Didilia sp. infecting *Phlebotomus stantoni* in Thailand

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Abstract. Nematode infection in wild caught Phlebotomine sand flies was investigated in Thailand. Light microscopy (LM) and scanning electron microscopy (SEM) were used to detect and morphologically characterize entomopathogenic nematodes that presented in the sand flies. *Didilia* sp. nematodes were found for the first time in the body cavity of wild caught male *Phlebotomus stantoni* sand flies. The *Didilia* sp. was identified based on the morphology of the adult nematodes, from their stylet and teeth at the anterior tip, body length, and egg shell sculpture. It was noted that every infected male sand fly had unrotated genitalia, which would not allow them to mate, thus leading to the loss of their offspring. This finding provided information that might lead to study on whether or not the *Didilia* sp. has the potential to control sand fly population.

Keywords. Sand flies; *Leishmania*; Nematode; *Didilia* sp.; Phlebotomine

INTRODUCTION

Phlebotomine sand flies are responsible for the spread of the medically important *Leishmania* parasites. Several methods for the control of adult sand flies are available, such as residual sprays, space sprays, barrier methods including treated netting/clothing, tropical repellents, and application of specific pesticides in reservoir burrows (reviewed by Claborn, 2010). Extensive work into entomoparasites of phlebotomine sand flies, including protozoa, nematodes, viruses, bacteria, fungi and mites, has been conducted and some of these are described as capable of killing their hosts (review by Warburg *et al*., 1991).

Entomopathogenic nematodes in the families: 1) Allantonematidae in Colombia (Poinar *et al*., 1993); 2) Splendidofilariinae in Israel (Bain *et al*., 1992) and South Africa (Hering-Hagenbeck *et al*., 2000); 3) Steinernematidae in Brazil (Secundino *et al*., 2002) and Turkey (Karakuş *et al*., 2013); 4) Tylenchidae in Argentina (Fernández *et al*., 2016), Pakistan (Kakarsulemankhel, 2003; Kakarsulemankhel & Yasinzai, 1999), and India (Srinvasan *et al*., 1992; Dinesh *et al*., 2013), and 5) Tetradonematidae in Pakistan (Kakarsulemankhel, 2003), Afghanistan (Killick-Kendrick *et al*., 1989; Tang *et al*., 1993; Tang *et al*., 1997), and Portugal (Pires *et al*., 1997) have been found in various sand flies, i.e., *P. sergenti*, *P. papatasi*, *P. salehi*, *P. alexandri*, *P. dubosqi*, *P. argentipes*, *P. tobbi*, and *Lutzomyia longipalpis*. To date, no sand fly infected with entomopathogenic nematodes has been reported in Thailand. Therefore, the objective of this study was to investigate for entomopathogenic nematodes in sand flies in Chiang Mai, Thailand.

MATERIALS AND METHODS

Sand fly collections and identification

Sand flies were collected from Chiang Mai, Thailand, from January to August 2016 using five CDC light-traps from 6 pm to 6 am (overnight). Collected sand flies were transferred to

the laboratory at the Department of Parasitology, Faculty of Medicine, Chiang Mai University. Sand fly identification was performed using the following keys and articles (Lewis, 1978, 1987).

Dissection of sand flies and measurement of nematodes

Each collected sand fly was examined for nematodes by dissecting under microscopy. When a nematode was found in the body cavity of a sand fly, the infected fly was opened carefully under a dissecting microscope. Then, the nematodes were observed under a light microscope and photographed using an OLYMPUS microscopy camera using DP2-SAL Firmware Ver.3.3.1.198 (Tokyo, Japan). Measurements were performed using ImageJ 1.46r software (Maryland, USA).

Scanning electron microscopy

SEM was used to observe the morphology of the nematodes from the sand flies. The nematodes were fixed in 2.5% glutaraldehyde solution, left overnight at 4°C and washed twice for 10 min in PBS (pH 7.2) on the following day. Thereafter, they were post fixed for 1 h with 1% osmium tetroxide in PBS, dehydrated in increasing grades of ethanol, and dried to critical point. The nematodes were glued to an SEM stub and coated with a 20-nm thick gold layer before being observed with a Zeiss DSM640 scanning electron microscope (Oberkochen, Germany).

Nematode identification

Nematodes were identified by comparing the body size, egg diameter, and morphological characteristics with those in previous studies of the Tetradonematid nematodes (Killick-Kendrick *et al*., 1989; Tang *et al*., 1993; Tang *et al*., 1997).

RESULTS

Eight *P. stantoni* males out of 661 collected sandflies (mixed gender and species) were found infected with a nematode. Male *P. stantoni* had a cibarium with spicules, spines on the pharynx, and palp extending further than antenna 3 (data not shown). The morphological characteristic of the male *P. stantoni* genitalia is shown in Figure 1. Figure 1a shows a genital pump and an ejaculatory duct. The genitalia of the male had two styles, with each having four long spines of which one is terminal, one subterminal, and two were near the middle. The male also had paramere with trilobed and the accessory spine of the aedeagus, was longer than the aedeagus (Fig. 1b).

Figures 2a and b show normal genitalia and unrotated genitalia of the male *P. stantoni* sand flies, respectively. One gravid female nematode was observed in the body cavity of each male sand fly, all of which had unrotated genitalia (Fig. 2b). Light microscopic analysis revealed that nematodes were filled entirely with round, light brown eggs with a sculptured surface (Fig. 2c and d). A comparison of body size and egg diameter of the nematodes to *Didilia ooglypta* in the previous work of Tang et al. (1993) is shown in Table 1. The SEM results showed spherical or oval shaped eggs with a thick shell and surface pattern divided into hexagonal plates (Fig. 2e and f). The position of the stylet was found at the anterior tip with a tooth opposite the stylet (Fig. 2g and h). According to their morphology, body size and egg diameter, the nematodes were identified as *Didilia* species.

DISCUSSION

In this study, male *P. stantoni* sand flies were found to be infected with nematodes. The mean egg diameter and the body width of the nematodes were similar to those found in previous studies of *D. ooglypta* from Afghanistan and Portugal (Tang *et al*., 1993; Pires *et al*., 1997)

but their body length was shorter. Because of their short body length it suggests that these nematodes were not *D. ooglypta*. However, due to the similarity in ultrastructural morphology of their eggs, teeth, and stylets compared with *D. ooglypta*, they were identified as *Didilia* species.

The Tetradonematidae nematode, *Didilia* sp., was found in Chiang Mai, Thailand for the first time whereas the Steinernematidae nematodes, *Steinernema khoisanae*, *Steinernema websteri* (Thanwisai *et al*., 2012), *Steinernema minutum* (Maneesakorn *et al*., 2010), *Steinernema siamkayi* (Stock *et al*., 1998), and other *Steinernema* have been reported in wax moths and Japanese beetles in Thailand (Tangchitsomkid & Sontirat, 1998; Noosidum *et al*., 2010; Vitta *et al*., 2015). Tang *et al*. (1997) have described the life cycle of *D. ooglypta*. Briefly, *D. ooglypta* eggs are ingested by first instar sand fly larvae. Female and male nematodes develop inside the larvae. The sex organs of both females and males are seen on day 20 of infection, they then mate. After mating, males die in 2 days. In the meantime, the sand fly larvae develop into the fourth stage. 37 days after infection the female nematodes are fully mature with fertilized eggs in the uterus. By the time the adult flies emerge from pupae the gravid nematodes use their teeth and stylet to break through the intersegmental membranes of the infected fly and bore a hole through the cuticle of the abdomen of the adult flies to expose the vulva and lay eggs. The gravid female nematodes affect the development of reproductive organs, delay preimaginal stages, and reduce the life span of the infected sand flies when compared with uninfected flies. Unrotated genitalia of infected male of *P. papatasi* are observed (Tang *et al*., 1997).

Our study revealed that the genitalia of the infected *P. stantoni* were also unrotated. Therefore, male infected sandflies are likely to be unable to mate because of their unrotated genitalia. Most infected flies survive approximately 6 to 8 days after the nematodes laid eggs and the infected flies emerge approximately 18 to 20 days later than uninfected ones (Tang *et*

al., 1997). The delay of sand fly development might be of benefit in controlling sand flies because when nematodes oviposit, it would be the same time that the first instar larvae of uninfected sand flies in the same generation are ready to ingest the nematode eggs. Thus, this would increase infection in the sandflies. The eggs also survive for two and a half years in humid larval feces, but almost always die within two weeks in water (Tang *et al*., 1997). Based on this information, further study on the potential of *Didilia* sp. for use in the control of phlebotomine sand fly populations should be considered.

In conclusion, male *P. stantoni* infected with nematodes were found for the first time. Based on the results of comparison of body size, pattern of egg cases and the position of stylets and teeth of the nematode to previous publication, the nematodes were identified as *Didilia* species. It is likely that *Didilia* sp. will affect the development of the genitalia of *P. stantoni* and therefore, infected male sandflies may be unable to mate because of their unrotated genitalia. These findings provide information and justification for further studies in using *Didilia* sp. to control sand flies and hence limit or decrease the spread of *Leishmanias*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

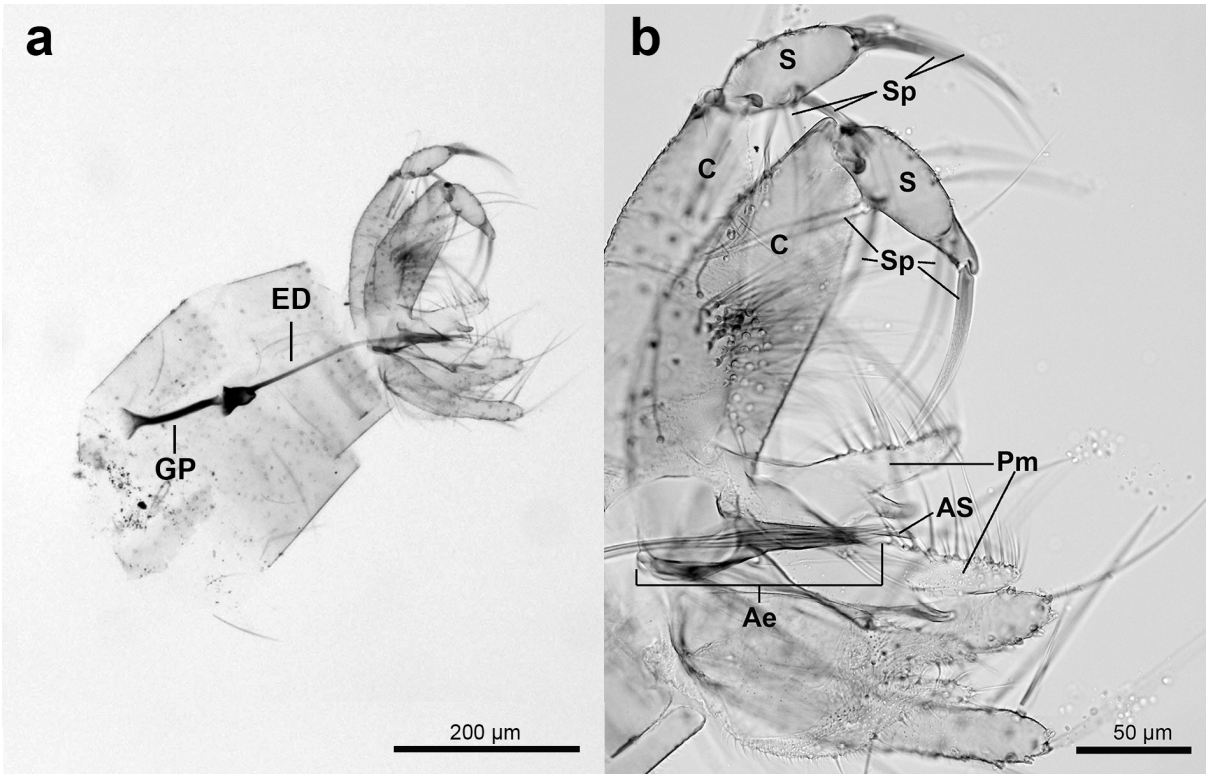

Figure 1. Light microscope image of male genitalia of *P. stantoni*. (a) Male genitalia with genital pump (GP) and ejaculatory duct (ED). (b) High magnification of aedeagus (Ae), accessory spine of aedeagus (AS), coxite (C), paramere (Pm), style (S), and spine of style (Sp).

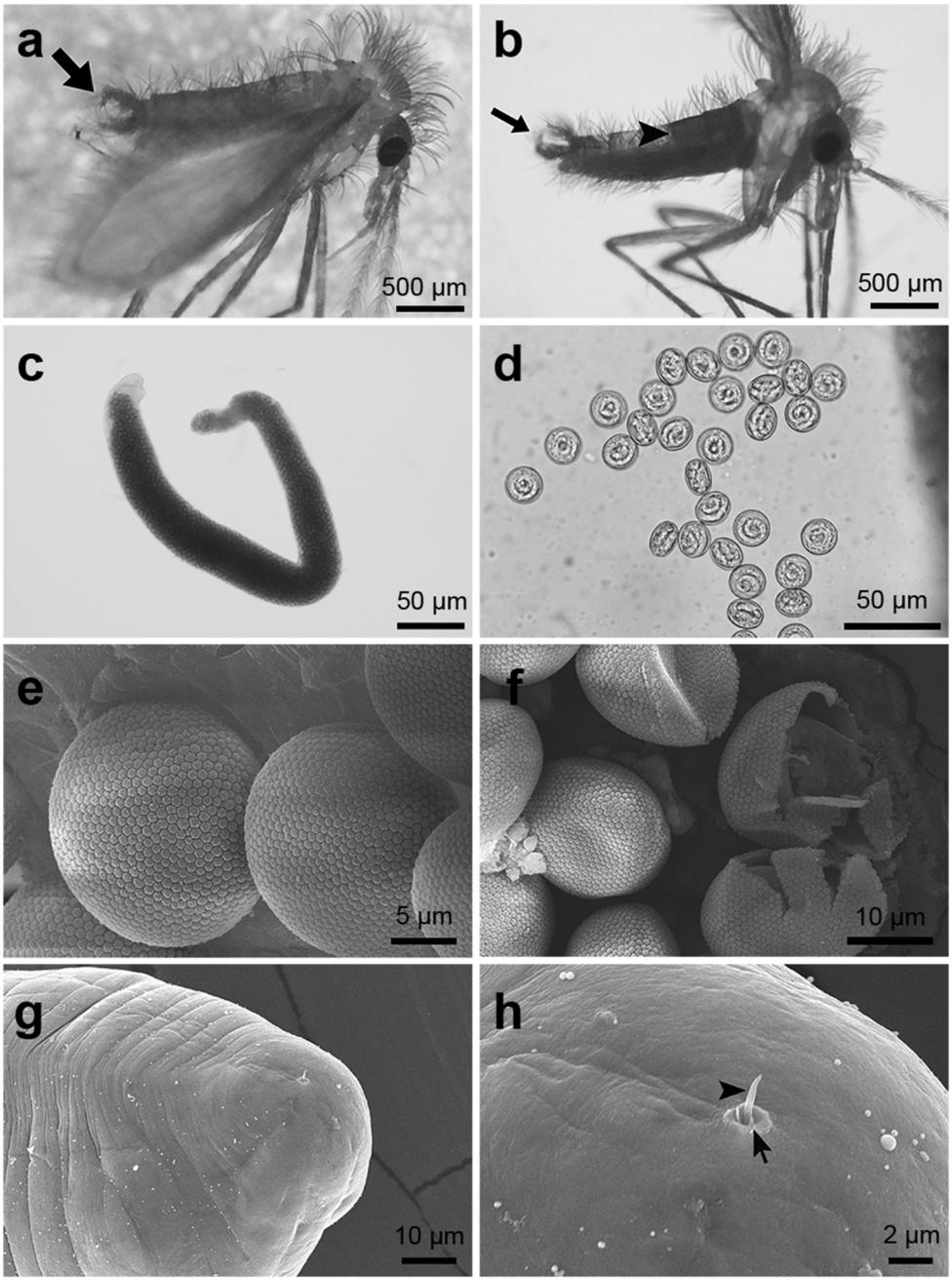

Figure 2. Female nematodes found in male *P. stantoni* Chiang Mai, Thailand (2016). (a) Arrow indicates normal genitalia of male. (b) Arrow indicates unrotated genitalia of male and arrowhead indicates gravid female nematode in sand fly. (c) A gravid female nematode from the body cavity of sand fly. (d) Eggs of nematode in PBS. (e) SEM of eggs of nematode. (f) SEM of incomplete eggs inside the nematode. (g) Anterior tip of female nematode showing stylet (h). Stylet (arrowhead) and teeth (arrow) at anterior tip of nematode.

Table 1. Comparison of body size and egg diameter of nematodes collected in Chiang Mai, Thailand and *D. ooglypta* from Afghanistan (Tang et al. 1993)

Nematode	Body length (µm)	Body width (µm)	Egg diameter (µm)
Chiang Mai 2016	1794.31 ±30 (N=8)	148.15 ±10 (N=8)	20.63 ± 5 (N=30)
Didilia oogypta	3113 ± 361 (N=17)	141 ± 16.4 (N=17)	27.70 ± 1.5 (N=17)