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First record of the pathogenic fungus *Entomophaga maimaiga* Humber, Shimazu, and Soper (Entomophthorales: Entomophthoraceae) within an outbreak populations of *Lymantria dispar* (Lepidoptera: Erebidae) in Croatia

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Abstract

Background and Purpose: *The last wave of a gypsy moth* (Lymantria dispar) *outbreak in Croatia and recent discoveries of its fungal pathogen* Entomophaga maimaiga *in eastern neighboring countries focused the attention of researchers on the possible presence of this pathogen in local gypsy moth populations. Since the first introduction of* E. maimaiga *in Bulgaria in 1999, several southeastern European countries confirmed its presence in subsequent years. One unchecked report made by a local forester "of dramatically high mortality in gypsy moth larvae at one locality at the easternmost part of Croatia in early summer 2012" raised the interest in whether* E. maimaiga *occurred in Croatia even higher. In spring 2013, the gypsy moth outbreak area grew even larger. Large areas were aerially sprayed with a* Bacillus thuringiensis kurstaki*-based bacterial insecticide and larval development was closely inspected in the field. One of the tasks was to check on the possible presence of* E. maimaiga *in gypsy moth populations in Croatia, and these results are presented here.*

Materials and Methods: *The research was conducted during June and July 2013 throughout the Eastern part of Croatia where gypsy moth populations entered into or continued an ongoing outbreak. From ten selected localities where excessive mortality was observed by local foresters, larval cadavers were sampled from tree trunks 0,5 – 1,5 m above the ground. Only older larval stages (L_4–L_6) were sampled due to the period of sampling. The cadavers were placed in Petri dishes on moistened filter paper discs, after which they were stored in the refrigerator on +4 °C for 48 – 72 h. Larval tissue samples were inspected under the light microscope. During the process many images were recorded by digital camera. Measurements of spores and vegetative stages were made via digital imaging software associated with a compound microscope. Selected samples of field collected dead larvae were stored in ETOH for further DNA molecular analysis.*

Results: *In nine out of ten field samples of dead gypsy moth larvae, either conidia or resting spores of* E. maimaiga *were confirmed. Depending on collection date, microscopic analysis confirmed both conidia and azygospores or azygospores only. The latter was typically the case when fully dry cadavers were collected towards mid July and later. Spore dimensions were*

as follows: pear-shaped conidia 24.2–35.8 μm crosswise and 29.5–43.6 μm lengthwise; azygosporees 31.7–47.1 μm diameter. Spore sizes and their general shape varied in accordance with gypsy moth cadaver age in days, time of year when collecting was done and general weather conditions that prevailed immediately prior to sampling. Macroscopic symptoms of E. maimaiga *attack were already clearly visible in the field, along with some signs of larval mortality caused by nucleopolyhedrovirus (NPV), but to a lesser extent. There were very few signs of parasitoid mortality and no visible presence of typical gypsy moth predators like* Calosoma sycophanta. *Larval mortality that could be assigned dominantly to* E. maimaiga *was highest in the easternmost localities. By the beginning of June thousands of larvae were hanging head down on tree trunks and no living larva or viable pupa could be found in the area.*

Conclusions: *Based on the field collections and microscopic analysis, it can be concluded that* E. maimaiga*, a pathogen of* L. dispar *introduced on the European continent, has been confirmed in Croatia. On the basis of some reports from the previous year, it is reasonable to assume that* E. maimaiga *appeared at least one year earlier (2012) but this cannot be proved now, in spite of the fact that the location of its possible presence in 2012 was somehow logical as it was reported from the far eastern border with Serbia and* E. maimaiga *already was known to occur in Serbia. Further targeted research in subsequent years, following the ongoing gypsy moth outbreak, should give a better picture of the spread and efficacy of this pathogen within Europe.*

INTRODUCTION

Lymantria dispar (Lepidoptera: Erebidae), widely known as gypsy moth, can rightfully be defined as one of the most important indigenous defoliators in Europe. Since the mid nineteenth century, following its introduction and later spread throughout North America, it became the major non indigenous forest pest defoliating huge areas of forested land in the eastern United States and causing large environmental disturbances *(1, 2)*. The ecological and economic importance of gypsy moth within its native range in Europe have not diminished through the decades. Periodically, gypsy moth populations enter into mid to large scale outbreaks and cause serious damage in some countries *(1, 3–5)*. Croatia, being a southeastern country, experiences its share of gypsy moth outbreaks that appear within the larger European region *(6–8)*. Population monitoring in the form of population density assessment, along with a multitude of suppression activities, was developed in the very beginning of organized forestry in this region *(9)*. In modern times, more selective and less environmentally harmful agents have been sought in order to minimize negative side effects of control practices. It is therefore logical that both foresters and scientists in general are eager to detect and further develop any possible contributing factor that may fit into this scheme.

Entomophaga maimaiga Humber, Shimazu and Soper (Entomophthorales: Entomophthoraceae) is a highly virulent fungal pathogen of gypsy moth. It was described as a host specific pathogen of *Lymantria dispar* (L.) in Japan, where it causes epizootics (10). In 1989 this pathogen was recovered in the northeastern USA where it caused epizootics in several states *(11)*. This field discovery of *E. maimaiga* in the USA was preceded by 2 unsuccessful attempts to artificially introduce it in 1910-1911 and 1985-1986 *(11–13, 26)*. In the USA, *E. maimaiga* has expanded its range by natural spread and targeted introductions in gypsy moth populations *(14–16)*. *E. maimaiga* can cause high larval mortality even at low gypsy moth densities and is capable of maintaining populations below damage thresholds. In addition, this pathogen is highly host specific and possesses great potential in biological control under a conservation or inoculation strategy *(17)*. In 1999-2000 *E. maimaiga* was successfully introduced in two gypsy moth populations in Bulgaria via inoculum from the US *(18)*. This is the first known and properly documented case of introduction of this pathogen on the European continent. Following a period of 10–12 years, *E. maimaiga* expanded its range (naturally and by introductions) and is now found throughout Bulgaria *(19)*. After the establishment in Bulgaria, first records of this pathogen appearing in neighboring countries followed. In 2005, *E. maimaiga* was recorded in Georgia which is additionally important because it is the first documented presence of this pathogen east of Black Sea *(20)*. In 2011 it was found both in the European part of Turkey and in Central Serbia *(21–22)*. A year later *E. maimaiga* was reported from Greece and the former Yugoslavian Republic of Macedonia (FYROM) *(23)*. In 2013 it was discovered in Hungary *(24),* and Slovakia (M. Zubrik 2013, pers. comm., 22 July).

During the latest outbreak of gypsy moth in Croatia, starting in 2012, extensive aerial treatments were applied, reaching more than 27,000 hectares in 2013. National legal restrictions enforced the use of biological insecticides, which in this case was *Bacillus thuringiensis kurstaki* (*B.t.k.*) based Foray 48B (Valent BioSciences). One unchecked report by local forester "of dramatically high mortality in gypsy moth larvae at one locality at the easternmost part of Croatia in early summer 2012" caught researchers' attention. During the field observations before, during and after aerial treatments, we focused on cases of exceptionally high larval mortality with well known *E. maimaiga* field symptoms *(11)* and collected dying or dead larvae for closer laboratory inspection. The results of these pathological inspections are presented in this paper.

MATERIALS AND METHODS

Field sampling

Targeted sampling of gypsy moth larvae in 2013 was done in the general area of a gypsy moth outbreak that started one year earlier. Dominating forests in this part of Croatia are characterized by large areas of *Quercus robur*

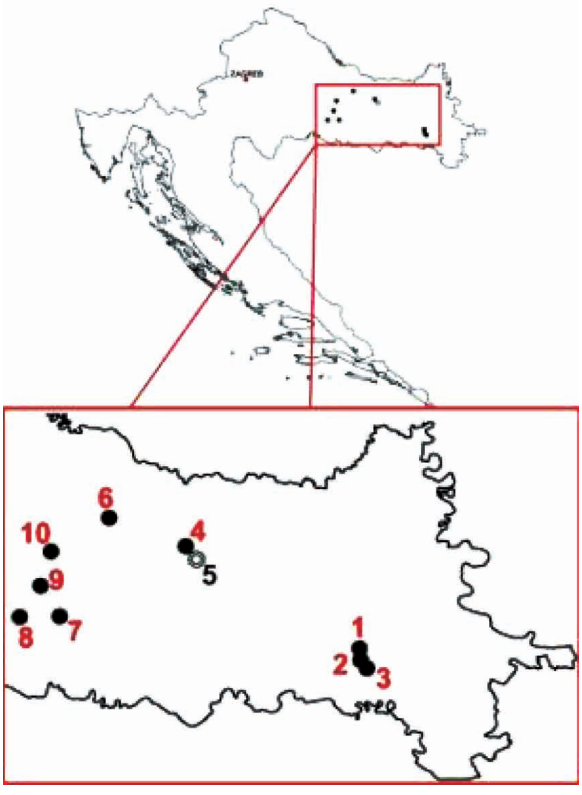

Figure 1. *Geographical distribution of sampling locations in eastern Croatia (black dots* – Entomophaga maimaiga *confirmed; white dot* – E. maimaiga *not found).*

and *Fraxinus angustifolia* floodplain stands growing in the Sava River basin and *Quercus petraea* and *Fagus sylvatica* stands covering hilly slopes of Psunj, Papuk, Krndija and the hills of the Panonian plain. Among the other commonest tree species in the area, the widely distributed *Carpinus betulus* must be mentioned. Only highly promising locations where high mortality of older larval instars was observed were chosen for sampling in order to heighten the chance of *E. maimaiga* confirmation. During the June-July 2013, based on the aforementioned criteria, 10 candidate locations were chosen to check for *E. maimaiga*. Larvae clinging head down on tree trunks were handpicked from the height of 0.5–1.5 m above the ground. Recently dead or already shrunken and fully to partially dried older L_4–L_6 cadavers were packed in plastic vials or paper bags and immediately transported to the laboratory cooler and kept at +4 °C until further analysis.

Laboratory analysis

Prior to microscopy, cadavers were placed in Petri dishes on moistened filter paper discs after which they remained in the refrigerator between 48 and 72 hours. Cadavers were inspected and tissue sampled under the stereo microscope (LEICA Leitz MZ8) and light stereo microscope (Motic SMZ – 168 TLED). The process was digitally documented by Olympus SP – 500UZ camera equipped with an Olympus QuickPHOTO CAMERA 2.3 microscopy imaging software. For closer observation

TABLE 1

Geographical characterisation of 10 spatially spread localities where gypsy moth larvae were sampled and tested for *Entomophaga maimaiga.*

Locality	Forestry administration unit	Forestry office	Management unit	Department	Altitude (m a.s.l.)	Latitude	Longitude	Sampling date	Btk treated	*E. maimaiga* confirmed
1●	Vinkovci	Cerna	Krivsko Ostrvo	23	89	45°12'4.08"N	18°35'8.92"E	June 6	YES	YES
2●	Vinkovci	Strizivojna	Orljak	3	91	45°10'58.84"N	18°34'52.06"E	June 6	YES	YES
3●	Vinkovci	Strizivojna	Orljak	26	88	45° 9'38.19"N	18°35'33.72"E	June 6	YES	YES
4●	Našice	Orahovica	Duzlučka Planina	18; 19	441	45°29'49.58"N	17°52'55.47"E	July 11	YES	YES
5○	Našice	Đurđenovac	Krndija Gazijska	61a	532	45°28'46.12"N	17°53'35.28"E	June 8	YES	NO
6●	Našice	Voćin	Jovanovica	38a	348	45°37'32.60"N	17°30'12.14"E	June 8	NO	YES
7●	Nova Gradiška	Nova Gradiška	Južni Psunj	32a; 33a	365	45°19'30.26"N	17°19'31.29"E	July 11	YES	YES
8●	Nova Gradiška	Okučani	Okučanska Brda	13a; 15a	264	45°18'34.54"N	17°11'8.94"E	July 9 and 10	YES	YES
9●	Bjelovar	Lipik	Rogoljica	8b	601	45°24'35.94"N	17°14'35.25"E	July 18	NO	YES
10●	Bjelovar	Pakrac	Pakračka Gora-Zapadni Papuk	14d	353	45°30'8.76"N	17°18'47.71"E	July 22	NO	YES

presence (graphical symbols in first column correspond with those in Figure 1).

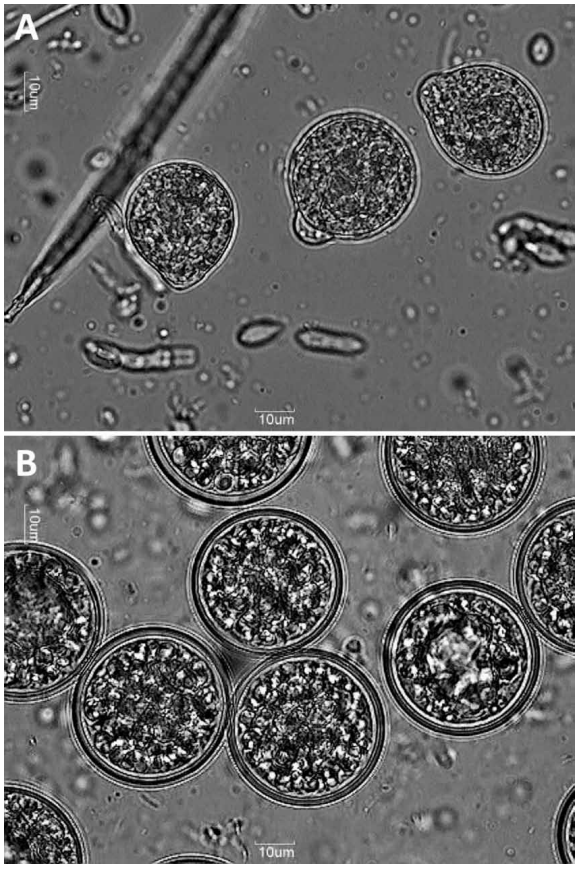

Figure 2. *Conidia (A) and azygospores (B) of* Entomophaga maimaiga *isolated from cadavers of gypsy moth larvae from eastern Croatia (localities No 2 and No 7 respectively).*

of sampled tissue which was needed for quantification of size, shape and structural characteristics of fungal pathogen, a compound microscope (Olympus BX53) coupled with a Motic MoticamPro 252A digital camera was used. Digital images were analyzed via Motic Images Plus 2.0 and Motic Images Advanced 3.2 microscopy imaging software. Selected field samples of dead larvae were stored in 95% ethanol for further DNA molecular analysis.

RESULTS

In nine out of ten chosen field localities where dead gypsy moth larvae were sampled, either conidia or resting spores of *E. maimaiga* were confirmed (Figure 1, Table 1). Depending on collection date, microscopic analysis confirmed both conidia and azygospores or azygospores only. The latter was typically the case when fully dry cadavers were collected later in season, towards mid July and later on. Spore dimension measurements were as follows: pear-shaped conidia (24.2–35.8 μm width and 29.5–43.6 μm length); azygospores (31.7–47.1 μm diameter) (Figure 2). Typical symptoms of *E. maimaiga* caused mortality, dead larvae hanging head-down on tree trunks, their prolegs spread sideways at a 90 degree angle *(27)* were already clearly visible in the field, along with some nucleopolyhedrovirus signs of larval mortality but to a much lesser extent (Figure 3). There were very little signs of parasitoid mortality and no visible presence of typical gypsy moth predators like *Calosoma sycophanta*. Larval mortality that could be assigned dominantly to *E. maimaiga* was highest in the easternmost localities. By the beginning of June thousands of larvae were hanging head down on tree trunks and no living larva or viable pupa could be found in that area.

DISCUSSION AND CONCLUSIONS

Based on the size, shape and other microscopic structural characteristics of observed fungal life forms (azygospores, conidia and mycelia) and well described and known narrowness of host selectivity of the pathogen *(17),* it can be concluded that *E. maimaiga* is present in the continental Croatian gypsy moth population. Coastal gypsy moth population showed no presence of this pathogen (results not presented in this paper). Spatially scattered and dramatically high mortality of late instar gypsy moth larvae with clear and distinguishing signs of *E. maimaiga* attack were not obscured by the effects of wide use of *B.t.k.* in the area. All observed field cases of extreme mortality with typical *E. maimaiga* symptomatology occurred later in the season (June–July) whereas *B.t.k.* treatments ended in mid May. Some of the sites were not even considered for spraying due to the lower gypsy moth infestation rates. It may be argued whether

Figure 3. *Masses of dead gypsy moth larvae clinging head down on a tree trunk – a typical field scene that indicated a possible presence of* Entomophaga maimaiga.

and how these treatments interfered with the intensity of *E. maimaiga* in the area but as far as this research had aimed, it is clear that the fungus is present and has established a strong foothold in Croatian territory. It was probably present a year or more earlier as suggested by some observations from the foresters that were involved in earlier gypsy moth suppression activities. One factor though, which complies with earlier findings *(25),* is that the moderate rainy period during May–June 2013 which affected part of the area of gypsy moth outbreak must have contributed the pathogen's high infection rates at the easternmost part of the affetcted area. Intensive screening in the following seasons should reveal the direction and speed of pathogen spread, at least on a local scale. In a wider European scale it will be a challenging task to explain how this pathogen appeared in such distant places almost simultaneously (Milan Zubrik, personal communication, July 2013).

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