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Detection of bacterial symbionts (*Wolbachia*, *Spiroplasma*) and eukaryotic pathogen (Microsporidia) in Japanese populations of gypsy moth species (*Lymantria* spp.)

Диагностика японских популяций непарного шелкопряда (*Lymantria* spp.) на заражённость бактериальными симбионтами (*Wolbachia* и *Spiroplasma*) и эукариотическим патогеном Microsporidia

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Ключевые слова: бактериальные симбионты, патогены, насекомые-филлофаги, заражённость, популяционная изменчивость.

Abstract. In the present study, Japanese populations of gypsy moth *Lymantria dispar japonica* (Motschulsky, 1860) and *L. postalba* (Inoue, 1956,) were screened for the presence of two maternally-inherited bacterial symbionts (*Wolbachia* and *Spiroplasma*) and Microsporidia. Both Microsporidia and *Wolbachia* were absent in all examined individuals (N = 99) while presence of *Spiroplasma* was registered at 3.9 % prevalence rate (N = 51) in *L. dispar japonica* population. We discuss the possible role of these microorganisms in gypsy moth population dynamics.

Резюме. Данная работа посвящена изучению заражённости матерински-наследуемыми бактериальными симбионтами (*Wolbachia* и *Spiroplasma*) и эукариотическими энтомопатогенными микроспоридиями популяций непарного шелкопряда (*Lymantria dispar japonica* (Motschulsky, 1860) и *L. postalba* (Inoue, 1956,)), населяющих японские острова. В результате исследования нами не вывялено факта присутствия микроспоридий и бактерий *Wolbachia* в исследуемых индивидуумах шелкопряда, однако выявлено присутствие бактерий *Spiroplasma* у 3,9 % особей популяции *L. dispar japonica*. В работе ставится вопрос о возможной роли данной бактерии для популяционной динамики непарного шелкопряда.

Introduction

The gypsy moth *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae) is a notorious pest of deciduous and coniferous forests of moderate latitudes of Holarctic [Giese, Schneider, 1979; Bakhvalov et al., 2010; Ponomarev et al., 2012]. Larvae of this species are able to defoliate forest plant species (over 300 taxa) on vast territories during pest outbreaks [Il'ynsky, Tropin, 1965; Qian, 2000]. Symbiotic microorganisms are able to significantly affect the populations of outbreaking insects [Graham et al., 2012]. For instance, certain species

of endosymbiotic bacteria can alter sex ratio in populations [Zakharov, 2015]. Other microorganisms, such as microsporidia, are directly pathogenic and may cause drastic density drops during epizootics [Becnel, Andreadis, 1999].

Maternally inherited bacteria of the *Wolbachia* genus (alphaproteobacteria) are intracellular symbionts of diverse arthropods, in particular of many Lepidoptera [Tagami, Miura, 2004; Russell et al., 2009; Salunke et al., 2012; Ahmed et al., 2016; Ilinsky, Kosterin, 2017], including economically important species [Kageyama et al., 2004; Sakamoto et al., 2008; Yudina et al., 2016; Tokarev et al. in press]. It is known that *Wolbachia* to induce reproductive abnormalities in host species [Charlat et al., 2003], to decrease [Teixeira et al., 2008] or, conversely, to increase [Graham et al., 2012] effects of viral infections, to suppress of harmful alleles of the host [Starr, Cline, 2002; Ikeya et al., 2009], and to provide hosts with essential metabolites [Foster et al., 2005; Raverdy et al., 2008; Haegeman et al., 2009]. However, the role of *Wolbachia* in the most of reported symbiotic associations remains unknown.

Previously, screenings *Wolbachia* infection were performed for gypsy moth *L. dispar japonica* (Motschulsky, 1860) for the mainland of Japan and *L. dispar praeterea* (Kardakoff, 1928) for Hokkaido in Japan [Higashiura et al., 2011] and *L. dispar asiatica* (Vnukovskij, 1926) in Western Siberia [Martemyanov et al., 2014], however infection was no found. In the present study, we examined populations of gypsy moth from the mainland of Japan (i.e. *L. dispar japonica* (Motschulsky, 1860) according to Higashiura et al., 2011) and also populations of gypsy moth form south Japan (Kyushu island) which was separated to another species *L. postalba* (Inoue, 1959) [Jikumaru, 2013]. It is known that Japanese populations of gypsy moth are genetically distant to Siberian populations [Wu et al., 2015] and demonstrate significant differences in ecology in terms of migratory activity, female oviposition site preference and preferred host plants [Pogue, Schaefer, 2007; Bakhvalov et al., 2010; Iwaizumi et al., 2010; Jikumaru, 2013].

Bacteria of the *Spiroplasma* genus (Mollicutes) are characterized by parasitic lifestyle, being found in insects and plants. Moreover, their possible role in human pathology is also discussed [Bastian et al., 2007]. They infect various orders of insects, including Lepidoptera [Jiggins et al., 2000; Tabata et al., 2011]. Like the *Wolbachia* symbiont, *Spiroplasma* may affect the reproduction biology of insect hosts and upregulate their adaptability. For instance, *Spiroplasma causes male killing* in *Ostrinia zaguliaevi* (Mutuura, Munroe, 1970) (Crambidae) [Tabata et al., 2011], and in *Danaus chrysippus* (Linnaeus, 1758) (Nympalidae) [Jiggins et al., 2000]. In addition, the last host is suffered the *Spiroplasma* effect on adult morphology [Herren et al., 2007]. *Spiroplasma* increases survival rate in some dipteran hosts, that are infected with parasitic ichneumonoids or nematodes [Jaenike et al., 2010; Xie et al., 2010]. It should be also noted that certain populations of *L. dispar japonica* and *L. dispar praeterea* subspecies from Japan were tested for *Spiroplasma*, however no infection was found [Higashiura et al., 2011]. In the present study the aforementioned populations of *L. dispar japonica* and *L. postalba* from Japan were also screened for *Spiroplasma.*

Phylum Microsporidia (Opisthosporidia, Holomycota, Opisthokontha) is a huge group of eukaryotes, obligate intracellular parasites of all major taxa of Metazoa and some protists (Gregarinida; Ciliata), being most abundant in arthropods and fishes [Wittner, 1999]. Due to having (as a group) broad host range and tight associations with hosts, microsporidia are widely dispersed in diverse natural and artificial habitats within the host area. Certain species of microsporidia induce mass epizootics of arthropods, sometimes devastating local host populations [Becnel, Andreadis, 1999]. Nevertheless, presence of microsporidia has been only registered in European population of gypsy moth, i.e. *L. dispar dispar*. There are *Nosema lymantriae* (Weiser, 1963), *Nosema portugal* (Maddox et al., 1999), *Vairimorpha disparis* (Timofejeva, 1956), *Nosema serbica* (Weiser, 1963) and *Endoreticulatus schubergi* (Zwölfer, 1927) [Pilarska et al., 1998; Maddox et al., 1999]. They are considered as natural regulators of pest density, and as potential biocontrol agents for North American populations of gypsy moth [Solter, Hajek, 2009; Solter et al., 2010].

Here we examine the Japanese populations of two closely related species of gypsy moth for microorganisms that can significantly affect the host sex ratio and population density.

Materials and methods

Sample collection. For detection of microorganisms, pupal abdomens were used from insects collected on the territory of three prefectures Fukushima, (37.25° N, 139.56° E) for *L. dispar japonica* during population outbreak in 2015; Kagoshima, (31.62° N, 130.51° E), and Miyazaki, (31.72° N, 131.20° E) for *L. postalba*.

Insect abdomens were halved and stored in 96 % ethanol at 23 °C for independent confirmation of positive cases.

Experimental procedures. For total DNA extraction, ethanol was removed and 300 µl of extracting buffer (10 mM TRIC-HCl (pH 8.0), 25 mM EDTA, 0.5 % SDS, 0.1 M NaCl) was added for homogenization and incubation of samples at 56 °C for 5 hrs. DNA was precipitated and dissolved in 100 µl of molecular grade water. Extracted DNA samples were checked by PCR using universal primers 28sF3633/R4076 flanking a region of *28S rRNA* gene [Choudhury, Werren, 2006]. In case of unsatisfactory results, samples were additionally purified, tested and further screened with «BioMaster HS-Taq PCR (2x)» (BioLabMix, Novosibirsk, Russia). Infections were tested by PCR using primers coxAF1/R1 targeting subunit A of cytochrome oxidase gene of *Wolbachia* [Baldo et al., 2006]; primers 63F [Mateos et al., 2006] and TKSSsp for *16S rRNA* gene of

Spiroplasma [Fukatsu, Nikoh, 2000] and primers 18f/1047r for small subunit rRNA gene of Microsporidia [Weiss, Vossbrinck, 1999]. Earlier characterized DNA insects samples that were infected by appropriate symbionts were used as positive control.

PCR cycles included initial denaturation for 2 min at 94 °C, 35 repeats of denaturation for 10 sec, annealing for 40 sec at 55 °C (for coxAF1/R1), 54 °C for 63F/TKSSsp) or 60 °C (for 18f/1047r) and elongation for 1 min at 72 °C, followed by final extension for 5 min. The obtained amplicons of expected size were purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) according to manufacturer's instructions and sequenced using an automatic capillary sequencer with PCR primers under BigDye® v. 3.1 protocol.

Results and Discussion

Results of screening Japanese populations of *Lymantria* species are shown in Table 1. Infection with microsporidia was not found. This result may be interpreted in two ways, first, microsporidia infection is absent in Japanese populations of gypsy moth, and, second, this infection has very low prevalence in the sampling period, since many parasite-host systems display seasonal and long-term dynamics of microsporidia prevalence rate fluctuations between 0 and 100 % [Issi, 1986].

Endosymbiotic bacterium *Wolbachia* was not found in studied populations of *L. dispar japonica* and *L. postalba*. No *Wolbachia* infection in Japanese *L. dispar* population (including the data of Higashiura et al., 2011) supports our previous conclusion [Martemyanov et al., 2014] that probability of *Wolbachia* revealing in gypsy moth populations is extremely low. However there is the great interest to examine the Chinese populations because they contain the major genetic diversity of *L. dispar*. Negative *Wolbachia* infection status of *L. postalba* populations was demonstrated for the first time. Because of the restricted distribution of *L. postalba* populations by south of Japan [Jikumaru, 2013] we suppose that this species is uninfected. It can not be ruled out that an unique case of *Wolbachia* infection in studied species will be found since *Wolbachia* strains are horizontally transferred between host species [Vavre et al., 1999; Baldo et al., 2006, Gerth et al., 2013; Ahmed et al., 2016; Ilinsky, Kosterin, 2017].

Spiroplasma infection was revealed in two samples in the population of Fukushima Prefecture, comprising 3.9 % prevalence with confidence interval of 0.5–13.5 % at p = 0.05. In the populations of *L. postalba* there were no positive *Spiroplasma* cases. Nucleotide sequences of *Spiroplasma* isolates were closely related to symbiont isolates from Arachnida and different orders of Insecta (Fig. 1). Since *Spiroplasma* induces reproductive

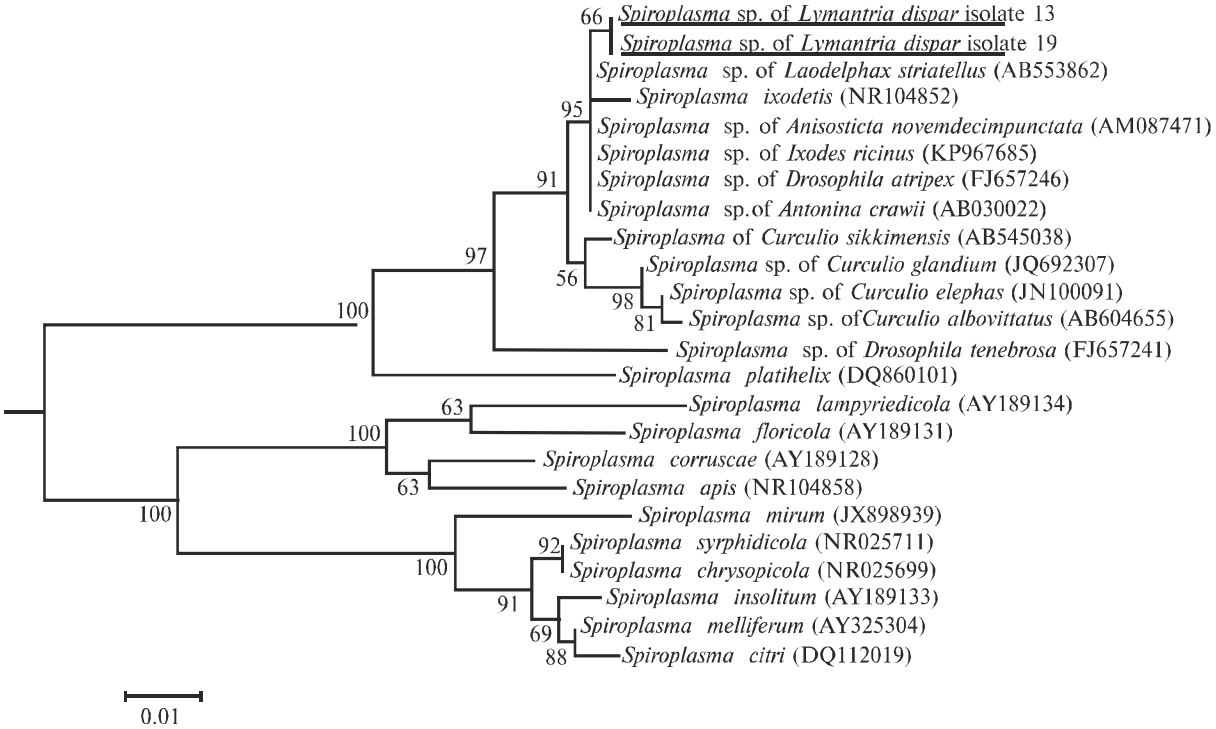

Fig. 1. Maximum-likelyhood phylogenetic tree of *Spiroplasma* isolates, based on 414 bp region of 16SrRNA. Sequences were aligned using the MUSCLE algorithm employing the K2p + Γ model of nucleotide substitutions, and bootstrap 1000 iterations. GenBank numbers, *Spiroplasma* species and/or hosts are provided, underlining indicates our findings.

Рис. 1. Филогенетическое дерево изолятов *Spiroplasma*, реконструированное методом максимального правдоподобия на основе анализа 414 пн локуса 16SrRNA. Последовательности были выровнены с помощью алгоритма MUSCLE, модель нуклеотидных замен K2p + Γ, статистическая оценка бутстреп 1000 итераций. Приведены номера GenBank, виды *Spiroplasma* и/или виды-хозяева, подчёркиванием выделены изоляты, обнаруженные в данном исследовании.

Table 1. The results of screening of Japanese populations of *Lymantria* species
Таблица 1. Результаты скрининга японских популяций представителей рода *Lymantria*

Region (Year of the collection) / species	N	Microsporidia	*Wolbachia*	*Spiroplasma*
Fukushima pref. (2015) / *L. dispar japonica*	51	0	0	2
Kagoshima pref. (2014) / *L. postalba*	37	0	0	0
Miyazaki pref. (2014) / *L. postalba*	11	0	0	0
Total	99	0	0	2

abnormalities and increases adaptability of hosts, the further investigation of this symbiont in gypsy moth is perspective. In particular, it is necessary to study a relation of *Spiroplasma* prevalence rates in gypsy moth and phases of host population cycle to check the role of the symbiont in the host population dynamics.

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