) represents the mean of four replications ± standard error. Means within each column followed by the same letter are not significantly different according to Duncan Multiple Range Test at P<0.05.

This study revealed that the eggs in their early stage with no sign of apparent juvenile shape being detected in the embryo were more susceptible to *P. lilacinum* A and *P. lilacinum* B infection than eggs at a later stage in which the embryo was already developed into an identifiable juvenile shape (Fig. 4b and 4c). In most cases, none of the juvenile 1 (J1) was detected in the infected eggs and the embryos seemed to be disintegrated (Fig. 4a, 4d and 4e), which reflected a parasitic effect of *P. lilacinum* on the developing embryos. In several eggs containing developing J2, emerging mycelium was also detected on the surface of these eggs and J1 appeared motionless. This has suggested that both *P. lilacinum* A and *P. lilacinum* B parasitized not only the immature eggs but also some mature eggs containing developing J1. Similar observations were made in the early studies on parasitized eggs of *Meloidogyne* spp. by Morgan-Jones et al. (1984), Irving & Kerry (1986), Jatala (1986), Lopez-Llorca & Duncan (1991), Holland et al (1999) and Eapen et al (2005). This attribute offers advantage in biological control since eggs of all *Meloidogyne* spp. are the major target of plant-parasitic fungus and can remain dormant in the soil for long periods of time.

Under microscopic observation, the hyphae of *P. lilacinum* form an extensive network were ramifying several eggs (Fig. 4f) but not growing on other adjacent eggs in the group. A simple, swollen, hyphal structure recognized as appressorium (Fig. 4f, AP) appeared at the end of hyphae is likely to be in contact with eggshells. Some incubated eggs appeared to be abnormal, deformed and shrunken may be due to the pressure exerted by the network of hyphae. The use of mechanical means to penetrate host was also reported by Holland et al (1999).

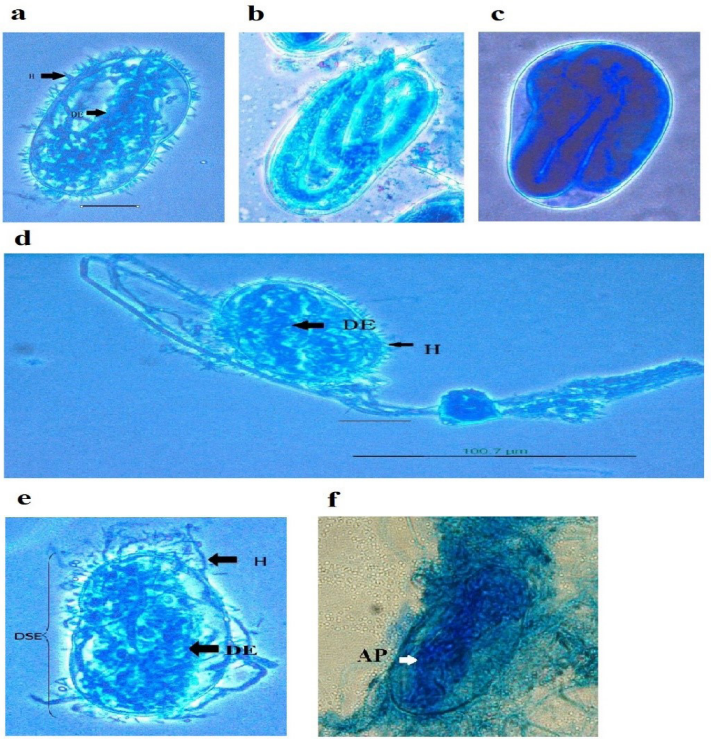

Fig. 4. Parasitism of *Purpureocillium lilacinum* on *Meloidogyne incognita* eggs. Hyphae (H) emerged from a deformed shape egg (DSE) with disintegrated embryo (DE) of *Meloidogyne incognita* after 4 days of incubation. It also penetrated into the egg and consumed the egg content (400x magnification, photo a, d, e & f). Photo b & c: uninfected egg in control treatment. Photo f: Appresorium (AP) pressing on the egg. ('–' is correspond to actual distance of 25 µm).

Once an egg is infected, the nutrient in the egg induces proliferation of hyphae on the egg, for enabling growth to adjacent eggs. In fact, eggshells of nematodes are the barriers to fungus infection (Morton, Hirsch, & Kerry, 2004). They consist of three layers, namely a vitelline layer predominantly composed of proteins, a chitin-protein complex as the middle layer, which is responsible for egg structural strength (Gortari & Hours, 2008) and lastly, an inner lipo-protein layer that protects egg from harmful chemical but loses its protective effect once the chitin layer is destroyed. According to Lopez-llorca, Olivares-Bernabeu, Salinas, Jansson, & Kolattukudy, (2002), Lopez-llorca, MaciÁ-Civente, & Jansson (2008), when a hypha encounters an egg surface, it forms appressoria (Fig. 4f) that subsequently adhere (using extracellular material on appressoria) to the egg for better binding of the fungus to the host (Lopez-llorca et al, 2008). From these appressoria, the fungus uses enzymatic and mechanical means to penetrate the host (Huang, Zhao, & Zhang, 2004; Gortari & Hours, 2008; Lopez-Llorca et al, 2008). The secretions of chitinase and proteases by *P. lilacinum* facilitate egg penetration by breaking down layers in eggshells (the barrier)

so that a narrow infection peg can push through (Morton et al, 2004). The combined effect of chitinase and protease produced by *P. lilacinum* in degrading eggshell layers of *M. javanica* was reported by Khan et al (2004). The lipid layer disappeared while the chitin layer was much reduced after enzyme treatment. Besides, the involvement of serine protease, an extracellular protease penetrating the eggshell has been reported by Mérillon & Ramawat (2012). This enzyme degrades vitellin component in immature eggs. They claimed that addition of chitin or vitellin to the fungal culture medium may induce proteolytic enzymes.

Impact of *P. lilacinum* on egg hatching of *M. incognita*

There are no significant differences in egg hatching inhibition rates (percentages) between all three *P. lilacinum* isolates (*P. lilacinum* M, *P. lilacinum* A and *P. lilacinum* B) with 89.4± 1.0%, 88.3± 1.4% and 88.2± 1.7%, respectively, although significant ($P<0.05$) differences were observed among the treatment and the inoculated control (Table 1). Most eggs in the control treatment appeared empty, with the presence of many J2, suggesting hatching of eggs had occurred whereas eggs treated with spore suspension appeared to be shrunken, deformed and with multiple vacuoles. These studies corroborate the findings by Costa, Campos, Pfenning, & Oliveira (2001) & Sun et al (2006) who reported that culture filtrate of *P. lilacinum* grown in Czapek broth greatly reduced egg hatching of *M. incognita* and average 58% egg hatch inhibition of *M. incognita* for their 186 *P. lilacinum* isolates and further confirm the higher rate of parasitized eggs and egg hatch inhibition of *M. incognita.* According to Mérillon & Ramawat (2012), hatching of eggs containing mature juveniles appeared to be stimulated when incubated in culture filtrate of *P. lilacinum* but development of immature eggs appeared to be disrupted. Under some conditions, egg hatch percentage of plant-parasitic nematode was reported by Chen, Dickson, & Mitchell (2000) to be higher in diluted culture filtrates of some fungi than in the media itself. They suggested that a hatching stimulant in the culture filtrate released by the fungi may gradually reduce the concentration of hatching inhibitor in the media by consuming the nutrient that contribute to a higher egg hatch percentage. This suggest that experimental conditions, fungal strains, culture media, and nematode species are among the different factors which may contribute to contradictory results.

Impact of *P. lilacinum* on mortality of 2nd stage juveniles (J2) of *M. incognita*

In this study, no significant differences in mortality effect on J2 of *M. incognita* between all three *P. lilacinum* isolates (*P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M) with low mortality of 6.0%, 5.5% and 5.7% respectively, although significant ($P<0.05$) differences were observed among the treatment and the inoculated control (2.3%) (Table 1). The results of present study confirmed the findings of Sun et al (2006) who reported a low average J2 mortality percentage of 16% for their 186 *P. lilacinum* isolates. Furthermore, it is in agreement with the reports by other researchers such as Jatala (1986), Bonant et al (1995), Singh & Mathur (2010) that *P. lilacinum* primarily parasitized eggs but not juveniles of *M. incognita*. However, *P. lilacinum*

strain YES-X-2-14 was reported in exhibiting high *in vitro* nematicidal effect on J2 of *Meloidogyne* spp. (Sun et al, 2006). Similarly, Al Kader (2008) reported a high nematicidal effect of their *P. lilacinum* culture filtrate on J2 of *M. incognita*, with 99% of J2 immobilized after 2 days of treatment. It is suggested that different strain of *P. lilacinum* can exhibit different nematicidal effect on J2. Throughout the years, metabolites in culture filtrate of *P. lilacinum* have been detected and screened for paecilotoxin (Singh, Pandey, & Goswami, 2013), acetic acid (Favre-Bonvin, Ponchet, Djian, Arpin, & Pijarowski, 1991), and leucinostatins (Park et al, 2004). These metabolites may potentially result in death on J2 of *M. incognita*. The low mortality effect on J2 of *M. incognita* by spore suspension of the three *P. lilacinum* isolates (namely *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M) in this study might be attributed to the absence of digestive enzyme secreted by the *P. lilacinum* or the amount of secreted enzyme was far below the threshold level to invade and penetrate the cuticle of J2 individuals. To infect J2 of *M. incognita*, *P. lilacinum* firstly needs to overcome the cuticle of nematode which is a non-cellular layer production of the hypodermis consisting of keratin, collagens and fibers (Huang et al, 2004). Once the cuticle is penetrated by fungal hyphae, the plant-parasitic nematodes *M. incognita* are then paralyzed, invaded and digested (Soares Sufiate, & de Queiroz, 2018).

Bacillus thuringiensis (Bt)'s parasporal crystal toxicity on 2nd stage juveniles (J2) of *M. incognita*

After 24 hours incubation, none of the treatments (3.12%-50.00% of harvested *B. thuringiensis* toxin, which includes cells, spores and crystals) affected the mobility of J2 (Table 2). *B. thuringiensis* crystal and endospores were ineffective in killing the J2 of *M. incognita*. The observation was continued for 48 hours and 77 hours, however, *B. thuringiensis* crystal proteins also did not demonstrate contact nematicidal effects on J2 of *M. incognita* for both periods of incubation. There was no significant difference on the percentage of paralyzed J2 among the treatments, which is in agreement with the finding of Devides & Rehberger (1992) that purified *B. thuringiensis* toxin did not manifest contact nematicidal activity toward J2 of *Meloidogyne* spp. but was 100% active against the free-living nematode, *Caenorhabditis elegans* (Maupas) (Rhabditidae). There are several reasons that explain the ineffectiveness of harvested parasporal crystals of *B. thuringiensis* in killing J2 of *Meloidogyne* spp. First, phytonematodes including *M. incognita* have a modified feeding structure (known as stylet) which is too small to engulf material actively from soil as compared to bacteriophagous nematodes and substrate ingestor nematode. Therefore, the low mortality effect of *B. thuringiensis* on J2 of *M. incognita* may be due to the inability of its stylets to ingest *B. thuringiensis* toxin (crystal protein) as reported by Mozgovaya et al (2002) and Yu et al (2008). In fact, the presence of specific crystal protein genes in *B. thuringiensis* does not guarantee its toxicity because the genes may not be actively expressed or expressed in a concentration below the threshold level or under the control of a promoter which is not efficient (as cited by Salehi Jouzani,

2008; Jansson et al, 1997; Ferrandis, Jua'rez-Pe'rez, Frutos, Bel, & Ferré, 1999). Moreover, the toxicity of *B. thuringiensis* towards plant-parasitic nematodes can also be affected by solubilization and activation of crystal proteins before ingestion by pests (as cited by van Frankenhuyzen, 2009). Without an alkaline pH, a crystal protein will not be able to dissolve in the midguts of nematodes, thus losing its toxicity effect on J2. Even though ingested crystal protein may show a certain level of toxicity, it can be proteolytically unstable after ingestion and thus not induce J2 mortality. Moreover, in the crystal proteins bioassay of Salehi Jouzani (2008), solubilization of *B. thuringiensis* crystal with 10 mmol/L of mercaptoethanol for 4 h at 37°C was included as one of the steps prior bioassay. In the present study, the technique of Carneiro et al (1998) was adopted without incorporating *B. thuringiensis* crystal solubilization as one of the steps before bioassay. It is speculated that the insolubility of *B. thuringiensis* crystal may also have contributed to its impact on J2 mortality in this study with *M. incognita* [although undiluted *B. thuringiensis* crystal (100% from crude harvest) was used in the bioassay]. Therefore, it is suggested that crystal protein solubilization should be included as an important step in all bioassays related to *B. thuringiensis* and *M. incognita*.

Table 2. Percentage of paralyzed J2 of *Meloidogyne incognita* after 24, 48 and 77 hours exposed to extract of spore-crystal mixtures of *B. thuringiensis*.

BT	24 Hours	48 Hours	77 Hours
50%	5.6 ± 1.0	13.0 ± 2.6	3.5 ± 1.0
20%	4.5 ± 1.5	6.0 ± 1.4	5.5 ± 0.5
12.5%	6.0 ± 1.4	5.6 ± 1.7	4.0 ± 0.3
6.25%	4.4 ± 0.7	4.4 ± 1.5	4.0 ± 1.2
3.12%	5.6 ± 1.5	4.0 ± 1.4	3.5 ± 1.0
Control	3.6 ± 1.2	3.6 ± 1.5	4.0 ± 0.0

Each value represents the mean (%) of five replicates of paralyzed J2± standard error, in the same column are not significantly different according to Duncan Multiple Range Test at $P < 0.05$, n=5.

In contrast to the above finding, Prasad, Tilsk, & Gollakota (1972) claimed that *B. thuringiensis* toxin at 10- fold dilution of fermentation beer, caused total mortality of J2 of *M. incognita* within 24 hours of incubation. Besides, Khan et al (2010) reported a 50% concentration of *B. thuringiensis* cell free culture filtrate significantly ($P<0.001$) increased mortality of J2 in their *in vitro* studies. Studies by other researchers have revealed that the toxicity of *B. thuringiensis* towards nematodes is due to the extracellular β-exotoxins produced in the supernatant of the culture medium (Carneiro et al, 1998; Palma, Muñoz, Berry, Murillo, & Caballero, 2014). Carneiro et al (1998) reported that sporulated cells and δ-endotoxin of *B. thuringiensis* had no nematicidal effect on J2 of *M. javanica*. Surprisingly, Mohammed et al (2008) reported that the spore/crystal proteins of two *B. thuringiensis* isolates (*Bt7N* and *BtDen*) induced 100% mortality on J2 of *M. incognita*. Also, crystal protein toxin of *B. thuringiensis* strain YBT-021 was claimed manifesting toxicity effect to phytonematodes: *M.*

hapla, Pratylenchus scribneri Steiner in Sherbakoff & Stanley (Pratylenchidae), *Tylenchorhynchus* sp. (Belonolaimidae), potato tuber nematode (*Ditylenchus destructor* Thorne (Anguinidae), and *Aphelenchoides* sp. (Aphelenchoididae) (Yu et al, 2008). Furthermore, Khyami-Horani et al (2003) revealed the toxicity of parasporal crystal protein of *B. thuringiensis* towards 3rd stage larvae of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), J2 of *M. javanica* and *M. incognita*. When *Meloidogyne* spp. was treated with fluorescent labelled crystal protein toxin, the fluorescent signal accumulated in the intestinal tissue was detected (Yu et al, 2008). However, no study has been established in the entry mechanism of crystal protein into the intestinal tissue (Yu et al, 2008). Salehi Jouzani et al (2008) reported that newly hatched J2 were not affected by the parasporal crystal proteins of *B. thuringiensis* isolates YD5 and KON4 at 2 x 10^8 CFU/mL concentration after 24 hours of incubation. However, after 3-4 days of incubation, egg hatching inhibition was detected at 46% and 45% respectively, besides manifesting mortality effect on *M. incognita* at 77% and 81% respectively.

CONCLUSION

In the laboratory bioassay, *P. lilacinum* A and *P. lilacinum* B were proven effective in parasitizing *M. incognita* females, eggs and J2 population as well as inhibiting egg hatch of *M. incognita*. Microscopic observation revealed that one of the antagonistic mechanisms of *P. lilacinum* is by proliferation of hyphae on the egg, which then penetrate the egg by mechanical and enzymatic means. Nevertheless, future study should be extended to screen and isolate each metabolite compound produced by *P. lilacinum* to further confirm the pathogenic effect of each respective metabolite towards different growth stage of *M. incognita* and other phytophagous nematodes (egg, juvenile or female).

On the other hand, *B. thuringiensis* crystal and endospores were found ineffective in killing the J2 of *M. incognita* in the laboratory bioassay. It was suspected that the feeding structure (stylet) of *M. incognita* is too small to ingest *B. thuringiensis* toxin or the specific crystal protein genes (Cry6 and Cry14) in *B. thuringiensis* might not be actively expressed or was expressed below the threshold level.

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Study on Humbleflies Species Biodiversity (Diptera: Bombyliidae) in the Western Margin of the Caspian Sea Coastline

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ABSTRACT

In order to evaluate the Bombyliid species diversity in Guilan province, statistical sampling was performed from designated stations in the desired areas. Totally 20 species belonging to 11 genera were collected as below;

Callostoma soror Loew, 1873*, Conophorus pseudaduncus* Paramonov, 1929, *Exoprosopa amseli* Oldroyd, 1961, *Exoprospora dispar* Loew, 1869, *Exoprosopa efflatounbeyi* Paramonov, 1928, *Exoprosopa grandis* (Wiedemann in Meigen, 1820), *Exoprosopa kirgizorum* Paramonov, 1928, *Exoprosopa pectoralis* Loew, 1862, *Hemipenthes subvelutinus* Zaitzev, 1966, *Heteralonia megerlie* (Meigen, 1820), *Heteralonia suffuse* Klug, 1832*, Thyridanthrax elegans* (Wiedemann in Meigen, 1820), *Thyridanthrax griseolus* (Klug, 1832), *Thyridanthrax punctum* (Loew, 1854), *Veribubo misellus,* Loew 1869, *Lomatia belzebul* (Fabricius, 1794), *Parageron lutescens* (Bezzi, 1925), *Usia bicolor* Macquart, 1855, *Phthiria pulicaria* (Mikan, 1796) and *Phthiria vagans* Loew, 1846.

Most of the collected species were from the genus *Exoprosopa* Macquart, 1840 with relative frequency 54.83 %. According to Shanon-winner index 1.97 the highest species diversity was related to the Darestan region.

Key words: Biological control, Darestan region, Guilan province, Caspian sea, insects, pollination.

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INTRODUCTION

Diptera is considering as one of the major as well as biggest orders in insects classification, with great importance in several fields like agriculture, ecology and veterinary medicine (Ghafouri Moghaddam & Gharali, 2014; Robertson et al, 2020). These insects have a variety of diets, including eating meat, vegetarianism, caries, and eating blood (Ramirez, 2018). From the biological pest control aspect, some families of diptera such as Tachinidae, Syrphidae, Cecidomyiidae are playing role as predator (Driesche & Bellows, 1996). On the other hand, some species of families Sciaridae, Anthomyiidae, Cecidomyiidae and Tephritidae are considered as important agricultural pests (Latibari, Moravvej, Heller, Rulik, & Namaghi, 2015).

The order diptera is divided into two sub-orders: Nematocera (mosquitoes) and Brachycera (flies). The Bombyliidae family is belonging to superfamily Asiloidea, suborder Brachycera (Evenhuis, 2002). The efficiency of some Bomyliidae species in biological control and IPM is remarkable. Most Bombyliids species are found in the Middle East and the Horn of Africa, where is native to migratory locusts (Hull, 1973). Most of Bombyliid species predating the locust's egg capsule, thus preventing their unnecessary outbreak (Shah, Godonou, Gbongboui, Hossou, & Lomer, 1998). It is one of their beneficial roles in the natural regulation of these insects (Yeates & Greathead, 1997). The Bombyliidae family devided to 100 genera included at least 45,000 described species worldwide. Unfortunately, there are few studies have been done on Bombyliidae family. However, more studies have been done in neighboring countries in Turkey, Oman, Iraq and Saudi Arabia (Greathead, 1980). Guilan province is located in the north part of Iran, in south-west margin of Caspian Sea, which is contain extensive green and first-class pastures with abundant vegetation (Akhani, Djamali, Ghorbanalizadeh, & Ramezani, 2010) Since a serious study on Bombyliidae biodiversity has not been done in this region so far, it is expected to find interesting data about this family in the mentioned area. Due to the important role of most Bombyliidae species both in the pollination and natural regulation of pests, considering the high agricultural capacity in Guilan province, not only investigation on Bombyliid fauna but also the determination of their species diversity will be useful, as a basis for other related entomological studies about Bombyliidae in the similar ecological regions. The first principle in entomological studies is the investigation of insect fauna and biodiversity in the region, which is more than usual for Bombyliidae flies, due to their wide efficiency in biological pests control and IPM (Iperti, 1999).

MATERIAL AND METHODS

Sampling and collection methods

Sampling was done mainly from rangelands in different locations in Guilan province (Table 1 and Fig. 1). To collect larger species, flowering plants were sweeping net, especially plants such as ox-tongue flowers (to collect the Bombyliidae; Latreille, 1802 species). Other genera, due to their spawning behavior in areas free of vegetation on the soil, were collected during spawning using a net and used to collect smaller species.

Table 1. Coordinates of sampling locations in Guilan province.

Locality	Geographical Coordinates	Date
Amlash	37°5'29.88"N, 50°11'12.98"E	2019.08.28, 2019.08.30
Bivarzen	36°40'57.19"N, 49°34'39.57"E	2019.04.17, 2019.04.21
Damash	38°26'32.45"N, 48°34'53.26"E	2019.06.26, 2019.06.29
Heiran Pass	36°42'12.85"N, 49°47'17.57"E	2019.05.27, 2019.05.31
Jirandeh	36°48'34.96"N, 49°24'57.98"E	2019.07.23, 2019.08.20
Rudbar- Darestan	36°48'34.96"N, 49°24'57.98"E	2019.08.07, 2019.08.09
Rudbar- Lockhee	37°5'29.88"N, 50°11'12.98"E	2019.06.04, 2019.06.07
Rustam Abad-Jokin	36°40'57.19"N, 49°34'39.57"E	2019.06.11, 2019.06.19

Fig. 1. The sampling localities in Guilan province.

Preparation and storage of samples

For larger species at the sampling site, to prevent hair loss and hair loss, they were killed with cyanide and these insects were cut with fine needles. Also, large species can be immersed in 96% alcohol and after transfer to the laboratory, they can be taken out of alcohol and dried and removed. For smaller species collected from pan traps, the samples were collected in 96% alcohol after collection from traps and stored in the freezer.

Species identification

The available scientific sources; Paramonov (1928), Engel (1932-1937), Zaitzev (1966), Linder (1975) and Greathead & Evenhuis (2001) have been consulted to identify species. The identifications have been confirmed by Dr. Rahim Abdollahi Mesbah (University of Tehran).

Statistical analysis

All of collected insets were sorted out according to their species/location. Their frequency data were analysed using Shannon's Weiner diversity index to compare abundance and species richness among different localities in the study areas.

RESULT AND DISCUSSION

Totally 20 species belong 11 genera were collected, identified and illustrated (Table 2). According to dynamic populations of collected samples, *P. vagans* with the highest occurrence frequency between collected flies, is considered as dominant species (Fig. 2). Also, the highest species biodiversity index; Shanon-winner have been seen in Darestan station (Fig. 3).

Table 2. Species frequency/Shanon-winner index in each location.

Species	Amlash	Bivarzen	Damash	Locations Heiran Pass	Jirandeh	Rudbar-Darestan	Rudbar- Lockhee	Rustam Abad-Jokin
Exoprosopa amseli					3	7		3
E. kirgizorum					6			15
E. efflatounbeyi						4		
E. pectoralis							3	
E. grandis						1		
E. dispar					2			2
Thyridanthrax punctum						1		
T. elegans	8		4					
T. griseolus						6		
Lomatia belzebul			5					
Usia bicolor	25			40		5		
Parageron lutescens							1	
Conophorus pseudaduncus				1				
Heteralonia megerlie				1				
H. suffuse							3	
Callostoma soror	5							
Veribubo misellus						3		
Hemipenthes subvelutinus					10	2		6
Phthiria vagans	20	100	100	30	20	10	20	10
Phthiria pulicaria				1				
Shanon Winner index	0	0.67	0.83	1.38	0.79	1.31	1.97	1.21

The results showed that the highest and lowest densities and biodiversity of Bombyliid flies in sampling related to Guilan province, in the western margin of the Caspian Sea coastline. The Bombyliid species are placed as important members of

biodiversity, playing an impressive role in the natural ecosystem counterbalance; as partly primary consumers, an essential part of food-chains with predation pests, and mostly with pollination. even though there are exerting unprecedented pressures like human-made ones, on ecosystems all over the world, and such pressures may affect all the species (El-Hawagry & Gilbert, 2014).

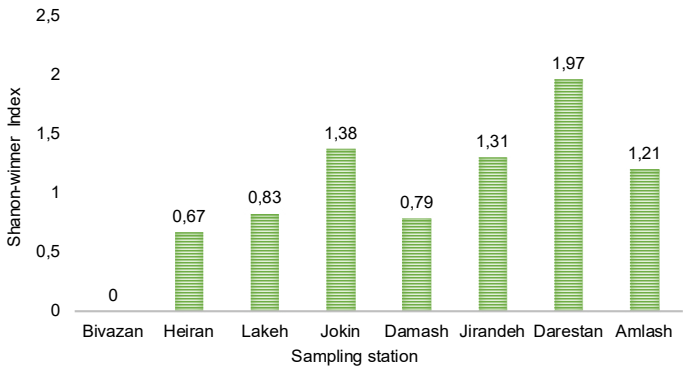

Fig. 2. Relative frequency for each species.

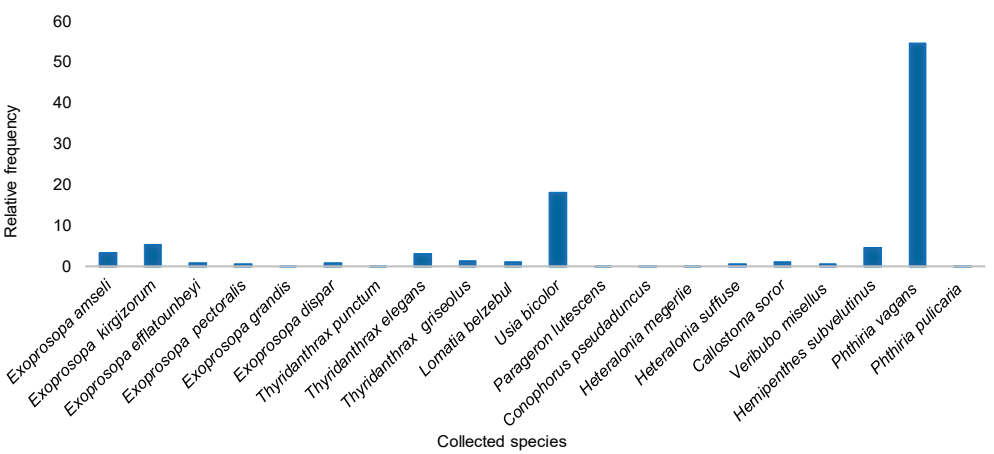

Fig. 3. Shanon-Winner Index amount for each station.

The greatest threats to beneficial insects (natural enemies) are habitat fragmentation and destruction, intensification of agricultural practice with over-use of pesticides and herbicides which is the main reason for increasing the pests population by supporting their resistance to their control methods (Tschumi et al, 2016; Heidari Latibari, Moravej, Ghafouri Moghaddam, Barahoei, & Hanley, 2020). Despite Guilan province being known for its rich biological diversity in flora and fauna, but the preservation of its habitats have received scant attention. Nevertheless, most of its biodiversity, including Bombyliidae species, is still unexplored because of a significant lack of

national research capacity. This study has substantially increased the knowledge of Bombyliidae diversity in an important agricultural region in Iran. Previous studies have listed limited species of the present species in Iran. However, the number of species found in our study was so lower than in comparable studies outside of Iran. This most likely reflects the region's relatively low diversity of this family. Based on our own results. Aim to increase our biodiversity knowledge about insect fauna, especially on beneficial groups like Bombyliidae, capacity building in the area at various levels is needed. we hypothesize that the diversity of Bombyliidae in the agroecosystems is substantially higher than is currently known. Lack of specialists and organized projects on insect biodiversity in the margin of the Caspian Sea, are among the identified gaps.

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An Effective Method for the Monitoring of Stag Beetle (*Lucanus cervus* (Linnaeus, 1758) (Coleoptera: Lucanidae)) larvae

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ABSTRACT

Lucanus cervus (Linnaeus, 1758) is involved in the Near Threatened (NT) danger category in the IUCN Red List. It plays an important role in the ecosystem, nutrient cycling, and the decomposition process of wood. Its larvae live under or in damp and decaying trees. We designed an equipment to ensure the detection and the long-term monitoring of *Lucanus cervus* and many other insect larvae that complete their larval stages in a wood tissue without destroying their habitats. The equipment developed is an audio listening device that amplifies the larval sounds. This device was designed as a 4-stage structure with a sensor stage, low and high pass filter stage, voltage gain sound amplifier stage, and output stage. This device was used for the first time in the larvae's natural environment. With this new equipment, listening was performed on decaying trunks in the Amanos Mountains, which are included in the spreading area of *Lucanus cervus akbesianus* Planet, 1896, located within the borders of Hatay province in June-July 2017. The presence of the larvae of *Lucanus cervus akbesianus* was detected in oak tree trunks, and sound recording was performed. Accordingly, the larvae made approximately 5 clicks in a series and left a 7-8 second gap between two series. It is considered that the audio listening device developed in this study is an effective method for the detection of other saproxylic beetles larvae in decaying wood.

Key words: Coleoptera, Stag Beetle Larva, Audio listening device, Piezo Sensor

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INTRODUCTION

Lucanus cervus (Linnaeus,1758) known as stag beetle, is one of the species of the largest known terrestrial insects in Turkey. The adults are 25-89 mm long, including mandible length (approximately 25-49 mm for females, approximately 30-89 mm for males) (Bardiani, Chiari, Maurizi, Tini, Toni, Zauli, Campanaro, Carpaneto & Audisio, 2017).

The stag beetle is an obligate saprophytic species. Its larvae are present at, under, or inside the section of the damp and decaying wood close to the soil surface, and usually in light soils. The adults feed on fruit and tree sap. They usually fly in the evening. While the larvae generally develop in the oak in Ukraine, they are present on large trunks of deciduous trees such as chestnut, oak, willow, ash, poplar, and linden on the European side of Turkey (IUCN, 2020).

The females lay their large eggs, which are 3 mm in length, one by one. However, they lay approximately 20 of them in dead bark cracks (Huerta & Rodriguez, 1988; Baraud, 1993).The eggs hatch within two to four weeks, and the larvae come out. The larval stage, which lasts for five or six years, passes by feeding in these tree trunks. This slow development is due to both the low nutrient quality (low nitrogen content) of decayed trees and the large size that needs to be achieved at maturity. After the last larval stage, during which it may exceed 10 cm in length, it enters the pupae in the tree or soil, near the log (Sánchez, 1983).

While the larval stages of *Lucanus cervus* do not differ significantly in shape, there is a significant increase in their size. Typical lucanid larvae are white-looking or creamy, soft, oligopod, and roughly “C-shaped” on the side view, and have transverse folds in their abdominal segments and a longitudinal anal cleft. The head capsule is reddish-brown to orange in color and more hardened compared to its body. There are no ocelli and ommatidium, the antennas are elongated, and pubescence developed only on the front part of the body (Bardiani et al, 2017). The larvae have a typical sound-producing apparatus formed by two sclerosing (hardened tissue) organs on the second and third pairs of legs, respectively. A pair of soft, convex, and translucent oval bumps are formed above the abdomen on the dorso-posterior side, and these structures are large and close to each other in the inner parts. Very dense, strong, and numerous short brownish setae are present on the ventral surface of the distal abdominal segment of the larvae. The mandible of *Lucanus* larvae is large and overly curved (Bardiani et al, 2017).

Larval stridulation is approximately continuous and may serve to inform adults about the location of young individuals (Ratcliffe, 1991).

Typical predators of stag beetles are bird species such as woodpecker, magpie, crow, kestrel, and owl. Wild pigs and badgers also prey on pupae on the ground. They may also fall prey to species such as hedgehogs, foxes, shrews, and mole (Reißmann, 2020).

Lucanus cervus is included in the Near Threatened (NT) danger category in the IUCN Red List (IUCN, 2020) and the NT danger category in the European Red List of

Saproxylic Beetles (Cálix, Alexander, Nieto, Dodelin, Soldati, Telnov, Vazquez-Albalate, Aleksandrowicz, Audisio, Istrate, Jansson, Legakis, Liberto, Makris, Merkl, Mugerwa Pettersson, Schlaghamersky, Bologna, Brustel, Buse, Novák & Purchart, 2018). Although the species is commonly found in Europe, a significant decline is observed in the north and central parts of the distribution area. It is assumed that future trends of European forests will pose serious threats to this species. Thus, the species will soon be qualified in the Vulnerable (VU) category (IUCN, 2020). Furthermore, the species is included in Appendix III (Protected Fauna Species) according to the BERN Convention on the Conservation of European Wildlife and Natural Habitats.

Stag beetle has national conservation status in England (Hylyman, 1992), and the species is also in large protected areas in Romania and Ukraine. In Spain, they are protected as species of 'special concern' (Verdú & Galante, 2004). The species is listed as Endangered in the British Red Data Book, Endangered in Germany and Sweden, Vulnerable in Denmark, and Critically Endangered in the Czech Republic (Nieto & Alexander, 2010).

Many action plans regarding the conservation of the *Lucanus cervus* were implemented in many countries in Europe (London Wildlife Trust, 2020a, 2020b; Hertfordshire Environmental Forum, 2020; Joint Nature Conservation Committee-JNCC, 2020; REF Impact Case Studies, 2020), and long-term monitoring studies were carried out as a result of these action plans (Harvey, Hawes, Gange, Finch, Chesmore & Farr, 2011; Čížek, 2006; Campanaro, Hardersen, Toni & Grasso, 2010).

In Turkey, an action plan study was carried out for the Akbez stag beetle (*Lucanus cervus akbesianus* Planet, 1896) in Hatay province by the Ministry of Forestry and Water Affairs, VII Regional Directorate, Hatay Department (Akman, Biler, Hasbenli, Demirel, Çiftçi, Çiftçi, Can & Muratlı, 2017).

Acoustic devices provide the non-destructive, remote, and automatic detection and monitoring of hidden insect infestations for the pest manager, regulators, and researchers. The effectiveness of acoustic devices in detecting cryptic insects, predicting population density and in distribution maps depends on many factors such as sensor type and frequency range, the substrate structure, interface between the sensor and substrate, the distance between insect and the sensors, and evaluation time, size and behavior of insect (Mankin, Hagstrum, Smith, Roda & Kairo, 2017).

Ultrasonic sensors are particularly effective for detecting wood-boring insects at their best because background noise is negligible at frequencies of > 20 kHz and ultrasonic signals are attenuated in wood much less than air. The problems in distinguishing sounds produced by target species from other sounds have prevented the use of acoustic devices. However, new devices and signal processing methods have significantly increased detection reliability. New methods take into account the spectral and temporal pattern features that are prominently visible in insect sounds but invisible in the background noise and vice versa. As reliability and ease of use increase and costs decrease, acoustic devices will take a significant place as cryptic insect detection and monitoring tools in the future (Mankin et al, 2017).

Since biting and trembling patterns of *Lucanus cervus* larvae can be detected in tree trunks, acoustic listening technique can be used to detect without destroying the habitat. Research planning, with acoustic listening and recording methods, can have a great potential use in habitat research aimed at providing information about rare species (Harvey et al, 2011). Monitoring of the improved acoustic series of *Lucanus cervus* larvae has been proposed, but field tests have not been conducted yet (Harvey et al, 2011).

This study aims to introduce detecting larvae using acoustics devices that will not damage the larval habitats and that will be effective in reducing the research workload for the detection and monitoring of *Lucanus cervus* and other saproxylic beetles.

MATERIALS AND METHODS

The field studies were carried out in the parts within the borders of Hatay province of the Amanos Mountains, which are included in the distribution area of *Lucanus cervus akbesianus* Planet, 1896 (Fig. 1), in June-July 2017. The black pine (*Pinus nigra*) forests mixed with deciduous trees are present in the study area. Also, The kermes oak (*Q. coccifera*), hairy oak (*Q. cerris*), taurus fir (*Abies cilicia spp. cilicica*), cedar (*Cedrus libani*) and red pine (*Pinus brutia*) forests grow in the Amanos Mountains (Lise, 2006).

Fig. 1. Akbez Stag Beetle (*Lucanus cervus akbesianus* Planet, 1896) male.

The field studies were by conducted two researchers and the larval sounds were searched in many rotting tree trunks (about 110 trees, especially oak trees), using the new audio electronic equipment. The technical specifications of the new audio electronic equipment are presented below (Fig. 2). Recordings were made for a period of three minutes for each tree. The biting sounds of larvae were recorded from 17 decaying trees. Only one of the decayed trees that was subjected to sound recording was was cut into pieces and the larvae were removed to verify whether it was a *Lucanus cervus* larvae. The detected sounds were recorded by a mobile phone. The recording was then copied onto a PC, studied, and edited with Adobe audition audio editing programme.

In the equipment developed, which is shown in Fig. 2, the part number (1) is the sensor, the part number (2) is the connection to the recorder, the part number (3) is

the earphone output, the part number (4) is the input signal adjustable potentiometer between the audio amplifier stage and output stage, and the part number (5) is the power button of the circuit. When the circuit is operated, the sounds can be recorded with the recording equipment connected to the output from the part number (2) while larval sounds are listened from the part number (3).

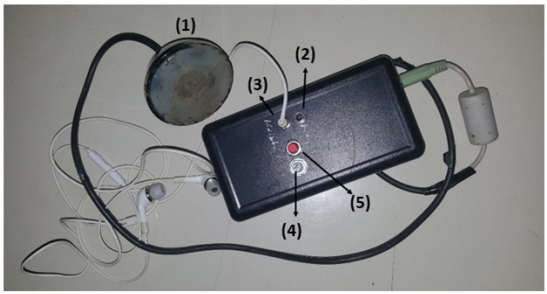

Fig. 2. Listening device that increases larval stridulation.

The electronic equipment developed in the study was designed as a 4-stage structure with a sensor stage (1st stage), low and high pass filter stage (2nd stage), voltage gain sound amplifier stage (3rd stage), and output stage (4th stage). The schematic representation of this 4-stage structure is presented in Fig. 3.

In the equipment designed, initially, the Piezo Sensor signal source was used as the receiving element. Then, the amplitude of the audio signal passing through the low pass and high pass passive filter stage was increased with a high gain signal amplifier. Along with the final amplification for the output stage, it was directly connected to an earphone or a computer or another similar device that can perform sound analysis.

Fig. 3. Schematic diagram of the electronic equipment designed.

Components of the listening device

Sensor stage (Piezo crystal)

Although the Piezo Sensor, also known as piezoelectric or piezo crystal elements, was discovered by Pierre Curie in 1880, the industrial use of this electronic element began after the 1950s. Piezoelectric sensors can convert mechanical stress into an electrical charge. The ability of a piezoelectric material to convert mechanical stress into an electrical charge is called the Piezoelectric Effect. The word piezoelectric was derived from the Greek word ‘piezein’ and means pushing, pressing, and squeezing. The piezoelectric effect is a reversible effect, which means that we get some electrical charge at the output when we apply mechanical stress to the piezoelectric material. When we provide an electrical charge to the sensor, the element is stressed or compressed. The working principle of the crystal is presented in Fig. 4 (Components 101, 2020).

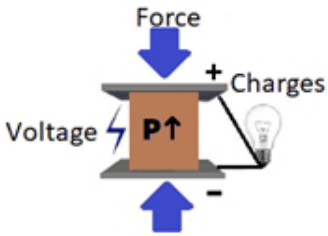

Fig. 4. Working principle of the piezoelectric material (Components 101, 2020).

Due to this feature of the piezoelectric effect, it is used for stress detection or stress generation. When the element is disengaged, they generate electrical signals starting from low levels due to environmental vibration or surface pressure (Murata Manufacturing Company Limited, 2020).

Piezoelectric sensors are used in many areas such as electrical power generation, contact microphone, ultrasonic receiver or transmitter, ultrasonic cleaner, instrument microphone, and water leak and flow detectors. Recently, they have also started to be used as bio-acoustic sensors. Through the contact of the piezoelectric sensors with the human body, biological data such as blood flow condition and heartbeat can be detected and processed electronically (Bhalla, Moharana, Talakokula & Kaur, 2017).

A standard 35-mm-diameter Piezoelectric sensor, which is very easily available in the electronics market and has a resonance sensitivity of 2-40 Khz, was preferred for the vibration sensor equipment. A sample piezoelectric crystal is presented in Fig. 5.

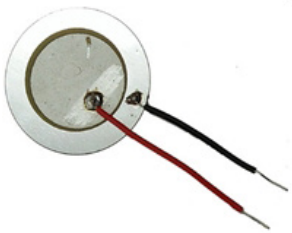

Fig. 5. Sample of 35-mm Piezo Crystal sensor (Components 101, 2020).

To prevent the Piezoelectric sensor, which is a sound receiving element, from being affected by environmental distortions, it was directly connected to the filter stage of our electronic circuit with a shielded 50-cm cable connected to the chassis ground and a 3.5-mm jack. The Piezo crystal was fixed with hot silicone sealant in a housing to increase the direct contact surface on log structures. Thus, it was aimed to have maximum contact on the log by obtaining a curved and softer surface compared to the crystal in the front part. The view of the piezo crystal in its housing is presented in Fig. 6.

Low and high pass filter

Since the sounds received through the circuit are subject to distortion by other sound-producing sources, quality sound reception is affected if this filter stage is not used. Therefore, audio signals were planned to transmit audio frequencies in the target

range before being subjected to amplification. Resistor-Condenser or Resistor-Coil pairs can be used for this process. We preferred to use the Resistor-Condenser pair (RC) in the filters for the equipment developed. The RC Low Pass Filter is a simple filter circuit that passes a low frequency band consisting of a resistor and condenser. The gain is constant between 0 Hz and the cutoff frequency (fc). At the cutoff frequency, the low frequency gain decreases by 3dB. The frequencies between 0 Hz and the cutoff frequency (fc) are band-pass frequency, and the frequencies higher than fc are band-quenching frequency. The gain in band-quenching frequency is very low. The frequency range can be calculated using the formula of fc = 1/(2.π.R.C) (Aspen Core Network, 2020a). Considering the sound range of stag beetles, in the calculation made to pass frequencies between 0-12 Khz without loss, the frequency cutoff value of 11483 Hz is achieved with a 220-ohm resistor and 63-nf condenser. The structure of low and high pass filters is presented in Fig. 7, and the operating performance chart of the low pass filter is presented in Fig. 8.

Fig. 6. View of the piezo crystal in its housing.

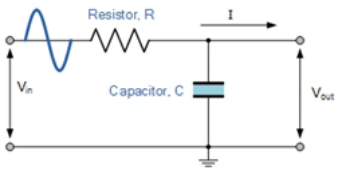

Fig. 7. Electronic schematic representation of the Low Pass Filter (Aspen Core Network, 2020a).

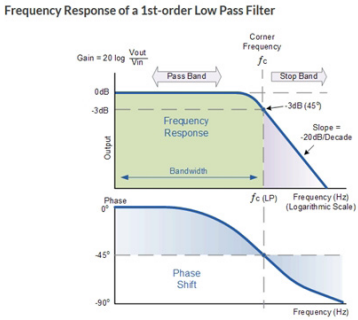

Fig. 8. Operating performance of the low pass filter (Aspen Core Network, 2020a).

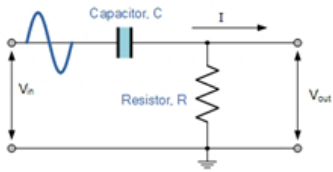

Fig. 9. Electronic scheme of the High Pass Filter (Aspen Core Network, 2020b).

High pass filter

With the high pass filter, which is the second filter stage, audio signals at a certain frequency are allowed to pass (Aspen Core Network, 2020a). The high pass filter structure is presented in Fig. 9 and the operating performance chart of the high pass filter is presented in Fig. 10.

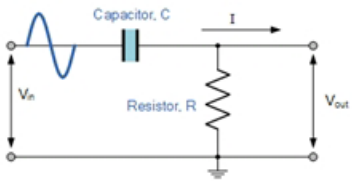

Fig.10. Operating performance of the high pass filter (Aspen Core Network, 2020b).

In the calculation made with the formula of fc = 1/(2.π.R.C) at this stage, it was calculated that the frequencies above 9645 Hz could pass with a 330-ohm resistor and a 50-nf condenser.

With the sequential use of low and high pass filters, a filter stage that allows the passage of the frequency band between 9645 and 11483 Hz by attenuating and suppressing the signals that are not in this frequency range was obtained. Due to this feature, it was possible to obtain maximum clean sound in the range of sound emitted by Stag beetle larvae.

Signal Amplifier

An amplifier stage was needed for the signal that was filtered at the desired bandwidth but still very weak to reach an audible and analytical level. Standard uses of opamp amplifiers are presented in Fig. 11 (Starecki & Wieczorek, 2017).

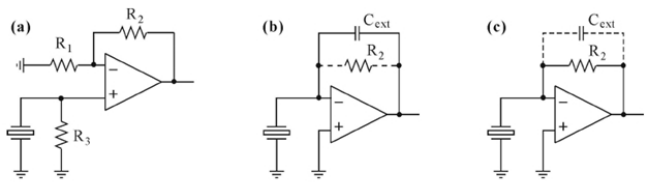

Fig. 11. Basic configurations of preamplifiers dedicated for use with piezoelectric sensors: (a) voltage amplifier, (b) charge amplifier, (c) transimpedance amplifier (taken from Starecki & Wieczorek, 2017).

The logic of the voltage booster circuit presented in Fig. 11a was used in our equipment. The gain on this amplifier is calculated with the formula gain= 1 + (R2/ R1). R1 resistor and R2 resistor were made variable by using a fixed value resistor and a multi-turn trimpot resistor, respectively, so that the gain settings of the circuit would be flexible.

In our study, the Opamp integrated amplifier circuit, which was used as an amplifier in the voltage gain mode, was selected due to the following features:

Since the device is portable, it should be able to draw low voltage and little current.

The gain rate should be adjustable.

The ratio of harmonic distortion should be very low.

The input level should have a low and wide impedance ratio.

The opamp series amplifier integrations with all these features together were examined, and it was found that NE5532 op-amp integrations, which are generally preferred in guitar microphone sound amplifier circuits, were suitable for this use.

The part of the sound amplifier stage made as a circuit is presented in Part 1 in Fig. 12. The sound amplifier stage was assembled in accordance with the standard amplifier circuit published by the NE5532 integrated manufacturers. One of the gain resistors was provided by using a multi-turn trimpot resistor so that the gain setting would be optimum. Part 2 on the audio listening circuit is the second amplifier stage, which was considered as the Output Stage (Fig. 12). These stage features are discussed in detail in the Output Stage section.

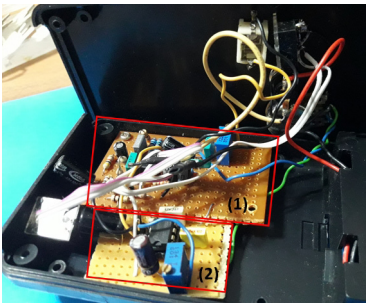

Fig. 12. Interior view of the Audio Listening Device.

Output stage

The part of the audio listening device assembled as the Output Stage is presented in Part 2 in Fig. 12. A second amplifier stage was used to bring the sounds directly to an audible level at the output stage. Thus**,** sounds can be heard with headphones or an external speaker. An interconnection was established before the output stage to allow the use of a computer or similar device for sound analysis.

At first, the electronic card NE5532 Op-amp integrator was assembled as a card in a way to be raised 100x as in the datasheet documents given by the manufacturer. The

output point of the circuit was connected to the recording equipment output connection and output stage input. LM386 integrator, one of the standard opamp integrators, was used as the output stage integration. The output stage audio output level was taken under control with the 10 Kohm adjustable potentiometer used in the input of the output stage. Since opamp was used in the circuit, the gain setting was adjusted to the optimum value using a multi-turn trimpot resistor in one of the gain resistors.

CONCLUSION AND DISCUSSION

In the present study conducted with equipment designed to ensure the detection and long-term monitoring of many insect larvae that complete their larval stages in a wood tissue without destroying their habitats, listening was performed on decaying trunks of such different trees as oak, Taurus fir, cedar and red pine. To detect the vibration and biting sounds produced on tree trunks by the larvae of Akbez Stag Beetle (*Lucanus cervus akbesianus*), listening was performed at the ground level and 0.5 m and 1 m above the ground level in order to measure the sensitivity of the device, and no significant decrease was observed in sound gain.

Akbez Stag Beetle larvae were detected on the trunks of decaying old oak trees. The detected sounds were recorded by a mobile phone and analyzed with Adobe audition audio editing programme. Consequently, the larvae made approximately 5 clicks in a series and left a 7-8 second gap between two series (Figs. 13-14).

Fig. 13. Stridulation spectrum of Akbez Stag beetle larvae (for 5 clicks).

Fig. 14. Stridulation spectrum of Akbez Stag beetle larvae (for 1 click).

For the determination of the presence of *L. cervus* larvae in decaying wood, Harvey et al. (2011) used terrarium set up in the laboratory to record the biting sound of the larvae. Their purpose was to detect the larvae in the wood without disturbing the larval habitat. For this purpose, a simple piezoelectric transducer and microphone were placed closed to the wood inside the terrarium and connected to an amplifier unit. They determined that the biting sound of *L. cervus* and other saproxylic species (*D. parallelipipedus*) could be determined and each species has a different and characteristic sound pattern. In our study, a sound listening device with piezoelectric sensors with sound amplifier was used. Thus, sound recording was made by amplifying the very low sounds in the decaying wood. Another difference from the other study is that the device trials were made in the natural environment of the larvae. Audio analysis of biting the wood sound of *L. cervus akbesiana* larvae recorded in natural environment is similar to the audio analysis of Harvey et al (2011) *in situ*. The data we have obtained as a result of the study show that the device is suitable and sufficient for use in the field. It is thought that this device can be used not only for the larvae of *Lucanus* species, but also for the detection of other saproxylic beetles larvae without destroying their habitats.

During in the field studies, precision and caution should be exercised when using Piezoelectric sensors. Piezoelectric sensors are an electronic circuit element that converts mechanical stress into electrical energy, and conversely, generates mechanical stress when there is electrical input. Therefore, the resulting signal amplitude increases in parallel with the stress rate applied to the sensor. The input sensitivity should be low, and the gain should be high for the sound amplifier circuit to have a very sensitive input. The signal to be generated by the piezoelectric sensor when it is contacted with the trunk with a hard impact while the circuit is working during the trunk observation is at a high level and damages the integration at the sound amplifier stage. To avoid this problem, observation should be made by energizing the listening circuit after the sensor is contacted with the log to be monitored before the circuit is energized.

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Billaea adelpha (Loew, 1873) (Diptera: Tachinidae), a New Parasitoid of *Xylotrechus sieversi baiocchii* Rapuzzi & Sama, 2018 (Coleoptera: Cerambycidae) from Turkey

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ABSTRACT

A new dipteran larval parasitoid of longhorn beetles (Coleoptera: Cerambycidae), *Billaea adelpha* (Loew) (Diptera: Tachinidae) was reared from *Xylotrechus sieversi baiocchii* Rapuzzi & Sama, 2018 on *Astragalus* plants in Karacağ Mountain of Diyarbakır, Turkey. *X. sieversi baiocchii* was recorded for the first time as host of this parasitoid. Some information on the host-parasitoid couple is given.

Key words: Billaea adelpha, new host record, *Xylotrechus sieversi baiocchii*, Turkey.

Bolu, H., Atay, T., Çelik, H., & Kara, K., (2021). *Billaea adelpha* (Loew, 1873) (Diptera: Tachinidae), a new Parasitoid of *Xylotrechus sieversi baiocchii* Rapuzzi & Sama, 2018 (Coleoptera: Cerambycidae) from Turkey, *Journal of the Entomological Research Society*, 23(1), 83-87.

INTRODUCTION

The Tachinidae is the largest and most important family of insect parasitic flies, with more than 8500 species in the world (O'Hara, Henderson, & Wood, 2019). There are currently 341 tachinid species known from Turkey (Kara, Tschorsnig, & Atay, 2020). All tachinids are endoparasitoids of a variety of insect, mostly phytophagous. There are many important pests of culture plants that are suppressed by tachinids. Some Tachinidae species have been used in classical biological control programmes against lepidopterous defoliators and sawflies, especially in the Nearctic and Neotropical regions (Grenier, 1988; Stireman, O'Hara, & Wood, 2006). In this context, it is important to determine the hosts of tachinids and their relations with their hosts. However, many of the hosts are still unknown. Kara & Tschorsnig (2003) and Tschorsnig (2017) have recently provided the most detailed host-parasitoid catalogue of Turkey and Palaearctic Region respectively.

The genus *Billaea* Robineau-Desvoidy, 1830 is represented with 12 species in Europe (O'Hara et al, 2019). There are two species of the *genus Billaea* in Turkey: *B. adelpha* and *B. irrorata* (Kara, 2001; Özbek, Tozlu, & Çoruh, 2009). Among these, only *B. irrorata* was reared from a host. Like other *Billaea* species, *B. adelpha* shows a strong parasitic preference on coleopteran larvae, primarily cerambycids (Tschorsnig, 2017). *B. adelpha* is the first record of a tachinid for *Xylotrechus sieversi baiocchii* Rapuzzi & Sama, 2018.

MATERIAL AND METHODS

Study area

Karacadağ Mountain, which is an extinct volcano that separates the Diyarbakır basin and the Şanlıurfa Plateau in the north-south direction, in the middle of the Southeastern Anatolia Region. Its highest point is 1952 m. The climate around Karacadağ is dominated by a local steppe climate, summers are hot and dry, winters are cold and rainy. Until recently, there were more forests on it, but the forests remain in secluded areas now. *Quercus* spp., *Celtis* spp., *Crataegus* spp., *Pistacia terebinthus*, and *Fraxinus* spp. are the tree species seen in the area. *Astragalus* plants cover the environment.

Cerambycid species

Xylotrechus sieversi baiocchii Rapuzzi & Sama, 2018. Length 14,0 mm, width 5 mm. The body is black except for elytra and antennae that are light brown, and all tibiae are dark brown (Fig. 1). All the specimens were reared from dead branches, drums, and roots of *Astragalus* cfr. *gummifer* (Fabaceae). The plants often were previously killed by the larvae of *Sphenoptera* sp. (Coleoptera, Buprestidae) (Rapuzzi & Sama, 2018).

Collecting of larvae and rearing of parasitoid

Astragalus plants with larval galleries and adult exit holes of *X. sieversi baiocchii* were collected in Diyarbakır Karacadağ Mountain in 2020. Stems were cut by secateurs

and brought to the laboratory. Field-collected larvae were reared at room temperature (25 ± 2 °C and 60 - 70% relative humidity) (Atay & Kara, 2014). Rearing containers were regularly checked and emergence date of parasitoids and some other observations (pupal duration and the appearance period of adult parasitoids) were noted.

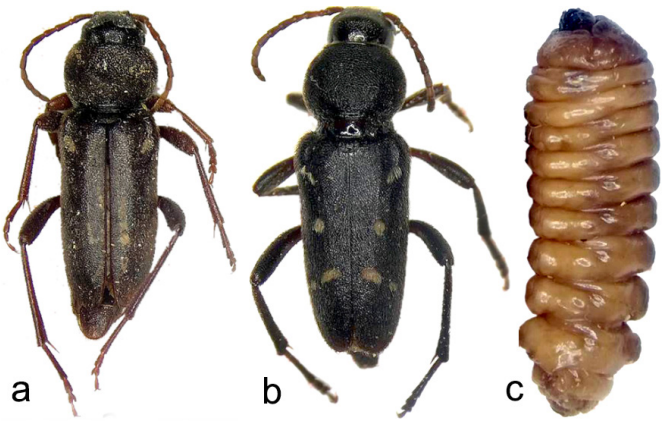

Fig. 1. *Xylotrechus sieversi baiocchii* Rapuzzi & Sama a. ♂ b. ♀ c. larva.

Identification

Tachinid identification was performed by using the identification keys of Tschorsnig & Herting (1994) and Tschorsnig & Richter (1998). The host was identified by Dr. Hüseyin Özdikmen (Gazi University, Faculty of Sciences, Department of Biology, Ankara, Turkey). Images were taken using a Leica MC 170 digital camera mounted on a Leica M205 C stereoscopic microscope and processed with Helicon Focus Pro software. The tachinid specimens were deposited at the Plant Protection Museum in Tokat Gaziosmanpaşa University, Agricultural Faculty, Tokat, Turkey.

RESULTS

The identification, distribution, hosts, and some additional information related to tachinid and host are as follows.

Host. *Xylotrechus sieversi baiocchii* Rapuzzi & Sama, 2018 (Coleoptera: Cerambycidae)

Material examined: The larvae of *X. sieversi baiocchii* were collected in Karacadağ mountain - Diyarbakır (Bağlar), 26.05.2020, 21.06.2020, N 37°44'52.69", E 39°52'45.11", 1600 m, on *Astragalus* cfr. *gummifer* (Fabaceae).

Billaea adelpha (Loew, 1873) (Diptera: Tachinidae: Dexiinae)

Reared Material: 02.06.2020, 1♀, 1♂; 29.06.2020, 1♀, 1♀.

Distribution: West Palaearctic (O'Hara et al., 2019). In Turkey: Tokat (Kara, 2001).

Description: Arista with hairs at least as wide as the 3rd antennal segment (Fig. 2a). Basicosta yellow, sternopleuron with 3 bristles (Fig. 2b). Females: Hind tibia anterodorsally with a regular bristle comb, at most with an intermediary bristle (Fig. 2c).

Remarks: Larvae of *Billaea adelpha* emerge from host and pupation occurs inside stem of the host plant (Fig. 2d). Pupal duration is approximately one week.

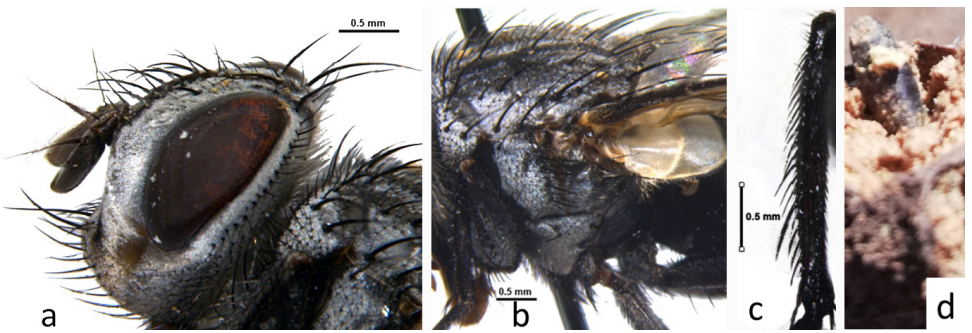

Fig. 2. Adult of *Billaea adelpha* a. Head- lateral view, b. Thorax- lateral view, c. Hind tibia d. Puparium.

DISCUSSION

The genus *Billaea* Robineau-Desvoidy belongs to the Dexiinae subfamily and is seen commonly in the world (O'Hara, Henderson, & Wood, 2019). *Billaea adelpha* has western Palearctic distribution extending to Azerbaijan (Herting, 1984). It prefers dry and hot areas (Tschorsnig & Herting, 1994). This species has been detected on Apiaceae plants and on the shadows of large stones in field studies (Tschorsnig, 1992). *B. adelpha* develops as a parasitoid especially on Cerambycidae (Coleoptera) larvae. Besides, it was reared from a buprestid and a scarabeid (Tschorsnig, 2017). There are two species belonging to the genus *Billaea* in Turkey. *B. adelpha* was obtained with sweeping net by (Kara, 2001) during fieldwork in Tokat, Turkey. *B. irrorata*, the other species belonging to this genus in Turkey, was reared from *Saperda populnea* (L.) (Col.: Cerambycidae) by Özbek et al. (2009). In this study, *B. adelpha* was first reared from *Xylotrechus sieversi baiocchii* Rapuzzi & Sama, 2018. The location where the host is found is Karacadağ, with hot and dry summers dominated by steppe climate. This information is similar to the given data by Tschorsnig & Herting (1994).

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Comparison of Microstructures on Elytral Disc of Some Species of the Genus *Acmaeoderella* Cobos, 1955 (Coleoptera: Buprestidae)

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ABSTRACT

The external morphological characters of integumental scales in four species of *Acmaeoderella* Cobos, 1955 (Coleoptera: Buprestidae) were examined using stereo microscope (Olympus SZX-12) and scanning electron microscope (JEOL JSM 6060 SEM). Dried museum materials that are deposited in ZMGU (Zoology Museum of Gazi University) were used in this study. Specimens were examined under the stereo microscope (Olympus SZX-12). Similarities and differences among the species were discussed. Although the scales look alike under the stereo microscope, they showed a rather different pattern under SEM. The fine structure of scales was shown to be useful for species diagnostics.

Key words: Coleoptera, Buprestidae, *Acmaeoderella,* elytral microstructures, scales, SEM.

Çağlar, Ü., (2021). Comparison of microstructures on elytral disc of some species of the genus *Acmaeoderella* Cobos, 1955 (Coleoptera: Buprestidae). *Journal of the Entomological Research Society*, 23(1), 89-95.

INTRODUCTION

The Buprestidae comprise about 14500 described species in 494 genera. (Volkovitsh 2001; Bellamy 2003). The genus *Acmaeoderella* Cobos, 1955 consists of six subgenera and more than 120 species in the Palaearctic region (Volkovitsh, Sakalian & Georgiev 2015). The family is one of the most species-rich families in the order Coleoptera (Muskovits & Hegyessy, 2002). The family Buprestidae is cosmopolitan and includes many species which are agricultural and forestry pests (Karagyan, Kuznetsova & Lachowska 2004). Buprestidae are mainly wood-boring insects damaging weakened trees, though some species may attack healthy trees (Lodos & Tezcan 1995). In addition, many buprestid species have received considerable interest from conservation biologists. Some species are red-listed in several countries, and buprestids are good indicators of biodiversity in the communities of saproxylic insects (Evans, Moraal & Pajares 2007). Their development takes place in the bark of dead, foliaged trees or the ligneous stalk of perennial herbaceous plants (Muskovits & Hegyessy, 2002).

Buprestidae are commonly known as jewel beetles (Muskovits & Hegyessy, 2002; Evans, Moraal & Pajares 2007).The external morphology of jewel beetles are often used in keys. In general, jewel beetles are heavily sclerotized beetles (Muskovits & Hegyessy, 2002). Abdomen of the most species is completely covered by the elytra which have longitudinal grooves, or rows of punctures, or longitudinal keels (Evans, Moraal & Pajares 2007). Scutellum is reduced.in *Acmaeoderella* and the species of this genus are mostly small cylindrical beetles covered with scale-like hairs or wide scales (Muskovits & Hegyessy, 2002).

Character searching is one of the main tasks for taxonomists and is always time-consuming; finding out new sets of diagnostic characters is especially important (Liu, Schönitzer & Yang, 2009). In this article, we present a detailed examination of the elytral surface structures and scales of some species of the *Acmaeoderella* genus using both stereo microscope and scanning electron microscope (SEM). Diagnostic value of scales for species identification can be best illustrated by utilizing the SEM.

MATERIAL AND METHODS

Four species of *Acmaeoderella* Cobos, 1955 were used in this study, which are *Acmaeoderella (Liogastria) chrysanthemi* (Chevrolat, 1854), *A. (Omphalothorax) despecta* (Baudi di Selve, 1870), *A. (O.) longissima* (Abeille de Perrin, 1904) and *A. (Carininota) mimonti* (Boieldieu, 1865). Specimens were studied from the various habitats and altitudes of the Asian part of Turkey collected by sweeping net between 2013 and 2014. All specimens are deposited in the Zoological Museum of Gazi University (ZMGU).

Dried museum samples were examined under stereo microscope (Olympus SZX-12). For examination under Scanning Electron Microscope, samples were cleaned, dried and mounted with double-sided carbon tape on SEM stubs, coated with gold in a Polaron SC 502 Sputter Coater, and examined with a JOEL JSM 6060

SEM operated at 10 kV. We paid special attention to the middle part of the elytral disc in all species and then investigated the external morphology of scales under the different magnifications.

RESULTS

Stereomicroscopic examination

The species (*Acmaeoderella chrysanthemi, A. despecta, A. longissima* and *A. mimonti*) were first investigated under a stereo microscope (Figs.1a-d). All species have irregular yellow-colored patterns on the elytra. While these patterns on the elytra are longitudinal in *A. crhysanthemi*, these patterns in the other three species (*A. despecta, A. longissima, A. mimonti*) are in the form of irregular transverse bands or spots (Figs.1a-d). In addition, it is seen that the scales on the elytra look similar in all species, but these scales are sparser in *A. mimonti* (Fig.1d) and more dense in other species (*A. chrysanthemi, A. despecta* and *A. longissima*) (Figs.1a-c).

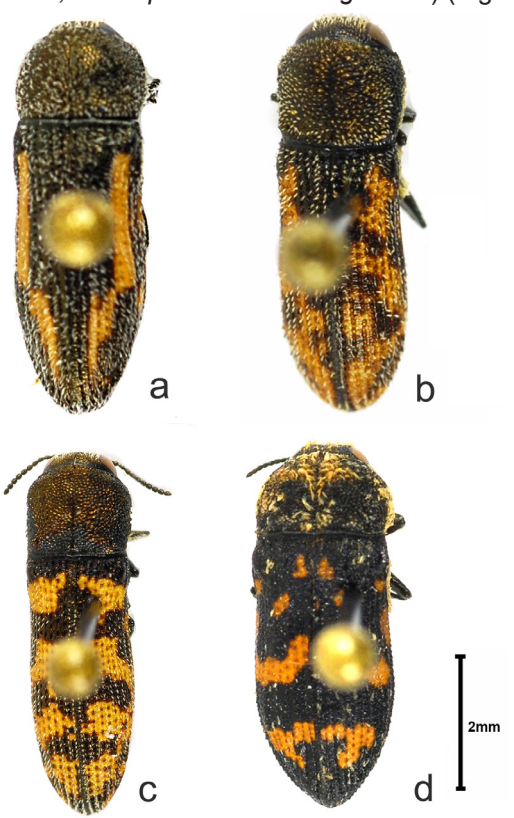

Fig. 1. Dorsal view of the *Acmaeoderella* species under a stereomicroscope; a) *A. chrysanthemi* (Chevrolat, 1854), b) *A. despecta* (Baudi di Selve, 1870), c) *A. longissima* (Abeille de Perrin, 1904), d) *A. mimonti* (Boieldieu, 1865).

SEM examination

The whole body, elytra surface and scales of all species were examined in detail under the scanning electron microscope. In *A. chrysanthemi, A. despecta*, *A. longissima* the body is cylindrical (Figs. 2a-c), in *A. mimonti*, the body is flattened dorso-ventrally (Fig. 2d).

We paid particular attention at the middle part of the elytral disc in all species. In *A. chrysanthemi* there are deep but not adjacent longitudinal strial punctures on the elytra.

Scales arise from piliferous micropunctures which in turn located on the bottom of larger punctures which are well visible on the photos. Usually iterstriae are alternatively wider and narrower; normally narrower interstriae bear uniseriate (1 row) scales, while wider ones - multiseriate (2, 3> rows) scales (Fig. 3a). In *A. despecta*, there are deep and sometimes adjacent punctures in the elytral striae. In the interstriae, the scales are arranged in a single row (Fig. 3b). In *A. longissima*, there are very deep adjacent strial punctures in the elytral striae. In the elevated area between these strial punctures, scales are arranged in a single row (Fig. 3c). In *A. mimonti*, there are shallow, narrow and longitudinally elongated strial punctures on the elytra. There are nearly oval scales and short setae in the interstriae (Fig. 3d).

The scales on the elytra were observed to they have different leaf shapes performed by scanning electron microscope. In *A. chrysanthemi*, the scales are lanceolate, the edges are serrate and they have a rib in the middle (Fig. 4a). In *A. despecta*, the scales are lanceolate, the surface with 2-3 ribs (Fig. 4b). In *A. longissima*, the scales are spatulated, the edges are serrate and the surface with 3-5 ribs (Fig. 4c). In *A. mimonti*, the scales are elliptical, and a saw-shaped rib extends in the middle. The edges of the scales are saw-like and the surface is covered with numerous spines (Fig.4d).

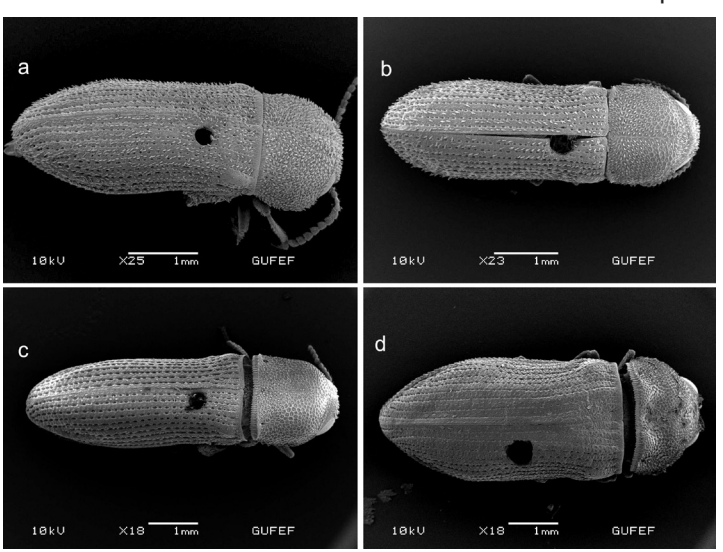

Fig. 2. Generel SEM photos of dorsal view of the *Acmaeoderella* species; a) *A. chrysanthemi* (Chevrolat, 1854), b) *A. despecta* (Baudi di Selve, 1870), c) *A. longissima* (Abeille de Perrin, 1904), d*) A. mimonti* (Boieldieu, 1865).

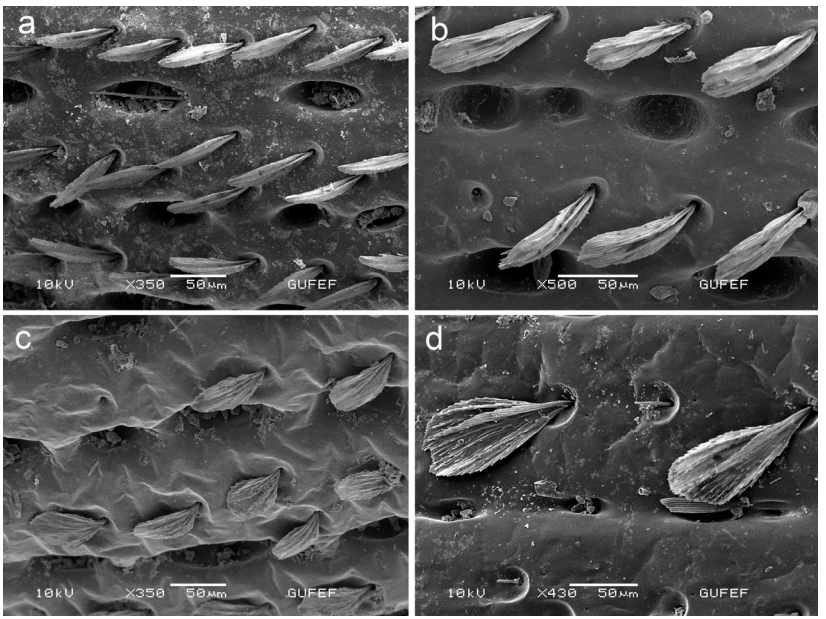

Fig. 3. Generel SEM photos of dorsal view of the *Acmaeoderella* species; a) *A. chrysanthemi* (Chevrolat, 1854), b) *A. despecta* (Baudi di Selve, 1870), c) *A. longissima* (Abeille de Perrin, 1904), d*) A. mimonti* (Boieldieu, 1865).

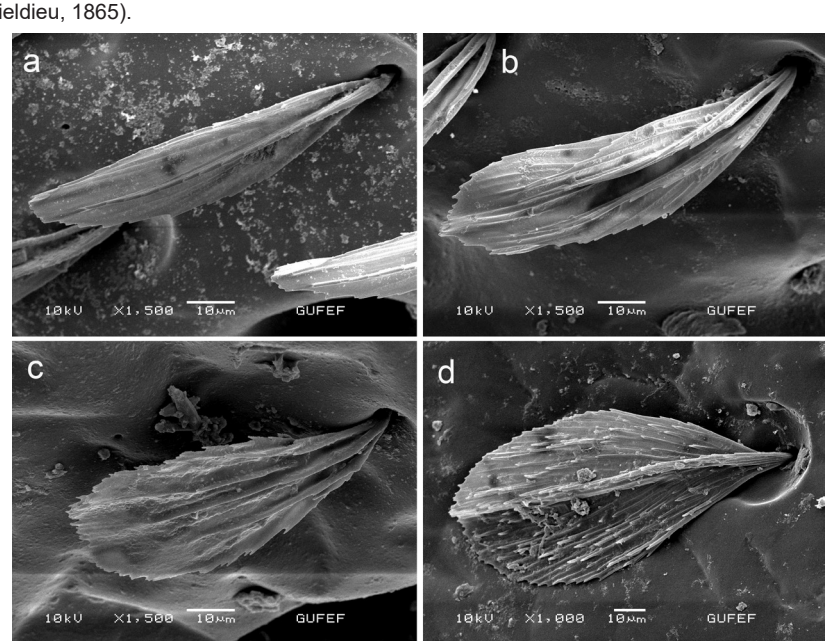

Fig. 4. SEM photos of scales of the *Acmaeoderella* species; a) *A. chrysanthemi* (Chevrolat, 1854), b) *A. despecta* (Baudi di Selve, 1870), c) *A. longissima* (Abeille de Perrin, 1904), d*) A. mimonti* (Boieldieu, 1865).

DISCUSSION

In this study the scales of four species of the genus *Acmaeoderella* (Coleoptera: Buprestidae) were investigated under a scanning electron microscope for the first time. The similarities and differences of some special microstructure characters among species were discussed.

Some morphological characters in taxonomy are very important such as hairs, setae, punctures, color and are used in diagnoses (Bílý, Kubáň, Volkovitsh & Kalashian, 2011; Booth, Cox, & Madge, 1990; Erbey & Candan, 2013, 2015; Hoffmann, 1954, Jendek, 2001; Manley, 1987; Sun & Bhushan, 2012; Volkovitsh,1979, 1983; Volkovitsh & Bílý 1979; Wagner, Neinhuis & Barthlott, 1996). Elytral colors are ecologically very important, and elytral microstructure is also very important as a model in technology and engineering. The structural colors of elytra are actually interference colors mixing partly to produce camouflage by matching the color of the environment. The study of the structure, functional and mechanical properties of beetle elytra gives an opportunity to understand their behavior and characteristics. This can form a good basis for the research and development of bioinspired materials, structures, and smart devices. (Sun & Bhushan, 2012).

While the colors of the elytra differ in the examinations under the stereo microscope, the scales look similar (Figs.1a-d). Under the scanning electron microscope, there is a difference in the general body shapes in low magnification examinations, while there is no significant difference in the structures on the elytra (Figs.2a-d). In addition, microstructures (scales and punctures) on the elytra in high magnification examinations under scanning electron microscopy are different in all species and have their own characteristics (Figs.3a-d, Figs.4a-d).

As a result, microstructures on the elytra surface can be used as taxonomic characters in species identification. To give more effective results, it was concluded that the populations of samples collected from different localities for each species should be compared. In addition, the investigation of the surface morphology of scales in insects may help in differentiation of similar species that have similar shape and color. Finally, when further investigated, elytral structures in beetles can help develop new bio-inspired designs in the fields of material science and technology.

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New Species of *Adoxomyia* (Kertész, 1907) (Diptera: Stratiomyidae) from Turkey

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ABSTRACT

A new species of soldier flies (Diptera: Stratiomyidae), *Adoxomyia hasbenlii* sp. nov., is described from Turkey. The characters of the new species were examined and photos are presented, and the species-specific male terminalia is illustrated and compared with related species, *Adoxomyia variabilis* Krivosheina, 2016, and *Adoxomyia ruficornis* (Loew, 1873). An identification key to the related species of *Adoxomyia* is presented.

Key words: Stratiomyidae, *Adoxomyi*a, new species, biodiversity, Turkey.

Üstüner, T. (2021). New Species of *Adoxomyia* (Kertész, 1907) (Diptera: Stratiomyidae) from Turkey, *Journal of the Entomological Research Society*, 23(1), 97-103.

INTRODUCTION

The genus *Adoxomyia* (Kertész, 1907) belongs to the subfamily Clitellariinae in the family Stratiomyidae and includes over 40 described species that are distributed all over the world, including 20 species in the Palearctic region (Lindner, 1938; Woodley, 2001; Hauser, 2002; Nartshuk, 2004; Üstüner, 2012; Krivosheina, 2016; 2017; 2018). Thus far, 7 species belonging to the genus have been identified from Turkey (Üstüner & Hasbenli, 2011; Üstüner, 2012). These species comprise *A. aurovittata* (Bigot, 1879); *A. begreliensis* Üstüner, 2012; *A. cinerascens* (Loew, 1873); *A. dahlii* (Meigen, 1830); *A. obscuripennis* (Loew, 1873); *A. sarudnyi* (Pleske, 1903); and *A. palaestinensis* Lindner, 1937.

The adult species of *Adoxomyia* are flies with a black body. The eyes, in males, are adjacent to each other. There is a very fine, hair-thin line between them; hence, they appear as though they are connected, while in females they are separated by a wide frons. The antennae, which have important distinguishing characters for the identification of the species, are thicker in the females than in the males. The flagellum of the antennae consists of 8 flagellomeres, but the shape and arrangement of the 4-6 flagellomeres differ significantly between species. In some species of this genus, the antennae are dark-colored, while some others are bi-colored. One of the important characters in the identification of the species of the genus is the structure of the genitalia. In the male genitalia, the middle protrusion (median process) of the synsternum and the shape of the dististylus differ according to the species.

MATERIAL AND METHODS

In this study, a new species belonging to the genus *Adoxomyia* was defined, based on 1 male and 2 female specimens. The specimens. The specimens were deposited in the collection of the Selçuk University, Department of Biology in Konya, Turkey. Illustrations of the specimens were made using a Leica stereomicroscope (Leica Microsystems, Wetzlar, Germany) with Integrated LED Ilumination and Digital 3 MP Camera Leica EZ4 D camera system and then imported into Adobe Photoshop CS9 (Adobe Inc., San Jose, CA, USA) for labeling and plate composition. One male and one female were dissected and the genitalia cleared in KOH overnight and placed in glass/plastic vials on the same pin as the specimen.

Adoxomyia hasbenlii sp. n.

Material examined: Holotype: ♂ Turkey, Kayseri, Yahyalı, Between Yahyalı and Dikme Village, elev. 1404 m, 38° 2′ N, 35° 23′ E, 14.08.2002, leg.: T. Üstüner.

Paratypes: 1♀Turkey: Kayseri, Yahyalı, Between Yahyalı and Dikme Village, elev. 1404 m, 38° 2′ N, 35° 23′ E, 14.VII.2002, leg.: T. Üstüner. 1♀Turkey: Konya, Bozkır, Karacahisar Village, Aygırdibi Location, elev. 1400 m, 17.08.2009, leg.: T. Üstüner.

Description: Male (Figs. 1-3, 7-9): Head semicircular in lateral view, completely black. Eyes contiguous, with erect, dense setae. Eyes pile black, almost as long as

scape. Frons and face black, covered with dense black setae as long as total length of the scape and pedicel. Frons with a fine longitudinal grove. Grove extends from the top corner of frons triangle to the bases of the antennae. Postocular band developed, its width is about as wide as the 7th flagellomere long in dorsal view. Postocular band is as wide as the total length of the 6th and 7th flagellomeres in the upper half of the head and as wide as the length of the first 2 antennal segments in the lower half of the head in lateral view. Postocular area covered with short, adpressed, silvery-white setae. Antenna (Fig. 3) as long as the length of the head and 2-colored, mainly darkened, but first 3 flagellomeres dark-reddish brown. The scape is about 1.5 times the length of the pedicel. Scape and pedicel with black hairs, approximately as long as 1.5 times the length of the pedicel. Three basal flagellomeres distinctly widened, their width slightly wider than the pedicel. From the fourth flagellomere on gradually narrowing. First flagellomere about 1.5 times as long as the 2nd flagellomere (15:10). Fifth flagellomere about 1.5 times as long as the 4th, 6th, and 7th flagellomeres (1.5:1:1:1). Last flagellomere as thick as the width of the 7th flagellomere, conical and equal in length to the 3 preceding flagellomeres combined. Height of the head nearly 1.5 times as long as the length of antennae (11.5:7.5).

Thorax black, with a small reddish-brown spot at the apex of humeral callus. Scutellum black with scutellar spines. Scutellar spines emerge from the corners of the posterior edge of the scutellum, about 1/3 of the length of the scutellum and 1/3 black basally, apical 2/3 light brown, glabrous, obtuse, slightly arcuately bent inwards. Mesonotum and scutellum are covered with two types of setae: black, erect setae, slightly longer than the length of the scutellar spines, and with more dense, oblique, short, whitish setae. Legs mainly dark brown to black, except apical part of trochanters, knees, and ventral surface of tarsi light brown. Wing brownish, with dark veins. Haltere pale yellow with a darkened stalk.

Abdomen black with silvery-white setae along lateral sides of terga 1-3, on posterior margin of tergum 4, and over the entire surface of tergum 5. Genitalia: epandrium elongate; synsternum wide, elongate with triangular median process, dististylus thick, short, and pointed apex. The aedeagus is as in fig. 9.

Length of male: 6 mm, wing 5 mm.

Female (Figs. 4-6, 10-11): Head black. Eyes with dense, dark brown, erect hairs, eye piles about as long as pedicel. Frons about 1/3 of head-width, slightly tapered towards the vertex. Postocular band strongly developed, its width is about as broad as the combined length of the 4th-7th flagellomeres measured in dorsal view. The postocular area black and covered with short, adpressed, and silvery-white hairs.

Antennae red and black (Fig. 6). Scape and pedicel black, first 3 flagellomeres bright reddish-brown. Scape slightly longer than pedicel (2:1.5). First 3 flagellomeres in female are wider than in male (1.5:1). First 3 flagellomeres of the flagellum distinctly widened, their width about 2 times the length of the pedicel. Last flagellomere slightly thickened at the base, narrowed and rounded at apex, equal in length to 3 preceding flagellomeres combined. Antennae are almost equal in length to the length of the head.

Mesonotum and scutellum with more densely oblique, short, and whitish hairs, but not covered with black erect hairs as in male. Abdomen as those in male. Margins of tergites with narrow light brown edging.

Length of female: 6.2 mm. Wing 4.5 mm.

Etymology: The species was named in honor of Prof. Dr. Abdullah Hasbenli, a prominent entomologist who contributed significantly to the knowledge of Turkish and Palearctic diptera.

Key to the East Mediterranean Species of the genus *Adoxomyia* (Kertész, 1907)

1. Legs mainly black ……..……...2

-Legs bicolored or mainly yellow……...……7

2. Antenna entirely bicolored.....3

-Antenna entirely black.……..……..5

3. Scutellum spines black basally, apically yellowish. *Postocular band in dorsal view wide in male.* Antennae of male dark……4

-Scutellum spines black. *Postocular band in dorsal view narrower in male.* Antennae of male light brown first 3 flagellomere. ……..……*A. ruficornis* (Loew, 1873)

4. Antennae of male shorter than length of head and usually black. The last flagellomere thin and approximately 1/5 of the total length of the flagellum…… ……..……*A. variabilis* Krivosheina, 2016

-Antennae of male almost equal to the length of the head and with the first three flagellomeres dark reddish-brown. The last flagellomere thick and approximately 1/4 of the total length of the flagellum. ……*A. hasbenlii* sp. n .

5. Scutellar spines short, slender and bare, basal 3-4 flagellomeres in female unusually broad……...**6**

-Scutellar spines longer, thickened and haired, basal 3-4 flagellomeres not as broad……..……..*A. obscuripennis* (Loew, 1873)

6. Female eyes black haired, postocular band wider than scape is long; male unknown ……..……..……..…...*A. hermonensis* Lindner, 1975

-Female eyes white haired, postocular band as wide as scape is long ……..……...*A. transcaucasica* Nartshuk, 2003

7. Legs entirely yellow ……*A. sarudnyi* (Pleske, 1903)

-Legs bicolored, at least femora black…...……8

8. Antenna black ……..……9

-Antenna partly brownish orange ……10

9. Body coarse and plump, abdomen with golden yellow hair patches..... .....……..……*A. aureovittata* (Bigot, 1879)

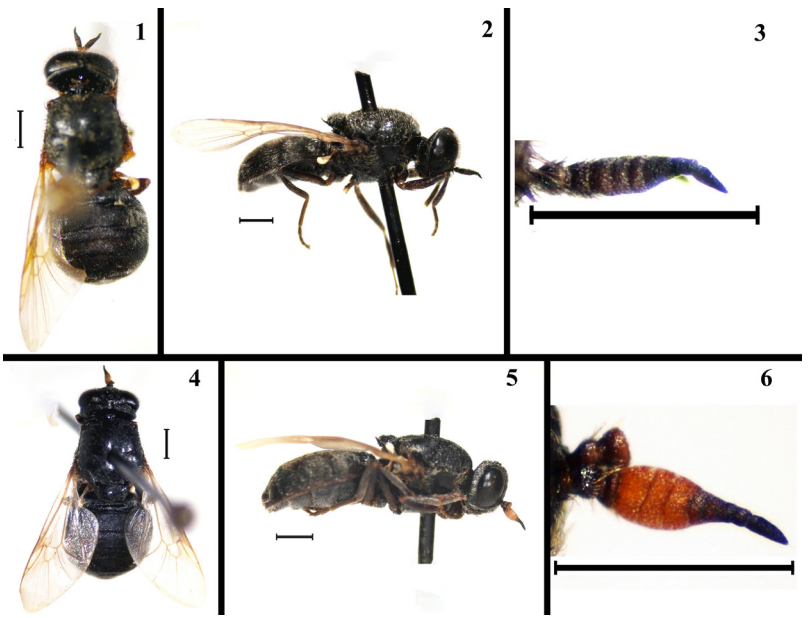
-Body slim and delicate, abdomen with silverish white hair patches.…… ……..……..……..*A. palaestinensis* Lindner, 1937

10. Scutellar spines black, third segment of antenna dark brown to black.....
.....*A. dahlii* (Meigen, 1830)

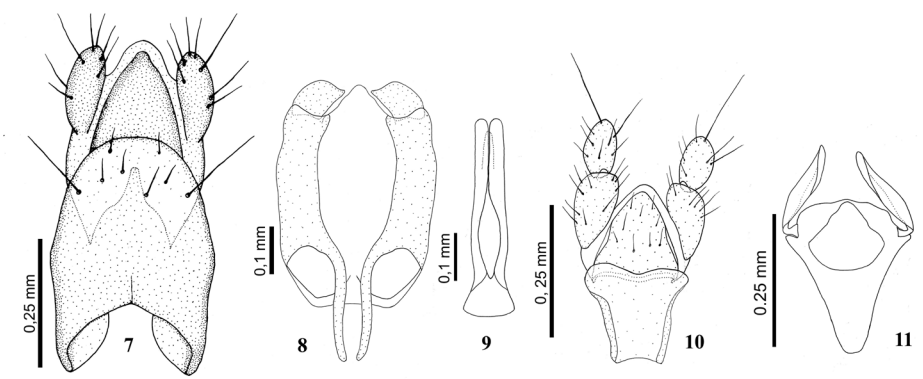
-Scutellar spines yellow, third segment of antenna orange ……11

11. Antenna 1.5 times as long as head, male flagellum cylindrical, female first three flagellomeres twice as broad as scape is long……...*A. begreliensis* Üstüner, 2012

-Antenna about as long as head, male and female flagellum distinctly swollen in middle……*A. cinerascens* (Loew, 1873)



Figs. 1-6: *Adoxomyia hasbenlii* sp.n., adult: 1. Male dorsal view; 2. Male lateral view; 3. Male antennae; 4. Female dorsal view; 5. Female lateral view; 6. Female antennae. Scale bar: 1mm



Figs. 7-11. *Adoxomyia hasbenlii* sp. n adult: 7. Male epandrium, dorsal view; 8. Male hypandrium view from inner side; 9. Male aedegus; 10. Female terminal dorsal view; 11. Female genital furca.

DISCUSSION

This new species is similar to *Adoxomyia variabilis* Krivosheina, 2016, described from Azerbaijan (Krivosheina, 2016). The length of the last flagellomere of *Adoxomyia variabilis* is approximately 1/5 of the total length of the flagellum, while the length of the last flagellomere of *Adoxomyia hasbenlii* is approximately 1/4 of the total length of the flagellum. The male antenna of *Adoxomyia variabilis* is slightly longer than the head and usually black, while that of *Adoxomyia hasbenlii* is almost equal to the length of the head, and the first 3 flagellomeres of the antennae flagellum are dark reddish-brown. The hypandrium of *Adoxomyia variabilis* is rectangular and its lateral edges are flat from the posterior to the anterior end, whereas the hypandrium of *Adoxomyia hasbenlii* is rectangular, its lateral edges are slightly inwardly recessed in the posterior part and slightly widen on the anterior part. The median process of the hypandrium of *Adoxomyia variabilis* is conical, rounded at the apical, and the dististylus of the hypandrium is a thick and pointed apex, while the median process of the hypandrium of *Adoxomyia hasbenlii* is conical, slightly pointed at the apically, and the dististylus is slightly thicker, stout, and pointed at the tip. In the female *Adoxomyia variabilis*, the ocellar tubercel is at the level of the posterior margin of the eye, while in the female *Adoxomyia hasbenlii*, the ocellar tubercel is slightly projected at the posterior edge of the eye. In the female *Adoxomyia hasbenlii*, the genital furca is different from that in *Adoxomyia variabilis*.

Adoxomyia hasbenlii is similar to *Adoxomyia ruficornis* in that its antennae are bi-colored. However, the antennae of *Adoxomyia ruficornis* are shorter than the head, the scutellar spines are black, and in dorsal view the postocular band in males is very thin (Krivosheina, 2017). *When A. ruficornis* male genital shape presented in the paper of Krivosheina (2017) is examined, it is seen that *Adoxomyia hasbenlii and Adoxomyia ruficornis* differ from each other by their different genital capsules.

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AUTHOR GUIDELINES

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For faunistic research follow this order, Distribution:., Material examined:…, Host plant:….etc.

Example:
Sphex oxianus Gussakovskij, 1928
Distribution: Central and South West Asia, Afghanistan, Iran, Israel, Turkey (Bohart and Menke, 1976; Menke and Pulawski, 2000; Kazenas, 2001), Turkey: Artvin (De Beaumont, 1967).
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 ♀♀
Host plant: Echinophora sp.
Please use ♀, ♂ symbols. Please write upper genus categories with capital letters.

Illustrations: Illustrations, graphs, their caption or legends should form a separate, and a self-explanatory unit. Abbreviations in the legends should be explained but if there are too many, they should be included into a separate list. The original drawing and photographs should not be more than twice as large as when printed.

Morphological illustrations should include a scale bar. Photographs and electron micrographs should be in high-resolution JPEG file format (300 dpi). Drawings (black and white type) should be in TIFF format and their size should be no more than 10 MB. Graphs should also be in black and white and submitted in excel file format. Tables should include headings and explanations, and should be numbered consecutively. Their approximate position in the text should be indicated in the margin. Legends and titles of the graphs and tables should be in Arial with 12 type size. Please do not embed the figures, graphs and table into the text, and send them as supplementary files. In the text attribution to the figures should be given in parenthesis and must be abbreviate like this; (Fig.1).Figs. 1-10. A. marriotti sp. n.. 1. Male (holotype), dorsal. 2. Female (paratype)

References:

Titles of manuscripts published in languages other than the major ones (English, German, French, Spanish, Portuguese, Turkish) should be an English translation (in parentheses) with an explanatory note at end, e.g. (in Russian). The list of references should be given at the end of the article and listed alphabetically, according to the following examples. All periodical names should be unabbreviated and italicized. In references, journal titles must be written in full (not abbreviated).

Journal Article

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Book

Steinmann, H.A. & Zombori, L. (1985). *An atlas of insect morphology*. (2nd ed.). Akadèmiai Kiadò, Budapest, Hungary.

Edited Book

Williams, S.T. (Ed.). (2015). *Referencing: A guide to citation rules* (3rd ed.). New York, NY: My Publisher

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Troy, B.N. (2015). APA citation rules. In S.T, Williams (Ed.). *A guide to citation rules* (2nd ed., pp. 50-95). New York, NY: Publishers.

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Mitchell, J.A. (2017, May 21). *How and when to reference*. Retrieved from https://www.howandwhentoreference.com.

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