



**ISOLATION OF FILAMENTOUS FUNGI ASSOCIATED WITH TWO COMMON
EDIBLE AQUATIC INSECTS, *HYDROPHILUS PICEUS* AND *DYTISCUS
MARGINALIS***

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ABSTRACT

Insects are widely used for their potential source of protein, lipids, carbohydrates and certain vitamins in many parts of the world. As in terrestrial ones, aquatic insects can also carry fungal structures. Therefore, in the present study, we evaluated microfungal flora of internal and external surface of *Hydrophilus piceus* and *Dytiscus marginalis* collected from their natural habitats in Erzurum (Turkey). We isolated total 19 different species of fungi belonging to *Penicillium*, *Alternaria*, *Beauveria*, *Trichoderma*, *Fusarium*, *Aspergillus*, *Acremonium*, *Paecilomyces* genera. The relationship between these fungi and edible insects was discussed further in the light of the existing literature. Among the isolated fungi, species that were recognized as pathogenic or toxigenic, and ones having biotechnological importance were found.

Keywords: edible aquatic insect, *Hydrophilus piceus*, *Dytiscus marginalis*, mycoflora

INTRODUCTION

The class *Insecta* consists of a large group of organisms with rich species diversity. There are approximately 750,000 species of insects and some estimates the number as high as 10 million (Novotny et al., 2002).

Insects have played an important role in the history of human nutrition in Africa, Australia, Asia and the America. Insects often contain more protein, fat, and carbohydrates than equal amounts of beef or fish, and a higher energy value than soybeans, maize, beef, fish, lentils, or other beans. As over 1500 different species of insects have been reported as being consumed or edible around the world (Defoliart, 1995; Food-Info, 2009). Of these species, *Hydrophilus piceus* Linnaeus, 1758 (*Coleoptera: Hydrophilidae*) and *Dytiscus marginalis* Linnaeus, 1758 (*Coleoptera: Dytiscidae*), investigated here, are widely used for human consumption in many countries. *H. piceus* is also used in alternative medicine in South–East Asia countries due to its anti-diuretic potential (Jäch, 2003; Rams–Elorduy, 1997; Morris, 2004; İncekara and Türkez, 2009).

Edible insects are constituted as a very common and important food source in many developing countries although these insects contain powerful pharmacologically active substances, which are known as vertebrate toxins (Akinnowo et al., 2002). Hence, eating of these insects may cause as serious harmful effects on humans. The consumption of non-toxic insects, therefore, have been encouraged by the various scientists and governments. In our previous studies, the potential toxic effects of some popular edible insects have been investigated by Incekara and Turkez (2009), Incekara et al. (2010) and Turkez et al. (2010, 2011).

Fungi are eukaryotic organisms and dependant on other organisms for their supply of nutrients. Most fungi are saprophytic, i.e. live on dead organic material and they play an important role in the ecosystem in the recycling of nutrients (Eduard, 2007). Invertebrates host a lot of microorganisms such as fungi or bacteria. Insect-associated microorganisms, particularly endosymbionts, are known to produce bioactive compounds that protect the host against adverse environmental conditions, predators or competitors; thus, they have been suggested as suitable for biotechnological applications (Chaves et al., 2009). Studies on microbial diversity can contribute to the discovery of new substances that can be used in the pharmaceutical and food industries (Harvey, 2000; Chaves et al., 2009).

Incekara and Sisman (2009) reported that *H. piceus* and *D. marginalis* species reached high population density at many wet lands in Turkey, also in these regions exist a great

number of different aquatic insect species that may be useful as a protein source. Moreover, these edible insect species can provide an additional source of income for local communities. Considering these points, we thought that the fungal flora of these edible insects should be investigated before they are converted to commercial products. Although *H. piceus* and *D. marginalis* are widely used for human consumption in many countries, no investigation has been carried out on the fungal flora of these two common edible aquatic insects. This is the first report on the fungal flora of *H. piceus* and *D. marginalis*.

MATERIAL AND METHODS

Sample collection

In the present study, insect samples were collected from their natural aquatic habitats in Erzurum province and its surroundings (East Anatolia) and killed without any chemical treatment. For analysis, adult and healthy insects were selected. Specimens were individually put into sterilized glass containers with screw tops and transported to the laboratory in boxes.

Isolation procedure

The two aquatic insects were transferred to a 0.9% saline solution (10 ml) and vortexed for 3 min. Then, 0.1 ml of this solution was transferred to Potato Dextrose Agar (PDA) plates containing chloramphenicol (0.05 g/L) to inhibit bacterial growth. Later, the collected insects were surface-sterilized by consecutive washing in sterile distilled water and 70% alcohol for 1 min (Pereira *et al.*, 2009). Each of insects was smashed using a mortar and a pestle in 10 ml of phosphate saline buffer. Dilutions of 10^{-4} of each sample (0.1 ml) were seeded onto PDA plates containing chloramphenicol. Plates were incubated at 28°C and examined every two days for two weeks. Isolation percentage was calculated using the formula:

$$\text{Isolation \%} = \frac{\text{Number of fungal isolates of a particular species} \times 100}{\text{Total number of isolates of all species}}$$

Identification of isolates

Cultures growing on PDA and Malt Extract Agar (Difco) were identified according to microscopic observations such as morphological characters of mycelium and conidia. Observations were made by staining the isolated fungus using lactophenol cotton blue and examination under low-power microscope. The species were identified according to **Von Arx (1981)**, **Hasenekoglu (1991)**, **Domsch et al. (1993)**, **Watanabe (2002)**. The organisms were maintained on PDA slants at 4 °C.

RESULTS AND DISCUSSION

A total of 230 fungal isolates were isolated from the adult aquatic insects. Table 1 shows the number and frequency of these fungi. Among the identified genera, the genus *Penicillium* included the greatest number of species (5): *P. frequentans* Westling, *P. brevicompactum* Dierckx, *P. expansum* Link ex Gray, *P. jensenii* W. Zalesky, *P. aurantiogriseum* Dierckx and *P. notatum* Westling. In this study, three species belonging to the genus *Aspergillus* were isolated: *A. niger* Tiegh., *A. versicolor* (Vuill.) Tirab. and *A. nidulans* (Eidam) G.Winter. Two species belonging to the genus *Cladosporium* were found: *C. C. cladosporioides* (Fresen.) B. de Vries and *C. herbarum* (Pers) Link ex Gray. We also identified *Beauveria bassiana* Vuill., *Trichoderma harzianum* Rifai, *Fusarium* sp., *Acremonium* sp., *Paecilomyces marquandii* (Masse) S. Hughes. After being transferred to different sporulation media, two different isolates did not have reproductive structures and were placed into the *Mycelia sterilia*.

P. frequentans was the most frequent and predominant species detected in the present study, followed by *A. niger*, *C. cladosporioides* and *T. harzianum* species for *D. marginalis*. For *H. piceus*, *P. frequentans* was also the most frequent and predominant species, followed by *A. niger*, *C. herbarum*, *T. harzianum* and *B. bassiana*. We found that *P. frequentans* and *A. niger* were associated with internal and external surface of each insect. In the composition of fungal complexes of the surveyed insects, we found four common species, namely *P. frequentans*, *A. niger*, *C. cladosporioides* and *T. harzianum*. Some differences in the taxonomic composition of fungi are probably due to different physiological and metabolic responses of the insects to the environmental conditions.

Table 1 Taxonomic composition and distribution of fungi in the internal and external surface of *H. piceus* and *D. marginalis*.

Isolated fungus	The number of isolates (%)			
	<i>D.marginalis</i> surface	<i>D.marginalis</i> internal	<i>H.piceus</i> surface	<i>H.piceus</i> internal
<i>Acremonium</i> sp.	1 (1.3)	0	0	0
<i>Aspergillus nidulans</i>	0	0	1 (1.56)	0
<i>Aspergillus niger</i>	14 (18.4)	10 (26.31)	12 (18.75)	10 (19.23)
<i>Aspergillus versicolor</i>	0	2 (5.26)	0	0
<i>Beauveria bassiana</i>	0	0	6 (9.37)	6 (11.53)
<i>Cladosporium cladosporioides</i>	12 (15.78)	8 (21.05)	10 (15.62)	0
<i>Cladosporium herbarum</i>	5 (6.57)	0	8 (12.5)	0
<i>Mycelia sterilia</i> 1	2 (2.6)	0	0	0
<i>Mycelia sterilia</i> 2	0	0	0	2 (3.84)
<i>Fusarium</i> sp.	0	0	3 (4.68)	2 (3.84)
<i>Paecilomyces marquandii</i>	0	2 (5.26)	0	0
<i>Penicillium brevicompactum</i>	0	4 (10.52)	0	0
<i>Penicillium expansum</i>	8 (10.5)	0	0	0
<i>Penicillium frequentans</i>	20 (26.31)	12(31.57)	22 (34.37)	16 (30.76)
<i>Penicillium jensenii</i>	5 (6.57)	0	0	2 (3.84)
<i>Penicillium notatum</i>	1 (1.3)	0	0	0
<i>Penicillium aurantiogriseum</i>	0	0	2 (3.12)	0
<i>Sphaeropsidales</i>	0	0	0	2 (3.84)
<i>Trichoderma harzianum</i>	8 (10.5)	0	0	12 (23.07)
Total	76	38	64	52

Legend: % = percentage of isolation

The association with a microbial flora of several insect groups, such as *Hymenoptera*, *Lepidoptera*, *Diptera*, among others, is widely exemplified by studies of insect-fungus interactions (Pereira et al., 2009; Kirpik et al., 2010; Braide et al., 2011). Kirpik et al. (2010), isolated the predominance of the genus *Penicillium* and *Alternaria* in *Apis mellifera caucasica*. These authors also reported the isolation of other species, such as *Aspergillus niger*, *A. fumigatus*, *Trichoderma harzianum*, *T. koningii*, *Ulocladium consortiale*, *Fusarium* sp. and *Mucor* sp. Braide et al., (2011) isolated and identified fungi from edible caterpillar of an emperor moth, *Bunaea alcinoe* and found *A. niger*, *Penicillium caseiocolum* and *Fusarium moniliforme*. In another work on isolation and identification of fungi in *Mosquito larvae*,

Pereira et al., (2009) reported various species, such as *Acremonium kiliense*, *Aspergillus sydowii*, *Fusarium sacchari* var. *sacchari*, *F. merismoides* var. *merismoides*, *Gliocladium viride*, *Paecilomyces* sp., *Penicillium citrinum*, *P. sclerotiorum*, *P. melinii* and *P. oxalicum*. These results demonstrated that especially *Aspergillus*, *Fusarium* and *Penicillium* species have been isolated from insects.

Some genera of fungi were associated with a specific type of substrate. Conidia (asexual spores) adhere to the surface of the host release extracellular enzymes including lipases, proteases and chitinase that help breach the host's chitinous exoskeleton. *B. bassiana* infects a variety of insects. It produces toxins which kill the host. After host death, the mycelium grows throughout the cadaver (**Bidochka and Khachatourians, 1987; Duo-Chuan, 2006; Shin et al., 2010**). Moreover, it has been reported that *Trichoderma* and *Aspergillus* have significantly reduced the survival and reproduction of spruce beetle (**Cardoza et al., 2006**).

The classification of mould metabolites as antibiotics or mycotoxins is on the basis of their toxicity or beneficial effect in treating diseases. Mycotoxin production is dependent on a number of factors, such as water activity, temperature, substrate, strain of mould, the presence of chemical preservatives and microbial interactions (**Chagas et al., 2000; Varga et al., 2005**). They are highly toxic secondary metabolic products of moulds mainly belonging to *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* species (**Schatzmayr et al., 2006**). Mycotoxins such as ochratoxin (*A. niger*), sterigmatocystin (*A. versicolor*), patulin (*P. expansum*), cyclopiazonic acid (*P. aurantiogriseum*), fumonisins (*Fusarium* species and *Alternaria alternata*), trichothecenes (*Fusarium* and *Trichoderma* species), beauvericin and oosporein (*B. bassiana*) are important as food-safety agents (**Sweeney and Dobson, 1998; Bennett and Klich, 2003; Valencia et al., 2011**). The various biological effects of mycotoxins such as organ toxicity, carcinogenicity, teratogenicity, allergic symptoms and immunosuppressant are well documented by *in vitro* and *in vivo* experiments (**Sweeney and Dobson, 1998; Bennett and Klich, 2003**).

Mycotoxins usually enter the body via ingestion of contaminated foods, inhalation of toxigenic spores and direct dermal contact. Various food commodities may be contaminated with mycotoxins, which even in small quantities have been detrimental effects on vertebrates' health (**Bennett and Klich, 2003**). The presence of toxigenic fungi in edible insects provides a real risk of poisoning if the infected insects are used for food (**Chagas et al., 2000; Bennet and Klich, 2003**). Many of the entomopathogenic fungi produce toxins which act as poisons for the insects (thereby are killed) and for the people who eat them.

Transport, storage and packaging are significant factors to be considered in ensuring the safety of the product. There is loss of proteins and lipids leading to reduction in nutritional value during fungal contamination. Most data indicate that baking, frying, roasting, microwave heating or vinegar and essential oils treatments cause reductions in mycotoxin levels in different food materials during processing and post-processing stages (**Nguefack et al., 2004; Kabak, 2009; Braide et al., 2011**).

Insect-associated microorganisms are known to produce bioactive compounds suitable for industrial or biotechnological applications. As cited above, some of the isolates have been reported in the literature as having biotechnological potential (**Kurbanoglu, 2004; Nicoletti et al., 2008**). Moreover according to toxicological analysis, single cell proteins proved to be a potentially good animal feed (**Sisman et al., 2012**).

CONCLUSION

The use of many insect species as an important food source has become so widespread in many parts of the world. Therefore, the consumption of non-toxic insects should be encouraged. Although edible insects have become so widespread in the world, very limited information is available concerning with their fungal biota in the literature. Ordinarily, insects are not used as emergency food to ward off starvation, however are included as a normal part of the diet throughout the year or when seasonally available. Eating insects have become more popular day by day around the world (**Memorial University, 2010**) and therefore, further investigations on the potential risks of these popular edible insects should be conducted. In promoting insects as human food, relative fungal biota and its risk potentials should be taken into consideration to provide the maximum benefit to human consumers. Based on the present findings, it was concluded that the studied insects can be consumed safely, but further investigations are needed.

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