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Ophryocystis anatoliensis sp. nov., a new neogregarine pathogen of the chrysomelid beetle *Chrysomela populi*

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Abstract

Chrysomela populi (Coleoptera: Chrysomelidae) is the most abundant and most important pest species that causes damage to poplar trees. Members of the family Chrysomelidae are frequently infected by protist pathogens but no neogregarine has been reported to date at the species level. In the present study we identify a new neogregarine pathogen from the chrysomelid *C. populi.* The infection was observed in the Malpighian tubules of adult beetles. A reddening of the Malpighian tubules was the most distinctive symptom of the infection. Single fusiform oocysts (9.8 × 4.7 μm) were formed within a gamontocyst. The polar plugs were very thin, varying from 380 to 525 nm in thickness. The oocyst wall was smooth and also quite thin (90–120 nm). Morphological and ultrastructural characteristics of the pathogen indicate that the described neogregarine in *C. populi* is clearly different from known *Ophryocystis* species which infect coleopterans. Therefore, the neogregarine pathogen was determined to be a newly discovered species and named *Ophryocystis anatoliensis* sp. nov.
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Keywords: Biological control; *Chrysomela* (=*Melasoma*) *populi*; Chrysomelidae; Entomopathogen; Neogregarine

Introduction

Poplars are commonly used plants in urban forestry and the wood industry (Gudurić et al. 2011; Plotnik 2009). They also play an important role in the rehabilitation of degraded forests and ecosystems and in the replacement of natural forests (FAO 2009; Yang et al. 2005). Despite their positive benefits, multiple biotic risk factors affect poplar breeding. The most important biotic factors hindering the efficiency of sustainable poplar breeding are pest insects which damage the plants (de Tillesse et al. 2007).

Chrysomela (=*Melasoma*) *populi* L. (Coleoptera; Chrysomelidae) is one of the most abundant beetle species that damages poplar trees. This species is known to cause a reduction in poplar biomass, and can kill young nursery plants (Urban 2006). Chemical control is the most widely known suppressive method against this beetle (de Tillesse et al. 2007), but it has many undesirable effects on the environment. Therefore, the use of chemicals is undesirable in urban forests. On the other hand, natural enemies are safe, sustainable and environmentally friendly control agents (Lukášová et al. 2014; Undeen and Vávra 1997; Yaman and Radek 2005). As natural enemies, entomopathogens have a great potential as biological control agents against insect pests, and are thus being developed worldwide for the control of many plant pests. However, a few entomopathogens such as microsporidia (Sidor 1979; Sidor and Jodal 1986), fungi (de Tillesse et al. 2007; Assaf et al. 2012) and *Bacillus thuringiensis* (Vriesen and Keller, 1994) have been stud-

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ied to find possible ecological control agents against *C. populi*, although chrysomelids are frequently infected by entomopathogenic organisms (Poinar 1988; Theodorides 1988; Toguebaye et al. 1988; Yaman and Radek 2003; Yaman et al. 2008, 2010, 2011). Among gregarines, only the neogregarines have a high pathogenic effect on their hosts by destroying the host's fat body and exhausting energy sources. Up to now, no neogregarine has been reported from *C. populi*, nor from any other member of the chrysomelids (Desportes 2013; Theodorides 1988). This study represents not only the first time that a pathogenic neogregarine species has been identified in a poplar pest (*C. populi*), but also that one species has been found in the family of the chrysomelids.

Material and Methods

Insect samples

Adults of *C. populi* were collected in the Samsun province (41°24′97″N, 36°58′15″E) in Turkey on April 23, 2013. They were kept alive at 26 °C, 40% humidity and a photoperiod of 18:6 h under laboratory conditions during the light microscopic examination with 2 days.

Microscopic examination

Fifty-one adults were dissected in Ringer's solution by using dissection pins, and then wet smears were examined for the presence of pathogens under a light microscope at a magnification of 400–1000× (Yaman et al. 2012). When an infection was found, the slides were air-dried and fixed with 100% methanol, then stained with freshly prepared 5% solution of Giemsa stain (stock solution, Carloerba, No. 6B712176C). Afterwards, the slides were washed in running tap water, air-dried and re-examined under the microscope (Undeen and Vávra 1997). Detected vegetative stages and oocysts of neogregarines were measured and photographed using an Olympus BX51 microscope with a DP-25 digital camera and a DP2-BSW Soft Imaging System.

Electron microscopy

Samples for transmission electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, rinsed in cacodylate buffer, post-fixed in 1% OsO_4 for 2 h, and rinsed in cacodylate buffer. After dehydration in an increasing ethanol series, the infected beetles were embedded in Spurr's resin (Spurr 1969). Ultra-thin sections were mounted on Pioloform-coated copper grids which were stained with saturated uranyl acetate and Reynold's lead citrate (Reynolds 1963). They were then examined with a Philips EM 208 transmission electron microscope. For scanning electron microscopy, tissue samples stored in distilled water were crushed in a drop of water, thinly spread on a cover glass and air dried. After sputtering with gold, the samples were observed in a FEI Quanta 200 scanning electron microscope.

Host specificity of the neogregarine pathogen

Bioassay experiments were performed to test the host specificity. For this, two other chrysomelid species, the Colorado potato beetle *Leptinotarsa decemlineata* and the willow flea beetle *Crepidodera aurata* were used. *C. aurata* lives in the same habitat as *C. populi*. For infection, a semi-purified oocyst suspension with a concentration of 2×10^7 oocysts/ml was prepared from homogenized insects. Ten μl of the suspension were applied to the surface of pieces of potato and poplar leaves (1 × 1 cm size) and 20 beetles in each group were allowed to feed on the leave pieces for 21 days. Then, the tested insects were dissected and microscopically examined for the presence of neogregarine oocysts.

Results

In the light microscopic wet smears of dissected *C. populi* we could find only one type of pathogen, i.e. a neogregarine infection. The adult beetles were infected; larvae were not included in the study. Nineteen of 51 examined beetles were infected by the pathogen, which corresponds to a prevalence of 37.2%. Only the Malpighian tubules were infected; we could not find any pathogen stages in other tissues. Several life stages of the pathogen were observed in the lumen of the Malpighian tubules (Figs 1–18). The Malpighian tubules were heavily infected, being completely full of neogregarine oocysts and other life stages (Figs 1, 2). Infected tubules became colorful, displaying colors from brown to red (Figs 1, 2). The reddening of the Malpighian tubules was the most distinctive symptom of the infection. In addition, the infected tubules were swollen (compared to the diameter of non-infected ones).

Mature oocysts of the pathogen were always fusiform in shape, but not uniform in size. Polar plugs were present at the two poles, but they were quite thin and integrated in the oocyst wall so that they were neither seen clearly under the light microscope nor in the scanning electron microscope (Figs 3, 4, 8–10, 12). Fusiform oocysts with plugs at both poles and containing sporozoites are typical for neogregarine infections. Fresh oocysts were 9.80 ± 0.58 (8.83–10.77) μm in length and 4.71 ± 0.41 (3.85–5.45) μm in width (n = 20). They were formed singly within a gamontocyst (Figs 3, 4, 8, 13). The wall of the gamontocysts was considerably thin measuring only 50–60 nm. Despite the thinness of the gamontocyst wall, it appeared to be quite resistant, so that mature oocysts were frequently observed within gamontocysts (Figs 3, 4, 8–11). The polar plugs at the poles could be clearly seen in the transmission electron microscope (Fig. 15). The polar plugs did

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