Parasite-host specificity: A cross-infection study of the parasite Ophryocystis elektroscirrha


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Parasite-host specificity: A cross-infection study of the parasite Ophryocystis elektroscirrha

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1. Introduction

Parasites are present in all kinds of organisms and affect host fitness in variable ways (Schmid Hempel, 2011). Parasitic infections can result in the decline of populations, especially under changing environmental conditions including global warming (Altizer et al., 2003; Wood et al., 2007). Most parasites of both plants and animals show host specificity (Little et al., 2006; Norton and Carpenter, 1998). With global warming, many parasites and their hosts can expand their geographic ranges, which likely affects host-parasite interactions (Hellgren et al., 2007; Pickles et al., 2013; Polley and Thompson, 2009). For example, parasites may infect closely related host species when introduced into new habitats, as they share similar immune defenses and physiological traits as the original host (Norton & Carpenter, 1998; Davies & Pedersen, 2008; Poulin et al., 2011). When host shifts occur, the virulence of parasites may change (Little et al., 2006). Interestingly, it remains unclear why some parasites are highly specialized on only one or few hosts and other parasites are shared across a wide range of host species (Cooper et al., 2012; Mácočová et al., 2018). Understanding the specific range of parasite infections is crucial to predicting future infectious diseases in plants and animals, including humans (Cooper et al., 2012; Keesing et al., 2010; Li et al., 2016).

The parasite Ophryocystis elektroscirrha (OE) is a neogregarine protozoan and has been thought to be an obligate parasite restricted to the monarch butterfly, Danaus plexippus, and closely related queen butterfly, D. glippus. This host specificity is deduced from the fact that OE was first observed on these host species in Florida (McLaughlin and Myers, 1970) and the life-cycle of OE is closely linked to its host (Altizer and Oberhauser, 1999). After early-instar larvae consume OE spores, they undergo asexual, vegetative replication in the larval gut (Altizer et al., 2000). Upon lysis, the spores form micronuclear schizonts which penetrate the gut wall, after which they migrate to the hypoderm where a combination of sexual and asexual replication commences (Vickerman et al., 1999). Sporulation occurs during host pupation and...
dormant spores emerge on the abdominal scales of adult butterflies (McLaughlin and Myers, 1970). OE infections have been found to be detrimental to host fitness in D. plexippus, reducing longevity, eclosion, mating success, fecundity and flight ability (Altizer and Oberhauser, 1999; Bradley and Altizer, 2005; De Roode et al., 2009).

Transmission of this parasite is predominantly vertical, as females scatter spores on their eggs or host plants during oviposition and egg shells are ingested by hatching larvae (Leong et al., 1992). However, paternal or horizontal transmission routes are possible, e.g., through mating or spores consumed by unrelated larvae (Altizer et al., 2004; De Roode et al., 2009). Recently, Barriga et al. (2016) firstly reported OE-like infections in the lesser wanderer butterfly, D. petilia, suggesting that the OE-like parasites could have a wider host range than previously assumed.

In checking field-collected samples of the cotton bollworm, Helicoverpa armigera (Hübner) (Lepidoptera, Noctuidae) in Australia to start laboratory rearing, we noticed that some adults did not fully emerge from their pupal case or could not expand their wings, a symptom indicative of OE infection (Leong et al., 1992). A check of adults indicated OE-like spores present. These observations led us to investigate the identity of the parasite infecting H. armigera by sequencing a subset of the 18S rRNA from parasites collected from field-collected H. armigera populations in China, Australia and Spain, as well as from the closely related species H. assulta in China and H. punctigera in Australia, and comparing these sequences to the D. plexippus OE. We further explored the OE or OE-like parasite host range by investigating its presence in butterfly species in the butterfly garden of Artis Zoo in Amsterdam, which are flown in biweekly as pupae from so-called butterfly farms in South-East Asia, Africa and South America. In addition to sequencing, we determined the host-specificity of OE and OE-like parasites by conducting cross-infection experiments with D. plexippus, H. armigera and Heliothis virescens, and we tested whether the susceptibility of OE-like infection varied between geographic origins of H. armigera populations. As H. armigera is highly polyphagous, feeding on a wide variety of host plants from at least 172 species within 68 families (Cunningham and Zalucki, 2014; Pitt, 1989), and is widely distributed throughout Africa, Europe, Asia, Oceania (Pitt et al., 1997) and recently South America (Czepak and Albernaz, 2013), these experiments give a first indication of the potential risk of OE-like parasite to spread to other closely related Helicoverpa and Heliothis species. (Pogue (2013) has recently resurrected the name Chloridea for a monophyletic genus containing virescens and subflexa, but for consistency with the older literature we continue to refer to Heliothis virescens here.)

2. Materials and methods

2.1. Occurrence of OE-like parasite in Lepidoptera other than Danaus

Three Helicoverpa moth species were collected from the field and checked for OE parasites: H. armigera from China, Australia and Spain, and H. assulta and H. punctigera in China and in Australia, respectively (see Table 1). The moths were collected by using a hand net or caught in pheromone traps, after which the moths were killed, placed individually in Eppendorf tubes and brought back to the laboratory at the University of Amsterdam for further diagnosis. To diagnose OE-like parasite infection, the abdomens of the moths were sampled using 2.5 cm diameter transparent tape and checked for parasite spores, as described in detail by Altizer et al. (2000). OE spores are approx. 14 μm long, 9 μm wide, dark when seen in scales or on the egg, but amber in transmitted light (McLaughlin and Myers, 1970). OE-like spores on H. armigera from Australia are morphologically similar as OE spores, with a dimension of approx. 12 μm length and 9 μm width.

To survey OE-like parasite occurrence in other butterfly species, specimens were collected from the butterfly garden at Artis Zoo, in Amsterdam, Netherlands in May–June 2018. In total 32 butterfly species, originating from Asia, Africa and South America, were sampled (see Supplementary Table 1). Butterfly specimens were placed individually in paper bags with the species identification and collected location. All specimens were brought back to the laboratory and checked OE-like parasite under the microscope, as described above.

2.2. Genetic similarity between OE and OE-like parasites

2.2.1. DNA extraction and PCR

To identify OE-like parasite that we found in the different species in the field and the Amsterdam Zoo, we extracted DNA from the collected spores from five different host species (Table 2). Specifically, the abdomen of infected adults was placed in a 1.5 ml Eppendorf tube with 100% ethanol. The tube was agitated for 1 min using a vortex mixer followed by a 5 min rest interval, and repeated three times. The abdomen was removed, after which the spore suspension was centrifuged for 10 min at 13,000 rpm and the supernatant discarded and 200 μl of 2% CTAB buffer with 5 μl proteinase-K were added to the tube with parasite spores. The spores were then ground with a clean pestle, and incubated for 1.5 h at 55 °C. After incubation, the supernatant was transferred into a sterile tube with chloroform: IAA (24:1) after centrifugation at 12,000 rpm for 5 min 300 μl of 100% ice-cold ethanol was added to precipitate the parasite DNA. The DNA sample was centrifuged at 13,000 for 10 min and the supernatant discarded. The DNA pellet was washed with 70% ethanol and dried in a speedvac for 10 min at 30 °C. The pellet was dissolved in 25 μl distilled H2O and stored at –20 °C.

To sequence the 18S rRNA, PCR reactions were prepared in 10 μl that included 3.5 μl distilled H2O, 2 μl 5x PCR buffer, 2 μl 5 mM dNTPs, 0.2 μl 10 mM primers, 0.1 μl Phire hotstart Taq and 2 μl DNA template. The PCR amplification was performed by 35 cycles of 30 s at 98 °C, 10 s at 56 °C, 2 min at 72 °C. The forward primer 5′-CCGGTTGTTGAGTCAAATTAG-3′ and reverse primer 5′-AGGGCAAGTCTGGTGCCAG-3′ were used to amplify a subset of the 18S rRNA gene in this study. The PCR products were examined on a 1.5% Agarose gel.

2.2.2. Phylogenetic analysis

The subset of the 18S rRNA sequences were checked manually using CodonCode Aligner (CodonCode Corp., USA), and were aligned to the sequence of O. elektroscirrha (AF129883) from GenBank, using MEGA version 10.0.5. The sequences in this study were deposited in GenBank (accession no. MK720123-MK720132). Outgroup species were selected based on previous phylogenetic studies by Carreno et al. (1999) and Bekircan et al. (2017), based on which the representative species As cogregaria cuculis, Aranciocystis muskarensis and Acarus siro were chosen.

To assess interspecific genetic distances of the OE and OE-like

<table>
<thead>
<tr>
<th>Species</th>
<th>Locations</th>
<th>Coordinates</th>
<th>Years</th>
<th>Number of Individuals</th>
<th>Infection rate (%)</th>
</tr>
</thead>
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<tr>
<td>H. armigera</td>
<td>Guadajira, Badajoz, Spain</td>
<td>38°51′08.8″N, 6°40′48.9″W</td>
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<td>27°32′11.6″S, 152°20′16.3″E</td>
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<td>19</td>
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<tr>
<td>H. armigera</td>
<td>Dali county, Shaanxi, China</td>
<td>34°45′01.5″N, 110°09′56.1″E</td>
<td>2017, 2018</td>
<td>66</td>
<td>2</td>
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<tr>
<td>H. assulta</td>
<td>Dali county, Shaanxi, China</td>
<td>34°45′01.9″N, 110°09′56.1″E</td>
<td>2017</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>H. punctigera</td>
<td>Gatton, Brisbane, Australia</td>
<td>27°32′11.6″S, 152°20′16.3″E</td>
<td>2017</td>
<td>45</td>
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</table>
Table 2
Samples used for phylogenetic analyses.

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<th>Species</th>
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<th>Host</th>
<th>Collection Location</th>
<th>Collection Date</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
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<tr>
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<td>Danaus plexippus</td>
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<td>Netherlands</td>
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<td>Parthenos sylvia</td>
<td>Asia</td>
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<td></td>
<td>Danaus plexippus</td>
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</tbody>
</table>

Table 3

parasites, Kimura-2-parameter distances (K2P distance) were calculated in MEGA. For phylogenetic analysis, a maximum likelihood (ML) tree was constructed using MEGA. The substitution model for ML tree was fits in MEGA. For phylogenetic analysis, a maximum likelihood (ML) tree was constructed using MEGA. The substitution model for ML tree was

- Kimura-2-parameter distances (K2P distance) were calculated for 122 ± 10 spores/μl.
- The abdomen was removed, and the spore suspension was then centrifuged for 5 min at 10,000 rpm. The supernatant was discarded and the remaining pellet was re-suspended in 1 ml distilled water. Third instar larvae of H. armigera from six families and H. virescens from four families were used in the experiments. All the larvae placed into the cups individually and observed to consume the diet completely, after which the larvae were transferred to the normal diet until pupation.

To infect the larvae of the monarch butterfly with OE-like spores from H. armigera, second instar caterpillars were infected with 1 μl of spore suspension (spores load: 76 ± 2 spores/μl) on a piece of 1 cm² A. incarnata. After consumption of the spores, the caterpillars were placed in 0.6 m³ mesh cages supplied with clean A. incarnata plants that were replaced every day until the caterpillars pupated. Pupae were individually transferred into clear plastic cups until eclosion.

Second, we used the OE parasite spores collected from D. plexippus to infect D. plexippus, H. armigera from Australia, and H. virescens. The OE spores were collected from newly hatched heavily infected D. plexippus at the butterfly garden in Harskamp, Netherlands. All the larvae were infected with 1 μl of spore suspension (spores load: 55 ± 3 spores/μl). As none of the moths were infected from D. plexippus OE spores, we repeated the experiment and infected H. armigera and H. virescens larvae with 1 μl of spore suspension (spores load: 135 ± 2 spores/μl) that we collected from D. plexippus. These two spore suspensions were prepared as described above, and all the larvae were infected in the same way. Third instar larvae of H. armigera from eight families and H. virescens from five families were used in the experiments. Upon eclosion, the adults of all species in the study were frozen in individual Eppendorf tubes or in paper bags, after which their abdomens were examined for infection of parasite spores as described above.

2.3. Host-specificity of OE

2.3.1. Cross-species infection experiment

To determine whether the OE or OE-like parasites can infect different host species, cross-infection experiments were conducted. For these cross-infection experiments, larvae of H. armigera were collected from Australia, Spain and China during 2017 and 2018, and reared individually on artificial pinto bean diet in the same climate chamber (60% relative humidity (RH); 25 °C; 14 h light: 10 h dark with lights off at 11.00 am) in the laboratory at the Institute for Biodiversity and Ecosystem and Dynamics, University of Amsterdam. Larvae of H. virescens used were also reared individually on artificial pinto bean diet.

Larvae of monarch butterfly D. plexippus were originally from five mated females collected at a butterfly garden in Harskamp, Netherlands in July 2018. Monarch larvae were reared on the milkweeds Asclepias incarnata. These plants were grown and supplied in the greenhouse at the University of Amsterdam.

First, we used the OE-like parasite spores collected from H. armigera to infect three species: H. armigera from Australia, a closely related species H. virescens and D. plexippus. The OE-like parasite spores used in the experiments were obtained from the lab-reared H. armigera from CSIRO, Black Mountain Laboratory, ACT, Australia. The spore inoculum was obtained using a method modified from Leong et al. (1992). Specifically, the abdomen of an infected H. armigera adult was placed in a 2 ml Eppendorf tube with a washing solution of 0.5 ml 0.05% wetting agent (Tween 20) and 1 ml distilled water. The tube was agitated for 1 min using a vortex mixer, followed by a 5 min rest interval, and repeated three times. The abdomen was removed, and the spore suspension was then centrifuged for 5 min at 10,000 rpm. The supernatant was discarded and the remaining pellet was re-suspended in 1 ml distilled water to ensure the spores were evenly dispersed. To estimate the spore load, 10 droplets of 1 μl spore suspension were randomly chosen and the number of spores in each droplet counted under the microscope. From these counts we calculated the average number of spores with standard error (SE) in 1 μl droplet of spore suspension.

To infect the larvae of H. armigera and H. virescens, 1 μl of spore suspension (spores load: 122 ± 10 spores/μl) was pipetted onto a piece of 1 cm² pinto bean-based artificial diet in a 37 ml cup. To prevent the diet from drying out before consumption, the cups were lined with cotton soaked in distilled water. Third instar larvae of H. armigera from

- Sequences download from GenBank.
- Collections from Artis Zoo, Amsterdam.
larvae were reared individually on pinto bean diet until eclosion. The adults were examined for infection. We also recorded the number of adults that were stuck in their pupal case, and the number of adults with crinkled wings that did not fully expand. To assess effects of OE parasite on the emergence success of H. armigera, we compared that the number of stuck in pupae and adults with crinkled wings between infected individuals from the larvae exposed to OE-like spores and uninfected individuals from larvae treated with distilled water.

2.5. Statistical analyses

To determine the difference of infection rate in host species in the cross-species infection experiment, we performed a chi-square test. The same test was used to determine the difference of OE-like infection rate among H. armigera populations from Australia, China and Spain, when treated with a low or high concentration of spores. To test the effect of OE-like parasite spore concentration on infection probability, a regression analysis was carried out. We used a chi-square test to determine the difference between infected and uninfected individuals of the number of pupae stuck and adults with crinkled wings in H. armigera. All statistical analyses were performed in R software, version 3.4.1 (R_Core_Team 2018).

3. Results

3.1. Occurrence of OE or OE-like parasites

In the field, the OE-like parasite was detected in all three Helicoverpa moths. We found OE-like infection in H. armigera populations in China and Australia, but not in Spain. We also found the OE-like infection in other closely related species, such as in H. assulta in China and H. punctigera in Australia (Table 1). The prevalence of OE-like parasite infection varied among the locations, with highest infection rate of 19% (9 out of 48 individuals) on H. armigera in Australia. From the specimens collected in the Amsterdam Zoo, two out of 32 butterfly species were found to carry OE or OE-like parasites. Specifically, 86% of D. plexippus (12 out of 14 individuals) that were originally from South America were infected and 58% of Parthenos sylvia (Nymphalidae) (15 out of 26 individuals) originating from Asia were infected (Table S1).

3.2. Genetic similarity between OE and OE-like parasites

The subset of the 18S rRNA sequences were 558 bp in length. Aligning all sequences, we found the interspecific K2P distance between D. plexippus and P. sylvia to be 1.5%, and the K2P distances between D. plexippus and H. armigera (also H. punctigera) was 4.8%. The mean K2P distance between OE-like spores collected within the Helicoverpa genus was < 0.2%.

All parasites collected from D. plexippus individuals, as well as all parasites collected from P. sylvia individuals, formed a monophyletic group with strong (99%) bootstrap support (Fig. 1). The remaining three Helicoverpa moths formed another monophyletic clade, with 100% bootstrap support.

3.3. Host specificity of OE or OE-like parasites

3.3.1. Cross-species infection experiment

When larvae from different species were treated with OE spores collected from D. plexippus, 97% (37 out of 38 adults) of D. plexippus were infected, while none of the adult H. armigera (n = 63) or H. virescens (n = 95) were infected ($\chi^2 = 194.8, df = 2, P < 0.0001$). In contrast, when larvae from the different species were treated with OE-like spores collected from H. armigera in Australia, the infection rate was 71% in D. plexippus (20 out of 28 adults) and 85% in H. armigera (93 out of 110 adults), but significantly lower in H. virescens, i.e., 48% (13 out of 27 adults) ($\chi^2 = 10.0, df = 2, P = 0.0067$) (Fig. 2).

3.3.2. Inter-population infection experiment

When we treated H. armigera larvae from different geographic locations with a low concentration (16 ± 1 spores/µl) of OE-like spores collected on H. armigera from Australia, 53% (9 out of 17 adults) of H. armigera from Spain were infected, 15% (3 out of 20 adults) from China were infected, while no (n = 10) adults from Australia were infected. The infection rate in individuals from Spain was significantly higher than that from China and Australia ($\chi^2 = 65.68, df = 2, P < 0.0001$).

When treated with a high concentration of spores (99 ± 3 spores/µl), 75% (18 out of 24 adults) of H. armigera from Spain were infected, while 73% (24 out of 33 adults) from China and 57% (13 out of 23 adults) from Australia were infected. There was no significant difference in the infection rate among the three H. armigera populations at this high dose ($\chi^2 = 2.99, df = 2, P = 0.22$) (Fig. 3).

3.4. Effect of OE-like parasite on moth emergence success

When larvae of H. armigera from Australia were infected with different concentrations of OE-like spores, the infection rates in adult moths best fitted a logarithmic regression line ($R^2 = 0.71, P = 0.0045$) (Fig. 4a). This logarithmic relationship suggests that the proportion of infected individuals increased with concentration of OE-like spores, reaching an asymptote. The number of pupae stuck ($\chi^2 = 4.63, df = 1, P = 0.031$) or adults with wings crinkled ($\chi^2 = 6.01, df = 1, P = 0.014$) from infected individuals were significantly higher than that from uninfected individuals (Fig. 4b).

4. Discussion

To our knowledge, this is the first report of OE-like parasite infections in species of Lepidoptera other than Danaus species. In the past, the protozoan OE and OE-like parasites were thought to be specialized on monarchs and closely related butterflies (Barriga et al., 2016; Mclaughlin and Myers, 1970). In our survey, OE-like parasites were found in three noctuid moth species H. armigera, H. assulta and H. punctigera collected in China and Australia, as well as in the butterfly P. sylvia (Nymphalidae) collected in Asia. This indicates that OE and OE-like parasites are more widespread and have more potential hosts in nature.

On the basis of partial 18S rRNA sequences in the different OE and OE-like spore collections, we found only 1.5% genetic distance between the butterflies P. sylvia and D. plexippus, indicating a high similarity. The OE-like parasite that we found in noctuid moth species (especially H. armigera and H. punctigera) exhibited a 4.8% genetic distance from D. plexippus OE. Our phylogenetic analysis did show an OE and OE-like parasites divergence into two groups, representing butterflies and moths. It strongly suggests that OE from D. plexippus and OE-like parasite from moths may be different species, which displays host specificity or coevolutionary processes of OE and OE-like parasites among different hosts.

As we found that OE from D. plexippus did not infect H. armigera or H. virescens, it suggests that OE populations present on monarch butterflies are more specialized than OE-like parasite that we found on H. armigera. Barriga et al. (2016) also found that monarch butterflies were most susceptible to their own natal OE parasite, while queen butterfly D. glippus was barely infected by monarch OE parasite. In contrast, both D. plexippus and D. glippus could be infected by OE-like parasite collected from lesser wanderer butterfly D. petilia. Similarly, OE-like parasite collected from H. armigera could not only infect the closely related host H. virescens, but were capable of infecting monarch butterflies. Such an asymmetrical distribution of a parasite between two or more hosts has been found in other parasites, e.g., helminths and copepods and indicates that local specialization of parasites to their hosts (Poulin et al., 2011).
Interestingly, there seems to be geographic variation in host susceptibility to OE-like parasite in *H. armigera*, as we found that *H. armigera* larvae from Spain were more susceptible to OE-like infections than *H. armigera* larvae from China and Australia. As the genetic variation of OE-like parasite was less than 0.2% within the *Helicoverpa* genus, the geographic variation is likely to be due to local environment conditions. Previous studies have found that environmental factors, such as temperature and humidity, could have a major impact on prevalence, transmission and intensity of parasites (Duncan and Little, 2007; Laine and Tellier, 2008; Poulin, 2006; Stromberg, 1997). Furthermore, the resistance to parasites could vary among the host populations from different geographic locations (Barriga et al., 2016; Kaltz et al., 1999; Sternberg et al., 2013). Since OE-like parasite was not found on any *H. armigera* individual collected from Spain, this population may be more susceptible to infection, while *H. armigera* populations in China and Australia could have developed some level of resistance or tolerance to OE-like infections.

Host migration could have a strong influence on the interaction

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Fig. 1. Phylogenetic relationship of OE and OE-like spores collected from different hosts. Maximum likelihood (ML) tree based on 558 bp 18s RNA gene sequences. Values above the branches indicate clade bootstrap support.

Fig. 2. Infection rate with OE spores collected from *D. plexippus* (black bar) or OE-like spores collected from *H. armigera* (gray bars). Significant differences are indicated by asterisks (***: \( P < 0.001 \)) and different letters (\( P < 0.05 \)).
between host and parasite (Altizer et al., 2011; Gandon et al., 1996; Kaltz and Shyko, 1998). For example, long-distance migration can help to reduce parasite transmission and spread host resistance genes (Bartel et al., 2011; Bradley and Altizer, 2005; Loehle, 1995). In D. plexippus, the prevalence of OE parasite has been found to be higher in resident than in migratory populations (Bartel et al., 2011). Helicoverpa armigera also has the ability to undergo seasonal migration covering long distances (Zalucki & Furlong, 2005; Feng et al., 2009), which could affect OE-like parasite transmission over spatial and temporal scales. Taking these factors into account, we can expect that the prevalence of OE-like parasite and host susceptibility or resistance varies among H. armigera populations.

Climate change can alter the dynamics of parasite transmission and increase the potential for host switching (Brooks and Hoberg, 2007; Cooper et al., 2012). Correspondingly, the OE or OE-like parasites could pose a potential risk to other Lepidoptera and expand their distributions into new areas, or even new hosts. As H. armigera has recently invaded in South America (Czepak and Albernaz, 2013), and we already found that OE-like parasites can infect closely related host species, non-pest moth species and butterflies in all continents may be at risk. Once the parasites are introduced into new areas or new hosts, a number of questions arise, such as: What are the major factors that drive host shifts in these parasites? How does OE or OE-like parasites affect the fitness of different host species and how do different host species respond to parasitic infections? As temperature and relative humidity affect many life history traits in plants and animals in general (Alcázar and Parker, 2011; Peng et al., 2019), therefore climate change likely also has profound consequences in coevolutionary process between hosts and parasites.

5. Conclusion

In summary, our study gives the first evidence of OE-like infections in Lepidoptera other than the genus Danaus. The genetic similarity between OE from D. plexippus and OE-like parasite from the moths H. armigera and H. punctigera was 95.2%. Cross-infection experiments showed a higher host specificity of OE collected from monarchs and lower host specificity from OE-like parasite collected from H. armigera. Interestingly, the H. armigera population in Spain, in which we did not find OE-like infections, showed higher sensitivity to OE-like infection than the H. armigera populations in Australia and China, indicating geographic variation in the level of susceptibility, resistance or tolerance to OE-like in H. armigera. Further studies should give insights into how parasitic OE or OE-like differs among lepidopteran hosts and the level and extent of parasite transmission over spatial and temporal scales.

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Declarations of Competing Interest

The authors declared that there is no conflict of interest.