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臺灣鐵線蟲生物多樣性及寄生對於寄主形態發育的操控

Biodiversity of the Taiwanese horsehair worms and the host morphological development manipulated by infection

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本論文係邱名鍾君（D99632002）在國立臺灣大學昆蟲學系.完成之博士學位論文，於民國 106 年 1 月 24 日承下列考試委員審查通過及口試及格，特此證明

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宣告

根據國際動物命名規約 (International Code of Zoological Nomenclature (ICZN, 2004)) 第 8 條，本論文中對新種鐵線蟲的命名非遵循正式的命名原則。因此本文中所提及的新種名稱 (*Acutogordius formosanus* n. sp.) 將在發表之後始正式生效。

Disclaimer

According to article 8 of International Code of Zoological Nomenclature (ICZN, 2004), the name of horsehair worms (*Acutogordius formosanus* n. sp.) in this dissertation will only become available by the referring publications.

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誌謝

從初步認識鐵線蟲到本文完成的這十年間，研究的重心都環繞在鐵線蟲這個類群身上，但鐵線蟲其實始終都不是我真正關心的對象。真正令我感到幸運與自豪的是能與不同領域的科學家一同在研究活動的過程中探索人類理解自然的方式。當然這個過程並不順利，特別是在當代的主流認知中尋求突破，無可避免去衝撞主流價值觀，並無止盡的懷疑與批判自己曾經相信的一切事物。這十年間有賴吳文哲老師的指導與容忍，特別是在我思考上過度理想化時不時引導我在現實與理想中尋求平衡。吳老師的支持也是這十年間研究得以持續的原因，特別是目前臺灣的環境幾乎不可能支持這類長期且基礎的研究。而這段期間內昆蟲生態室的成員，從一開始進入實驗室時指導我進行實驗的周倖瑜學姐，到黃旌集學長，鄧雅文學姐，與許雅均學姐，王瑞中學長，均讓我在昆蟲生態室的研究工作得以順利進行。博士班後期的研究則有賴昆蟲系統分類研究室的支援，特別是蕭旭峰老師的指導與簡維君小姐在電顯上的技術支援，簡小姐至今仍然是我所遇過對掃描式電子顯微鏡最為熟悉的技術人員。在博士班研究的最後一年藉由千里馬計畫的支持到神戶大學溪流生態實驗室參與一年的合作計畫，讓我經歷了一個完全不同於以往的實驗室氣氛，並在佐藤拓哉老師與佐倉綠老師的支持下與木村文，佐田恭子，相樂理嘉等學生初步建立了鐵線蟲的研究系統，為將來的研究打下基礎，並在這一年中認識了許多有趣的朋友。

除了這些主要經歷的實驗室之外，臺灣大學的臨床醫學研究所的王弘毅老師，昆蟲族群研究室的奧山利規老師，生命科學系的施秀惠老師，長庚醫學大學寄生蟲研究所的陳維鈞老師，與屏東科技大學植物醫學系的鄭光哲老師，均在研究的過程中給予極大的支援與鼓勵。樣本的採及有賴大野康邦，土岐和多瑠，蔡緯毅，劉人豪，方華德學長，臺北市立動物園的黃龍椿學長，蕭忠義學長的協助。實驗的技巧和分析有賴謝佳宏學長，許弘瑋，張倫賢學長的協助。並特別感謝博士班後期生病期間協助我度過的朋友，楊世綵學姐，何俊頤學長，林彥成學長，王庭碩，呂易澤，廖朝盛，吳宗學，于品馨，陳柏諺，王沿竣等壘球隊的朋友。

最後感謝在求學階段始終在背後支援我的父母與家人，持續不斷的恩澤往往讓人理所當然的忽略，然而它卻是工作與學業上最重要的基石。我有幸能夠通過這段時間的訓練，並有機會延續研究生涯。在失去學校的庇蔭後未來將面臨更大的挑戰。但和許多的科學家一樣，我們始終對於走在一條崎嶇的道路上而感到幸運與自豪，幸運的是我們仍然有選擇的權利，自豪的是我們能經由這條路來實踐各自的理念。希望在下一個十年後回頭看到這段文字，仍然能夠為自己的選擇而感到驕傲，並不辜負曾經接受過的幫助。

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中文摘要

鐵線蟲在生態上的獨特性使其在寄生蟲的研究中佔有一定的地位。儘管牠在應用上的價值不高，長久以來的傳說以及對鐵線蟲的誤解推動了大部分的相關研究。鐵線蟲的生活史橫跨水陸兩域，幼蟲在水中孵化並寄生在水生的保幼寄主體內形成包囊。受感染的保幼寄主被陸生最終寄主攝食後胞囊得以進入最終寄主體內發育，並在成熟後鑽出回到水中繁殖。在我們的採集中，臺灣目前發現三種鐵線蟲，分別是臺灣索鐵線蟲 (*Chordodes formosanus*)，臺灣尖尾鐵線蟲 (*Acutogordius formosanus* n. sp.)，原鐵線蟲 (*Gordius* sp.)，並在水生保幼寄主與淡水魚體內各別發現一種形態與上述種類均不相符的鐵線蟲。三種已知的鐵線蟲除了原鐵線蟲的最終寄主尚不明瞭之外，臺灣索鐵線蟲的主要寄主為斧螳 (*Hierodula* spp.)，而少部分個體也曾在花螳螂科 (Hymenopodidae) 及螽斯科 (Tettigoniidae) 的體內發現；臺灣尖尾鐵線蟲則廣泛的被發現寄生在直翅目 (Orthoptera) 的不同科之中。在臺灣索鐵線蟲的發育過程中，牠會影響最終寄主的形態發育並造成形態上的異速生長與間性。這兩個現象可能有利於寄生蟲提高對寄主資源掠奪的效率。間性現象也廣泛的被發現存在不同的寄生關係中，但至今其機制仍然不明瞭。昆蟲的性徵分化一直以來被認為不會受到細胞外的因子所調控，這包含寄生蟲的影響在內。因此藉由幼年化的方式阻斷寄主的性徵發育，使其停留在分化之前被認為是造成昆蟲間性的原因。然而在受到臺灣索鐵線蟲感染的寬腹螳螂 (*H. patellifera*) 身上，寄生蟲造成的影響卻不支持幼年化的假說，反而偏向寄主的雌雄二型性性分化過程受到干擾。儘管目前我們尚未收集到足夠多的樣本來支持這個假說，鐵線蟲的研究卻很有可能在當代的昆蟲性徵發育領域中帶來新的觀點。

關鍵詞：線形動物們，臺灣索鐵線蟲，臺灣尖尾鐵線蟲，斧螳屬，間性，幼年化，昆蟲性徵分化

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Abstract

The horsehair worm is the parasite which is famous for its unique properties instead of its applicability in human lives. Its life cycle typically goes through aquatic and terrestrial environment, with two parasitic phases in aquatic paratenic host and terrestrial definitive host. There are three known horsehair worm species in Taiwan (*Chordodes formosanus*, *Acutogordius formosanus* n. sp., *Gordius* sp.) and two undetermined species, respectively, detected from aquatic paratenic hosts and a fresh water fish. Except *Gordius* sp. whose definitive host is still unknown, the host range of *C. formosanus*, which is specific to the *Hierodula* mantids (despite it can be found to parasitize Hymenopodidae mantids and katydids), is much narrower than that of the *Acutogordius formosanus* n. sp. which is frequently found in different family of the orthopteran hosts. During the definitive host phase, *C. formosana* manipulates the morphological development of its mantid host for increasing the efficiency of resource exploitation, and consequently cause the morphological allometry and intersexuality on the infected host. Mechanism of the parasitic intersexuality is unclear. Since the insect sexual differentiation has long been believed to be the cell-autonomous process which lacks regulation outside a cell, it is generally believed that the parasitic intersexuality is caused by the inhibition of ontogeny, which is known as juvenilization. However, our study of the horsehair worm effect on *H. patellifera* is likely to conflict to the hypothesis of juvenilization and suggests the alternative hypothesis of the interference in insect sexual differentiation. Despite the sample size is still less to confidently support this conclusion, studies of the horsehair worms might provide the new light in the understanding of the developmental process of the insect sexual differentiation.

Key words: Nematomorpha, *Chordodes formosanus*, *Acutogordius formosanus* n. sp. *Hierodula*, intersexuality, juvenilization, insect sexual differentiation.

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1 General introduction: the parasitic property of horsehair worms

1.1 Horsehair worms in the myths

The horsehair worm is the parasite which is famous for its unique properties instead of the relationship with human activities. Instead of the applicability, people's misunderstanding or scares promote scientific studies of the horsehair worm. One early study conducted by Leidy (1850) is to clarify the "origin" of the horsehair worm. The "horsehair worm", as the most frequently used common name, is not only named after its long, dark, and slender body shape, but also implied how it is created: the fallen horse hair in the water. Since the adult worm was often found in water filling a wagon rut or the drinking trough of horses, it is believed to be the horse hair which has become "vivified" in the water (Leidy, 1850). Leidy (1850) argued this "abiogenetical myth" by putting horse hair in the water and introduced the reproductive behavior of horsehair worms by describing its egg developmental process. Before the end of the 19th century, the horsehair worm's life history with both a parasitic and a free-living phase has basically been established (Schmidt-Rhaesa, 2012). And in the early 20th century, the adoption of pseudoparasitism to describe the existence of horsehair worms in human also reflects the well-understanding of the parasitic property of horsehair worms (Hall, 1912; Schmidt-Rhaesa, 2012). However, people's negative impression of horsehair worms did not stop after their life history was revealed. Horsehair worms has long been thought to be poisonous and lethal to the livestock (Baylis, 1944 reviewed in Schmidt-Rhaesa, 2012) but they are likely to cause only mechanical damages to animal's intestine (Hanelt *et al.*, 2005). The fear and importance of the horsehair worms in veterinary is now approximately erased since the real livestock parasites were found, but the adult horsehair worms in the

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trout recently inspire the bright new hypothesis of the parasite-mediated allochthonous input from the terrestrial environment to the water (Sato *et al.*, 2008, 2011).

To date, most studies surrounding horsehair worms still comes from the old myth: host suicide induced by its parasites (Heinze, 1941 reviewed in Schmidt-Rhaesa and Ehrmann, 2001). And other than the host behavior manipulation, our recent studies in the horsehair worm's effect on host developmental manipulation might give a new light in the "myth" of the cell-autonomous insect sexual differentiation (Chiu *et al.*, 2015). In contrast to most of the parasitological studies, studies surrounding the horsehair worm are generally not that develops for practical applications, but improves and challenges the current understanding of natural or other phenomena. Horsehair worms are grouped as the micro-parasite. This category involves how as scientists consider the parasite, the micro-parasite, and the parasitic properties of the horsehair worm. As the part less mentioned in the study of parasite, it might be worth to begin from the definition of a parasite.

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1.2 The definition of a parasite

1.2.1 Parasite: a special consumer

All species of horsehair worms are generally acknowledged to be parasites (Hanelt *et al.*, 2005), included the species found emerging from their hosts and nearly half of the species which are "believed to be" parasites since their hosts have not yet been known (Poinar and Chandler, 2004; Schmidt-Rhaesa, 2012). The term "parasite" is widely used and it is commonly accepted that a parasite adopts one of the most unique strategy to be a consumer. Nevertheless, parasitism, despite it has been suggested for more than one century from 1879 (Zelmer, 1998), is not a constant concept in each assay. As the opposite concept of the free-living organism, parasites are generally considered to be the organisms live in their hosts. To date, using the host as both source of nourishment and habitat is the most common definition of parasitism (Zelmer, 1998; Poulin, 2007). However, this rule groups various parasitic organisms which is much more than people image. As almost all organisms have other organisms living in it (including parasites infected by other parasites, termed hyperparasitism), the free-living strategy may be more unique than parasitism (Matthews, 1998). Thus, parasite discussed is always further defined by the limited taxa or cases (Matthews, 1998), despite parasitism is more likely to be an ecological concept instead a systematic group (Eggleton and Belshaw, 1992; Zelmer, 1998).

The predator is always considered to be most closely related to the parasite. The most significant difference between parasite and predators is that if the prey are alive during being consuming. This property makes parasitoids, which consume the live hosts but finally kill them, become the special parasitic organisms. There are two properties mainly applied in characterizing a parasitoid: 1) the free-living stage in the life cycle (Eggleton and Gaston, 1990; Zelmer, 1998) and killing the host in the end of

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the parasitic stage (Eggleton and Gaston, 1990; Zelmer, 1998; Kuris and Lafferty, 2000). The former property emphasizes the habitat role of the host to a parasite and makes the parasitoid more likely to be a predator which, comparing with the normal predators, spend longer time on exploiting the food resource from its prey (Zelmer, 1998). This concept also rules out the "insidious and consumptive predators" usually has been considered ecto-parasites like mosquitos, which do not really live on their hosts. Since the emphasis on the habitat role of the host, whether the host is killed is not the essential property to characterize a parasitoid. This point of view is not much of the problem in the parasitoid wasps, but is debatable in the parasitoid flies. In the early concept, the parasitoid is suggested to specially describe the insect parasite especially the parasitoid wasps (Eggleton and Gaston, 1990). In many literatures to date, the parasitoid is still usually equal to the "parasitic" Hymenoptera (Eggleton and Belshaw, 1992). With the highly host specific, the parasitoid wasps generally parasitized with insect hosts and kill it in the end of the parasitic stage (Eggleton and Belshaw, 1992). However, the flies, as also common "parasitic" insects, highlights the non-essential property of killing host by its diverse host taxa. As the different way in evolving the parasitic life style, host range of the "parasitic flies" is much wider than the parasitoid wasp which can be invertebrates or vertebrates (Eggleton and Belshaw, 1992). In the fly species parasitizing insect hosts, the "parasitic flies" usually kill their hosts before pupation whose behavior is similar to the parasitoid wasps. Whereas the obligatory myiasis (e.g., screw worms, bot flies and warbles) which lay eggs on mammal hosts is usually less harmful to cause the lethal damage (Eggleton and Belshaw, 1992). Whether the host die or not among the parasitism of flies and wasps might be mainly due to the host relative size (Zelmer, 1998). Thus, death of the hosts is more likely to be an extended property but not a defining character. Since

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considering the host as a prey instead of habitat, the parasitoids frequently kill their hosts by the extreme virulence or damage, which tends to be reduced in the parasites (Alizon *et al.*, 2009).

The high virulence in the parasitoids, than the parasites, indicates their diverged way in exploiting their hosts. In contrast to the definition emphasizing on the habitat role of hosts, the host death as the character makes the parasitism more close to be a foraging strategy. The foraging model causes one special dichotomy for dividing the parasite from other consumers: the number of host/prey attacked during the particular life-history phase (Kuris and Lafferty, 2000). The functional response, which is the important component of the most widely known Lotka-Volterra predator prey model, describe how predators eat "many" prey (Kuris and Lafferty, 2000). This model is obviously not applicable in describing the parasite-host system as a parasite generally attacks one host in a particular life-history phase. Under this rule, typical parasites, the parasite contains typical parasites, parasitoids, and micro-predators (e.g., ecto-parasites, insect herbivores) whose size are smaller than their victims. However, comparing with the disease model mainly based on epidemiology, parasitoid models are derived more from predator-prey theory (Kuris and Lafferty, 2000). One of the example is the Nicholson-Bailey model. This model describes how the competition of female parasitoids search and lay eggs in the hosts, which is also known as the competition model (Royama, 1971). This model emphasizes the predator property of parasitoids in the free-living stage and implies the parasitic larval stage as the mean of handling the prey. Thus, the key difference between parasitoids and parasites, killing the host, could be considered as the predator property of the mother parasitoids. The separation of the free-living adult phase and the parasitic larval phase makes it more clear to characterize the foraging strategies in different developmental stages.

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Although it is less mentioned in the parasitoid model, during the parasitic larval phase, parasitoids still show the high similarity with typical parasite.

During evolutionary processes, the parasitic strategy independently evolved in wide phylogenetic taxa. It can be fixed in the population under different environmental conditions. Thus, it might be difficult and impractical to group all the parasitism evolved with different adaptations by a signal or few properties. Zelmer (1998) summarized different definitions of parasitism into a property that all parasitic organisms have the ability to evade the immune response of the host. However, this definition is rarely applied and actually does not highlight the significance of parasitism from other foraging strategies or ecological relationship. In the contrast, many characters of the parasitic organisms including the smaller size, feeding alive organisms, evading the host immune response, are all related to the habitat role of hosts. Thus, it is not surprisingly that "the habitat and nourishment role" become two critical parasite's properties most commonly applied, despite there are still a gray area between the parasitic organism and others.

1.2.2 Macroparasite and microparastie

The epidemiological model for parasitism not only suggests the fundamental difference from predators, but also leads to the classical dichotomy of microparasite/macroparasite (Kuris and Lafferty, 2000). One role to separate micro- and macro-parasite is if the parasite multiply in the host (Begon, 2009). For most of the macroparasites, they grow in the host without increasing their number. Whereas the microparasites often increase rapidly after establishing in the hosts (Begon, 2009; Rosà *et al.*, 2006). Multiplying of the microparasites makes the infected hosts go into dichotomized conditions: "infectious hosts" which contain enough parasites to infect other hosts, and "non-infectious hosts" with few or no parasites, which are suppressed

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by immune response. In this situation, prevalence, the probability of infected host (infectious hosts) in the host population, become the most and only considerable parameter in modeling the transmission of microparasites. This concept is applied in the original epidemiological model known as SIR model which characterize the host individuals into susceptible (S), infectious (I), and recovered (R). To date, the SIR or its extended models are still applied in estimating the outbreak of some human disease (e.g. Jansen and Stollenwerk, 2005). Considering the host as transmission unit implies the reproductive potential of microparasites dependents on the ratio of the infectious hosts. However, for the macroparaites, especially that reproduce outside of the hosts, the reproductive potential is highly related to the total parasite number. Moreover, the unequal distribution of parasite individual in each host (Kuris and Lafferty, 2000; Rosà *et al.*, 2006) makes the total parasite number unable to be estimated by the infected hosts. Anderson and May (1978) suggested the model which adds intensity (number of parasite in an infected host) as a parameter known as intensity-dependent model (Kuris and Lafferty, 2000; Rosà *et al.*, 2006). In contrast to the SIR model which takes only host as variables, variables in the intensity-dependent model contains "the density of hosts" as well as "the parasite burden" (distribution of adult macroparasites in the hosts) and " the density of free-living larvae of parasite" (Rosà *et al.*, 2006). Both of these models (SIR model/microparasite model and intensity-dependent model/macroparasite model) are now widely applied in the description of parasite-host dynamics and makes the three indicators (prevalence, mean intensity, mean abundance (prevalence times mean intensity)) become the most important parameters in description the parasite's aggregation in their hosts (Poulin, 2007).

The dichotomy of micro- and macro-parasite also implies the parasite size (or

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relative size to the host) as one important property in their infection. As it is showed literally, "small parasites" (protozoa and smaller microbes) generally fit the microparasite model whereas the "large parasites" (helminths and arthropods) generally fit the macroparasite model (Lafferty and Kuris, 2002). However, some exceptions (e.g. ciliates or coccidians is better to be modeled as macroparasites; and larval digeneans in molluscs and rhizocephalan barnacles are better to be modeled as microparasites) makes the parasite size is originally argued to be associated with the trophic strategy (Lafferty and Kuris, 2002). It is understandable that some macroparasite (e.g. cercaria of *Pleurogonius malaclemys* in the snail hosts) asexually reproduce in their hosts (Hunter, 1967) while the host infectious level is not always constant in the parasitic bacteria, *Pasteuria ramosa*, due to the various spore number in a host (Vale and Little, 2012). However, Lafferty and Kuris (2002) reviewed the current known host-parasite systems and suggest the inconsiderable relationship in parasite size and the trophic strategy. Especially in the parasitoids and castrators, their extreme large body size (mass), which approach the same mass with the hosts, is hard to be excluded as a possible factor in promoting the evolution of their specific trophic strategies (Lafferty and Kuris, 2002).

As the argument in separating "the parasite" from other consumer, it is hard to define a parasite as the micro- or macro-parasite by one or few properties. In fact, a parasitic life history is the combination of several characters multiply evolved in adapting to diverse environment. Each model or role is applied to describe a combination of properties instead of a real parasite. Horsehair worms, as parasites with large size and long-term development inside the host, show the specific living strategy in exploiting hosts. They leave their hosts at the end of the infection and finally kill the host. From any aspect, they match the definition of the parasitoid, but it

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is undoubtedly that the living style of horsehair worms are much different from other insect parasitoids. It might be hard and not necessary to characterize them as any category created, especially in some definition, the horsehair worm might be excluded from a parasite. Thus, instead of defining the horsehair worm as a parasite type, it might be better to focus on the adaptation of its trophic strategy and host effect, and compare that with the organisms with similar properties.

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1.3 The parasitic properties of the horsehair worm

Horsehair worms adopt parasitic life strategy, but its parasitic properties change drastically in each developmental stages. The parasitic life history of a horsehair worm is typically composed of two parasitic phases: paratenic host phase and definitive host phase. Eggs of the horsehair worm hatch in the aquatic environment. Larvae stay on the water bottom and encyst in the aquatic animals after being swallowed. The worm cysts in these aquatic animals, which are known as paratenic hosts, are carried to the terrestrial environment and enter terrestrial definitive hosts after the infected paratenic host is consumed as prey. The worm larvae inside the definitive host excyst and enter host hemocoel to develop into wormlike juveniles. The juvenile worms grow and become adult inside the haemocoel of the definitive host. Nearly the end of parasitic phase, the adult worms might manipulate the host behavior to enter aquatic environments and finally emerge to reproduce in the water (Hanelt *et al.*, 2005; Bolek *et al.*, 2015).

1.3.1 Paratenic host phase

The paratenic host, which is not required for the parasite's proper development (Baer, 1951 reviewed in Hanelt and Janovy, 2004a), carries the worm cysts to the terrestrial environment. Serving as vehicles in horsehair worm's life history, they are believed to interact less with the horsehair worm cysts. Few studies showed the negative effects of the cyst to hosts including inducing internal defense reaction (Inoue, 1962; Hanelt and Janovy, 2003, 2004a), reducing the wing pad size and inhibiting pupation in some aquatic insects (White, 1969). In our experiments, we also found the mortality of the snails and larval chironomids tended to increase with exploring high density of the worm larvae (data not showed).

The host range of the horsehair worm's paratenic host is extreme wide. The

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worm cysts have been found in phylogenetically far taxa of hosts from invertebrates (aquatic insects, crustaceans, water snails, trematodea) to vertebrates (fish) (Cort, 1915; Schmidt-Rhaesa and Ehrmann, 2001; Hanelt and Janovy, 2003). Hanelt and Janovy (2003) suggested the foraging behavior might be the most important factor affecting the infection of paratenic hosts since larval horsehair worms are able to infect almost all animals swallowed them. This suggestion is also supported by the unequal infection in different functional groups of larval chiromids (Chiu *et al.*, 2016a). This extreme wide host range is rare among parasites which might be caused by the costs incurred from the ability to exploit multiple hosts or decreased abilities to exploit hosts rarely encountered (Arbiv *et al.*, 2012). In this view, the true generalists indirectly support the larval horsehair worms do not evolve tight interaction with their hosts. It makes the relationship in horsehair worms and paratenic hosts is more likely to be passengers to carriers instead of parasites to hosts. Such passengers-carriers relationship is commonly seen in the phoretic mite attaching on the body surface of larger, flying insects for dispersal (Schwarz and Müller, 1992; Palevsky *et al.*, 2001). Nevertheless, it is worth to note that the paratenic hosts not only bring the larval worms to the definitive hosts, but also increase their infectiousness (Inoue, 1962; Schmidt-Rhaesa and Ehrmann, 2001; Hanelt *et al.*, 2005), longevity (Hanelt and Janovy, 2004b), and ability to survival the freezing (Bolek *et al.*, 2013b). These phenomena were found in the rearing experiment since Inoue (1962) found the mantids fed with larval worms had lower infection rate than that fed with infected insects contains worm cysts and Hanelt and Janovy (2004b) found the larval worms in the water are infectious for two days after being hatched but the cysts in the paratenic hosts can be infectious for more than six months. The mechanism to change the physiological properties from larvae to cysts are still unknown, but it might makes the

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paratenic hosts are not only ecologically but also physiologically essential in completing a horsehair worm's life history. In some literatures, the use of parasitic host indicates a host which facilitates the transmission but does not always appear in a parasite's life history (e.g. Jezewski *et al.*, 2013). This concept might be different from that of the paratenic host in horsehair worm's life history. In some cases, the term "intermediate host" is also seen in describing these "aquatic carriers" of horsehair worms (e.g. Schmidt-Rhaesa and Ehrmann, 2001).

The wide host range insures the larval horsehair worms to be encysted in the aquatic animals ingesting them, but not all species of the paratenic hosts take the worm cysts to their definitive hosts. Water snails are common paratenic hosts in nature which were frequently found to harbor cysts of horsehair worms (Hanelt *et al.*, 2001; Bolek *et al.*, 2013a; Harkins *et al.*, 2016), but they are rarely preyed by the crickets, which is the definitive hosts of horsehair worms in Northern America (Hanelt and Janovy, 2004b). These paratenic hosts servering as carriers instead of transmitters are called spurious paratenic hosts (Hanelt and Janovy, 2004a). Cysts in the spurious paratenic hosts is unable to directly finish their life histories, but can be transmitted to the definitive hosts via the horizontal transmission to the (true) paratenic hosts. Such horizontal transmission is called paratenesis. A horsehair worm cyst proceeds paratenesis to be encysted again in the new paratenic host which ingests the previous paratenic host (Hanelt and Janovy, 2004a). Paratenesis enable the horsehair worm cysts to be transmitted in through the food web, and theoretically make the cyst density to be concentrated in the predator hosts (Moravec and Skor´ıkova´, 1998; Brown *et al.*, 2001). However, the predator concentration was not found in the infection of different functional groups of larval chironomids (Chiu *et al.*, 2016a).

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Now the wide host range makes the cyst stage of the horsehair worms has recently been attended by the potential in the investigations of horsehair worm's geographic distribution, species composition, and seasonality (Hanelt *et al.*, 2001; Bolek *et al.*, 2013a; Chiu *et al.*, 2016a; Harkins *et al.*, 2016). The hosts used in these studies (snails or larval chironomids) might be not the main host bring the cysts to the definitive hosts, but they might well reflect the present and relative amount of the horsehair worm's larvae in the environment. In this dissertation, we also develop the technique to increase the efficiency in examine the infection of cysts in aquatic insects and hope the accumulation of field data will promote the understanding of the epidemiological properties of cyst in horsehair worm's life history.

1.3.2 Definitive host phase

The main post-embryonic development of a horsehair worm is launched from the cyst is ingested by the definitive host and finishes when the adult worm leave the host. During the time, a horsehair worm proceeds metamorphosis from a cyst to become the wormlike juveniles (Hanelt and Janovy, 2005), rapid increase of the body size (Schmidt-Rhaesa, 2005), and finally become a mature worm (Schmidt-Rhaesa, 2005). These three stages are respectively associated with three interactions with the host: determination of the host specificity, parasite's trophic strategy with altering host development, and host behavior manipulation.

The metamorphosis of the cyst is the first step to determine a host to be the definitive host or another paratenic host. Unfortunately, it has never been mentioned about the factors triggering the excysted larvae to proceed metamorphosis. In fact, definitive host data are still lacked in many horsehair worm species since they were frequently described only by the free-living adult collected in the environment (Poinar and Chandler, 2004) or were artificially infected to the laboratory-reared insects by

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field-collected cysts (Bolek *et al.*, 2013a). Unlikely the wide host range in the paratenic host, the definitive hosts of horsehair worms are limited to arthropods mainly including millipedes, orthopterans (crickets, grasshoppers, etc.), beetles, cockroaches, and mantids in freshwater environments (Schmidt-Rhaesa, 2012; Bolek *et al.*, 2015) and crustaceans in the oceans (McDermott *et al.*, 2010; Schmidt-Rhaesa, 2012). In each species, despite only a few surveys mentioned about the host specificity (Schmidt-Rhaesa, 2012; Chiu *et al.*, 2011), a horsehair worm seems to only able to develop in one or few species of definitive hosts.

After the metamorphosis to become wormlike juvenile, a horsehair worm go through the rapid growth in host's body cavity. This is the only known trophic stage of a horsehair worm to exploit resource from it host. It is believed that a horsehair worm consumes host non-essential fat and reproductive organs to support its growth (Lafferty and Kuris, 2012) since these two tissues in the infected hosts are commonly atrophied (Lafferty and Kuris, 2012; Chiu *et al.*, 2015; but in our observation, the fat body might be not always reduced in the infected mantids). Nevertheless, there is not yet an evidence confirmed how a horsehair worm gains nourishment from these tissues. By comparing the morphology of the horsehair worm developing for different time inside the definitive hosts, Schmidt-Rhaesa (2005) suggested the intestine is present and occupies half of the body cavity in the early development of the juvenile. The intestine maintains its size in the first third of its developmental period and then become smaller (Schmidt-Rhaesa, 2005). In the adult worms, the intestine is almost degenerated (Schmidt-Rhaesa, 2005). The degeneration of the intestine not only support the hypothesis that the horsehair worms emerges from the hosts as non-trophic adult (Schmidt-Rhaesa, 2005), but also suggests the juvenile might digest the nutrition through intestine. However, Schmidt-Rhaesa (2005) in the same report

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also suggested the alternative possibility that the horsehair worm directly absorb host nutrition from the "larval cuticle", which covers the juvenile and is much thinner than the fibrous adult cuticle. If the juvenile holding up the resource in the host body flow, the reduction of fat and reproductive organ of host might be not caused by the parasite consumes it but due to the loss of energy or altered development.

No matter how a horsehair worm intake the resource from the host, several reports suggest the infected host suffers the destruction of gonad tissues (Wülker, 1964; Lafferty and Kuris, 2009; Chiu *et al.*, 2015). This effect on the host also makes the horsehair worm to be categorized as the parasitoids with "prior castration" (Lafferty and Kuris, 2009). This trophic strategy castrating the host is frequently seen in the parasitic helminths and insects (Wülker, 1964; Baudoin, 1975; Hurd, 2001; Lafferty and Kuris, 2009) whose body size is larger than the usual parasites (Lafferty and Kuris, 2009). It is believed that the rapid growth of these parasites until almost occupied all the host body cavities is supported by the intensive exploitation of host resource, and thus promotes them consumes the energy invested in host reproductive system to insure their survival, both hosts and parasites (Obrebski, 1975; Lafferty and Kuris, 2009). The effort to maintain the host survival is the common adaptation of every parasite, but castrating a semelparous host will deprive it of the heredity contribution to its own population, which is equal to evolutionarily kill it. It is the reason why many of the parasitic castrators finally switch to be a parasitoid and kill the "snatched body". In the parasitism of the horsehair worm, the parasite adopts a unique way to kill and leave the host: forcing the suicide behavior.

Adult horsehair worms reproduce in the water but they are unable to move themselves on the ground. The only way is emerging from the terrestrial host fallen in the water. Parasite-manipulated host behavior is the hot spot of the horsehair worm's

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study. It has been noted for more than one century (Heinze, 1941 reviewed in Schmidt-Rhaesa and Ehrmann, 2001) but has been systematically researched in the recent 10 years. The "suicide behavior" in the horsehair worm-infected host has been frequently reported as field observation until the first control experiment was conducted by Thomas *et al.* (2002). It has long been believed that the horsehair worms cause the hosts thirsty and promote them to approach water (Schmidt-Rhaesa and Ehrmann, 2001), but the behavior of "searching the water" was not supported in the Y-maze (Thomas *et al.*, 2002). Thomas *et al.* (2002) found both infected and non-infected crickets were randomly choosing one direction of the Y-maze whatever if the arm contained the trough filled with water or not. The similar behavior was also found in the infected crickets which were brought from their forest habitat to the open area near a pool, while only half of individuals moved toward the pool (Thomas *et al.*, 2002). These two experiments suggested the infected host just moving around instead of searching and heading the water, but this behavior is still abnormal for a cricket since it always quickly hides itself back to the dark area or forest habitat (Thomas *et al.*, 2002). This "erratic behavior" is the reason why the infected crickets are always found in the non-habitat area, like parking area or ground in the house (Thomas *et al.*, 2002), and further suggested as the first step of the host behavior manipulation caused by the horsehair worm (Sánchez *et al.*, 2008b). The second step is to make the infected host to "suicide" when it encounters the water. Thomas *et al.* (2002) in the same Y maze experiment found that all the infected crickets, although they showed no tendency to the direction with water, jumped into the water if they randomly go into the arm with water. Whereas most of the non-infected crickets (11/12) just stopped near the edge of the trough. This phenomenon confirms the "suicide behavior" long spread as a legend. After ten years, Ponton *et al.* (2011) found the tendency of

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approaching reflection of light in the infected crickets, which suggests the "suicide behavior" is likely to be the attraction of light reflected by the water.

The two-step behavior manipulation composed of "erratic behavior", which causes the infected host leaves its habitat, and "suicide behavior", which promotes the host jump into the water, has been generally established. Now scientists are still striving for realizing the physiological mechanism behind these behavior changes. Several physiological changes have been noted. Thomas *et al.* (2003) found the infected crickets displayed abnormal concentration of several amino acids and increasing mitosis in mushroom bodies. Biron *et al.* (2005a) and Biron *et al.* (2006) noted the infected katydids and crickets showed the similar proteomic changes when performing the erratic behavior. These proteins are related to insect's neurogenesis, circadian rhythm and neurotransmitter activities. To date, expression of at least six protein families whose function are related to the physiological processes of insect's central nervous system have been known to change during the behavior manipulation (Libersat *et al.*, 2009). Two of the proteins belonged to Wnt family is likely to be the special case of molecular mimicry since the protein sequences in the horsehair worm is similar to that in insects instead of the phylogenetically close nematodes (Biron *et al.*, 2005a, 2006; Biron and Loxdale, 2013). It is once believed we are nearly to the answer of the molecular cross-talk between horsehair worms and their hosts, but the linkage of these physiological changes to the host behavior is still unclear (Biron and Loxdale, 2013). In addition, more parasitic effects induced by the horsehair worms found in the recent years including the nymphal appearance on the infected female adult crickets (Biron *et al.*, 2005b), morphological allometry and intersexuality in the infected adult mantids (Chiu *et al.*, 2015). The coevolution of the horsehair worms with their definitive hosts has proceeded since early Early Cretaceous (Poinar and

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Buckley, 2006; Bolek *et al.*, 2015) and creates the complex interaction to each other. Many of the old myths have been understood now, but more will be created with the future studies.

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1.4 The studies in this dissertation

There are two main topics discussed in this dissertation: 1) Biodiversity of the horsehair worm in Taiwan and 2) Horsehair worm-induced host developmental manipulation.

Biodiversity is one of the most basic understanding of living organisms. Horsehair worms attract scientists' attention since 19th century, but the related study is lacked in Taiwan. Although they are frequently witnessed to emerge from manitds, the horsehair worm in Taiwan has never been scientifically described until Chiu *et al.* (2011). To date, *Chordodes formosanus* Chiu *et al.*, 2011 is still the first and only one horsehair worm species ever had been reported, despite many evidences suggest several species are still existing in Taiwan. In the following context, five species of the horsehair worms collected from Taiwan are introduced, including the advanced researches of the seasonality and host specificity of the most common species, *C. formosanus*.

Horsehair worm-induced host developmental manipulation is another issue about the interaction of *Chordodes* and its mantid host. The altered developmental process in creating the abnormal morphology on the infected mantid. The abnormal morphology induced by the horsehair worm has ever been reported in early 20th century in the infected katydid (Ebner, 1940 reviewed in Wülker, 1964) and recently reported in an infected mantid (Roy, 2003). In the both cases, the infected host showed the abnormal characters resemble that in the opposite sex which is known as intersexuality (Wülker, 1964). These abnormal characteristics represent the special adaptation evolved in the co-evolution but have not yet been advanced discussed. In the following context, this issue contains the abnormal characteristics found in the *Hierodula* mantids infected by the *Chordodes* horsehair worms, the adaptation

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promoting the evolution of this manipulation, the possible physiological mechanism, and the new evidence to challenge the current understanding of insect sexual differentiation.

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2 Materials and methods

2.1 Description of the horsehair worm's morphology (adult)

Description of the adult horsehair worms was applied for the species identification and the examination of inter- and intra-species morphological variation.

2.1.1 Sample preservation

Adult worms collected directly from the environment (free-living) or induced by water from definitive hosts were killed by hot water (80–90°C). The dead worms were fixed in a 75% alcohol solution for several days and then kept in a 95% alcohol solution to preserve the DNA. The worms in the 95% alcohol solution were preserved under room temperature for future examination.

2.1.2 Morphological examination of adult horsehair worms

The anterior and posterior ends of worms were cut and examined directly under a stereomicroscope. Cuticle of the mid-body was removed for about 1 cm long and cut longitudinally from the lateral side. Soft tissue under the cuticle was removed by dipping the fragment into a 1% KOH solution for 30–180 min under room temperature until the soft tissue becomes semitransparent. The cuticles were washed with a 5% alcohol solution and then placed on a microslide for the observation under a light microscope at a magnification of 40–200×. The cuticle of the mid-body persevered as permanent slides were further dehydrated with a series of 75%, 95%, 100% ethanol solutions, and xylene for 15 min respectively and then fixed in Canada balsam (Entellan®).

The preparation protocol for the observation under SEM followed that of Schmidt-Rhaesa (2002b). Fragments of the anterior end, mid-body, and posterior end of preserved adult and larval specimens were dehydrated with a series of 75%, 95%, and 100% ethanol solutions and then replaced by acetone after using a series of

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alcohol/acetone mixtures of 2:1, 1:1, 1:2, and 0:1. Samples were then critical-point-dried, gold-sputter-coated, and examined under an SEM (JEOL JSM-5600, Tokyo, Japan) at a magnification of 100–15,000×.

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2.2 Description of the horsehair worm's morphology (Egg and larva)

Descriptions of the eggs and larval horsehair worms were applied for the advanced description of the immature stages of the horsehair worms from Taiwan and as the reference for identifying the cysts from field-collected paratenic hosts.

Each couple of adult horsehair worms for laying eggs were reared with 800 ml aerated tap water and under 28–30°C for *Acutogordius* and *Chordodes*, and 16°C for *Gordius*. Males were removed one days after they stick sperm drops on females' tails. Females were kept in the water for laying eggs for 2–4 weeks and then removed. Egg strings (*Chordodes, Acutogordius* and *Gordius*) found on the water bottom or stuck on the substances were reared under the same condition with the adults and change the water for every 4-5 days until hatched.

The live eggs and larvae were examined under a light microscope. Larvae for measurement were first killed by the heat water (90–100°C) and photographed. For SEM, egg strings were first crashed in a microtube fixed by a 75% alcohol solution for 15 min. The series of dehydration for SEM is the same with that of the adult worms by exchanging upper phase of the liquid with the crashed eggs after the centrifugation for few seconds. Samples were then critical-point-dried, gold-sputter-coated, and examined under an SEM (JEOL JSM-5600, Tokyo, Japan) at a magnification of 100–15,000×.

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2.3 DNA amplification and phylogenetic analysis

DNA amplification and phylogenetic analysis is the main evidence applied to justify the conspecific status of the horsehair worms collected from the field in Taiwan.

Genomic DNA of adult and larval horsehair worms was extracted using an ALS Tissue Genomic DNA Extraction Kit (Kaohsiung, Taiwan). A set of universal primers (LCO1490 and HC02198) (Folmer *et al.*, 1994) were applied to amplify and sequence the partial COI sequence. For some of the *Acutogordius* and *Gordius* samples which are not able to be amplified by the universal primer set, new primers were designed from the conserve region of the sequences of *Acutogordius* and *Gordius*, respectively. The new sets of primers are AcCOiF (TGAGCTGCCTTTTTAG) and AcCOiR (TGTATTAATGTTTCGGTC) for amplifying sequences of *Acutogordius*, and GoCOiF-1 (TTAGGAACTGCTTTAAG) and GoCOiR-1 (ATAGGGTCAAAGAAGGAGG) for that of *Gordius*. The PCR with the primer sets were initiated at 95°C for 5 min, and the amplification was conducted for 40 cycles, at 95°C for 1 min, 50 °C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min.

For the phylogenetic analysis, the pairwise genetic distances and a phylogenic tree reconstructed by the Neighbor-joining (NJ) method based on the Kimura 2-parameter model were used. The COI sequences with high sequencing quality were first aligned using CLUSTALX 2.0.10 (Thompson *et al.*, 1997) and the analysis was conducted by using MEGA 6.0 (Tamura *et al.*, 2013). The support for the topology of the NJ tree was estimated by bootstrapping using 1000 replicates.

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2.4 Examination of the cysts in the paratenic hosts

Examination of the cysts in the paratenic hosts was applied for the investigation of the distribution, the species components, and the seasonality of the horsehair worms in Taiwan.

Horsehair worm cysts inside paratenic hosts were examined under a light microscope. The examination protocol for checking snail hosts was followed that of Hanelt *et al.* (2001). The snail shells were removed first and the soft tissue was flatted by two glass slides, then the slides were examined under a light microscope at 200× magnification.

Aquatic insect hosts examined were first cleared tissues by heating the samples in a lactophenol solution (75 ml of lactic acid, 14 ml of acetic acid, 7 ml of phenol, and 4 ml of distilled water; Winterbourn, 2005) or Nesbitt's fluid (40 g of chloral hydrate, 25 ml of distilled water and 2.5 ml of concentrated hydrochloric acid; Walter and Krantz, 2009) for 15–20 min at 60°C or 40°C, respectively. The treated aquatic insect host was flattened between a glass slide and a coverslip and examined under a light microscope. In the observation of the folded larvae in *Acutogordius* and *Gordius* cysts, additional treatment by 1–5% KOH solution for 3–6 hr under room temperature was performed to release the folding.

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2.5 Artificial infection

Artificial infections of the horsehair worms, *Chordodes* spp., to their paratenic hosts, physid snail (*Physa* sp.), larval chironomids (*Chironomus* sp.), and larval mosquitoes (*Aedes albopictus*), and definitive host, mantid (*Hierodula patellifera*) were applied for estimating the egg developmental time of *C. formosanus* and further examination of sexual differentiation and parasitic effect on the mantid hosts.

2.5.1 Mantid rearing

The mantids were reared from the egg. The eggs were collected from the field (Kobe University, Kobe, Japan) or laid by the reared mantids (Miaoli District Agricultural Research and Extension Station, Council of Agricultural, Executive Yuan, Taiwan). Each newly hatched nymph was reared alone in a box under 29–30°C, 50–70% RH. Each mantid was fed by *Drosophila* sp. (1^{st}–5^{th} instar) and *Tenebrio molitor* (6^{th} instar to adult) 3 times a week.

Each mantids were recorded the time of hatching, molting, and eclosion.

2.5.2 Infection of paratenic hosts

The worm larvae for infection were hatched from the egg strings collected from Yamaguchi prefecture, Japan (*Chordodes* sp.) or laid by the female horsehair worms (*C. formosanus*) in Taiwan. The egg strings were reared in aerated tap water under the temperature of 26–28°C. The eggs nearly hatching turn dark in color and were cut into a 2-3 mm piece for the infection.

The infection of physid snail and larval chironomids were used in the further infection of the definitive mantid host. In the infection of the snail, each snail was exposed to an egg piece (*Chordodes* sp.) for 4 nights in a well of the 24–well culture plate. Then all the snails were reared together in aerated tap water and fed with the cabbage. The snails survived for 15–20 days (71 of the 236 infected snails) were

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randomly sampled 23 individuals for examining the infection. Their shells were removed and the soft tissue was flatted by two glass slides before counting the cyst number under light microscope. All these 23 snails were all infected with 153 ± 136 (19–616) cysts. The examined snails were all preserved under –80°C until fed to the mantid host (Bolek *et al.*, 2013b). In the infection of the larval chironomid, each larval chironomid (3rd instar) was exposed to an egg piece (*C. formosanus*) in a well of the 24–well culture plate for two nights. In the three times of the infection, 24 larval chironomids exposed were randomly chosen for checking the infection by **Method 2.4**. Twenty-three of them (infection rate: 95.83%) were infected with 43 ± 33 (0, 12–141) cysts. The remaining larval chironomids were then used for the infection of the mantids.

The infection of the larval mosquitoes were used for estimating the egg developmental time of the horsehair worm (*C. formosanus*). The mosquito eggs were laid by female mosquitoes reared in the Department of Public Health and Parasitology, Chang Gung University and preserved under 4°C until used in the experiment. The larval mosquitoes were hatched one night after the eggs were dipped in the water under room temperature. In each infection, 24 newly hatched larval mosquitoes were isolated individually in each well of the 24–well culture plate and exposed to an egg piece of horsehair worm contained 623.94 ± 102.12 (413–833) eggs. The infection were conducted for 12 times with the horsehair worm eggs developing for different days respectively (18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, and 59 days) from being laid and replicated for twice. The larval mosquitoes (both dead and live individuals) were flatted by a glass slide and a coverslip and count the cysts under a light microscope. The motility rate and infection rate of the hosts at 24 hours post exposure were recorded.

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2.5.3 Infection of definitive hosts

Infection of definitive hosts was applied for examining parasitic effects of the horsehair worms.

The infected snails preserved under –80°C harboring cysts of *Chordodes* sp. were used to infect the mantid host (hatched from the ootheca collected from Yamaguchi prefecture, Japan). The infected snails were separated into 2–3 pieces which contained 65 ± 60 (6–308) cysts in a piece. Each piece of the infected snail was mixed with the fat body of *T. molitor* and touched the mouth part of a mantid until the mantid consumed all the snail part. The infected larval chironomids with the cysts of *C. formosanus* inside (two chironomids in each infection contained 85 ± 65 (0, 24–282)) were directly mixed with the fat body of *T. molitor* and fed to the mantids.

The manitds (4^{th} to 8^{th} instar) fed with infected snails or chironomids were all reared to adult and collect the molting skins and the adult antennae for further examination. The infection of the mantids were determined by the presence of juvenile and adult horsehair worms in the mantids dissected.

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2.6 Host morphological examination

Measurements of the host morphologies were applied for comparing the sexual dimorphism, the developmental trajectory in both sexes of the mantids, and the parasitic effect on the mantids hosts, *H. formosana* (field-collected) and *H. patellifera* (reared).

2.6.1 Measurement of wing, leg and pronotum characteristics

Wings, legs and pronota of the field-collected *H. formosana* were removed from specimens and preserved in 75% ethanol solution. The wings were unfolded, flattened and covered by a transparent plastic slide. All dissected parts were photographed using a camera with a scale of 1 mm.

Pronotum and leg lengths were measured according to the description provided by Prete *et al.* (1990, 2002). The pronotum was measured from the rostral-most to the caudal-most midpoint of the prothoracic tergum. The leg was measured from the coxa to the end of the tarsus (the length of the tarsus was not included in the analysis of raptorial legs). The wings were measured according to sclerotized wing venation, as described by Roy (2005). The length of the forewing was measured from the base of the costal vein to the end of the second radial vein, which is the visually longest wing length. The length of the hindwing was measured from the base of the costal vein to the end of the radial posterior vein. The measurements were performed using the segmented line function in ImageJ 1.47 (Rasband, 1997-2016), and calibrated spatially to the scale included in each picture.

The forewing shape index was computed using the following formula: (BR−AR)/(BR+AR), where AR is the area above the radial vein and BR is the area below the radial vein. A high shape index indicates the relatively large area below the radial vein. The area of each part was measured using the polygon selection function

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in ImageJ 1.47.

Detailed methods is described in Chiu *et al.* (2015).

2.6.2 Measurement of antennal characteristics

The antennae were collected and preserved in a 75% alcohol solution before being examined. Each molting skin was preserved in a small tube and rinsed by 75% ethanol solution before being examined.

The comparison of the field-collected *H. formosana* is to examine the sexual dimorphism and parasitic effect on the field-collected mantids. Three antennal characteristics including 1) density of the grooved basiconic sensillum on antennae, 2) the first segment bearing grooved basiconic sensilla, and 3) the total number of antennal segments were examined using SEM (same method in the sample preparation with **Method 2.1–2.2**) or a light microscope. The density of grooved basiconic sensilla was calculated using the average number of sensilla per 25 × 25μm^2 on each selected antennal flagella. One antennal flagellum was selected for every 10 segments from the 10th to 100th segment; thus, 10 antennal segments were selected from each sample. The narrow median strip of each antennal segment was first divided into 16–96 cells of 25 × 25μm^2. The total number of grooved basiconic sensilla in this narrow median strip was counted and then divided by the number of square areas. In addition to the sensillum density, the first segment bearing grooved basiconic sensilla and the total number of flagellum segments were also recorded (samples with incomplete or broken antennae were not included in the analysis of the total number of segments). Detailed methods is described in Chiu *et al.*, (2015).

The comparisons of antennae in different instars of *H. patellifera* which were free from horsehair worm's infection is to examine the developmental trajectories and sexual differentiation in both sexes of the mantids. All the antennae (adults) and the

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antennal part of the molting skin (nymphs) were examined under SEM. For every flagellum segments, we counted number of the large trichoid sensilla, small trichoid sensilla, and groove basiconic sensilla, and measured the length and the wide of each segment. According to these measurements, the total number of large trichoid sensilla, the total number of small trichoid sensilla, the total number of groove basiconic sensilla, the total number of flagellum segment, antennal surface area of each flagellum segment, the first flagellum segment bearing the grooved basiconic sensilla, and the total number of flagellum segment bearing the grooved basiconic sensilla ("the total number of flagellum segment" minus " the first flagellum segment bearing the grooved basiconic sensilla") were compared between the sexes of the adult, the first instar nymph, the 8th instar nymph, and the 9th (last) instar nymph. The antennal surface area ("the length" times "the wide") of each flagellum segment were also compared between the sexes of the adult, the first instar nymph, and the 9th (last) instar nymph. According to the first flagellum segment bearing the grooved basiconic sensilla (BS) and the total number of flagellum segment (TS) in the different sexes, an antenna was separated into three zones: male BS to female BS (Zone1), female BS to female TS (Zone2), and female TS to male TS (Zone3) (see chapter 4.3.1, Table 2). Total number of the grooved basiconic sensilla on each of these three zones were compared between the sexes of adult mantids. The antennal surface area of the Zone2 was also compared between the sexes of the adult, the first instar nymph, and the 9th (last) instar nymph.

The examination of the antennae on the male adult mantids artificially infected by *Chordodes* sp. was to investigate the relationship of the host morphological change and horsehair worm's developmental time. The antennae were examined by a light microscope and recorded the first flagellum segment bearing more than three grooved

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basiconic sensilla. This value was first compared between the infected mantids (n = 26) and the control mantids (n = 11). The values of the infected male adults were separated into "manipulated group" (n = 13) which is outside of two standard deviations of the control mantids and "non-manipulated group" (n = 10) which is inside of one standard deviation of the control ones. These two categories were coded as 0 (Non-manipulated group) and 1 (Manipulated group) and analyzed by logistic regression model against the horsehair worm's developmental time before the last molting of the host to get the time when 50% and 90% of the infected mantids showed the manipulated antennal characteristic.

The examinations of antennal characteristics in the infected hosts of the adult female and the last instar male were to test the hypothesis of the juvenilization. The antennae of the adult female examined were collected from the female adults artificially infected by *C. formosanus*. The antennal part of the molting skin of the last instar male examined were collected from the control mantids and the mantids infected by *Chordodes* sp. All the infected mantids harbored the horsehair worms more than 30 days (when 50% of the infected host start to show the manipulated morphology, see chapter 4.3.2). The first flagellum segment bearing more than three grooved basiconic sensilla were applied to compare the difference between 1) the infected and control host of the last instar male, 2) the infected and control host of the adult female, and 3) the infected adult female and its last instar nymph.

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3 Biodiversity of the horsehair worm in Taiwan

3.1 Phylogenetic relationships of phylum Nematomorpha

Phylum Nematomorpha, commonly known as horsehair worm or gordiid, is now generally accepted as a monophyletic taxa (Hanelt *et al.*, 2005; Schmidt-Rhaesa, 2012; Bolek *et al.*, 2015). Before applying the molecular data in restructuring the phylogeny, Nematomorpha was considered as a subtaxon of (phylum) Nemathelminthes which is composed of Gastrotricha, Nematoda, Nematomorpha, Rotifera, Acanthocephala, Kinorhyncha, Loricifera and Priapulida (Ehlers *et al.*, 1996). Now these pseudocoelomate groups in Nemathelminthes are thought to be less phylogenetically related and Nematomorpha is now re-grouped by the data of 18s rDNA in the taxon Ecdysozoa, which is composed of the molting animals including Arthropoda, Tactopoda, Onychophora, Nematoda, Nematomorpha, Kinorhyncha, and Priapulida (Aguinaldo *et al.*, 1997).

Nematoda is the most closed taxon of Nematomorpha. Their sister group relationship is supported by both morphological (Ehlers *et al.*, 1996) and molecular data (Auinaldo *et al.*, 1997; Bleidorn *et al.*, 2002; Petrov and Vladychenskaya, 2005, but see Sørensen *et al.*, 2008). They are similar in the body shape, the presence of longitudinal dorsal and ventral epidermal cords with unpaired nerve cords, the absence of ring musculature, the absence of protonephridia and the cloaca in males (Schmidt-Rhaesa, 2012). This similar morphologies make the horsehair worms to be originally put together especially with its ecologically similar Mermithida, but several considerable differences still indicate the monophyly of Nematomorpha. These autapomorphic characteristics, including the degenerated intestine (Schmidt-Rhaesa, 2005), the subpharyngeal brain, the reduction of pharyngeal musculature, the distinctive larva (Schmidt-Rhaesa, 1996 reviewed in Bleidorn *et al.*, 2002), the

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generally dark cuticle, the round or bilobed end (pointed in mermithids), the smaller egg size in the adult females (Schmidt-Rhaesa, 2012) suggest Nematomorpha and Nematoda are sister groups united in a taxon Nematoida (Schmidt-Rhaesa, 1998 reviewed in Schmidt-Rhaesa, 2012). Such debate has been raised since mid 19th century (Meissner, 1856 reviewed in Schmidt-Rhaesa, 2012), and then further confirmed and generally accepted by the modern molecular studies (Auinaldo *et al.*, 1997; Bleidorn *et al.*, 2002; Petrov and Vladychenskaya, 2005).

Among Nematomorpha, more than 350 species belonged to 21 genera (including two extinct genera, *Cretachordodes* and *Paleochordodes*) have been decribed (Poinar, 2008; Bolek *et al.*, 2015). Two main taxa among the 19 extant genera are freshwater hairworms (Gordiaceae) and marine hairworms (Nectonematoidea) (Bleidorn *et al.*, 2002; Poinar, 2008). The marine hairworms are composed of 5 known species of genus *Nectonema* which mainly parasitize with marine crustaceans such as hermit crabs or crabs (Oku *et al.*, 1983; Poinar and Brockerhoff, 2001; McDermott *et al.*, 2010). Their life cycle is hypothesized to be different from the typical life cycle of freshwater hairworms since the larval horsehair worms might directly parasitize the definitive hosts instead of via the paratenic hosts (McDermott *et al.*, 2010).

The freshwater hairworm contains most species of Nematomorpha. Most of them adopt the typical complex life cycle with the aquatic paratenic host and the terrestrial definitive host, while few species might directly parasitize their aquatic definitive hosts (Schmidt-Rhaesa and Kristensen, 2006) as that in the marine hairworm. These 18 freshwater genera were once classified as four families: Gordiidae, Spinochordodidae, Lanochordodidae, and Chordodidae (Bleidorn *et al.*, 2002), but only two (Gordiidae, Chordodidae) are accepted in the recent studies (Poinar, 2008). The taxon Gordiidae contains only two genera (*Gordius* and *Acutogordius*) which

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share the postcloacal crescent on the male tail as the autapomorphy. The left 16 genera belong to Chordodidae were morphologically classified into three subfamilies Chorclodinae (*Chordodes*, *Euchordodes*, *Lanochordodes*, *Neochordodes*, *Noteochordodes*, *Pseudochordodes*, *Spinochordodes*), Paragordiinae (*Paragordius*, *Digordius*, *Progordius*, *Pseudogordius*), Parachordodinae (*Parachordodes*, *Cordionus*, *Beatogordius*, *Paragordiunus*, and *Semigordionus*) (Bleidorn *et al.*, 2002; Schmidt-Rhaesa, 2002, 2012), but few studies has conducted to further examine this suggestion.

After the last genus (*Noteochordodes*) was suggested in 2000 (Schmidt-Rhaesa, 2002a), the 19 genera of Nematomorpha are generally accepted and not changed in the recent years. Whereas, more than 50 new species have been newly described in recent decades since the new method applied, especially the sequencing data. The monotonous morphology of the horsehair worms causes two main challenges in the morphological identification: 1) finding a stable diagnosis characters and 2) setting a cutting point at the boundary of intra- and inter-species variation. Body surface of the horsehair worm is usually smooth which lacks diagnostic characteristics in many horsehair worm species. The male tail is relatively complex and frequently used in the classification (Schmidt-Rhaesa, 2002a), but it is still hard to distinguish all the 350 species by the few cuticlar characteristics. Application of scanning electronic microscope (SEM) since 1970s (Chandler and Wells, 1989) solved part of this problem and currently becomes the standard method (Schmidt-Rhaesa, 2001). In addition, the recent molecular studies further challenge the current morphological identification by exposing the cryptic species among the samples with similar diagnostic characteristics (Bolek *et al.*, 2015; Hanelt *et al.*, 2015).

Despite the new methods solve the problem of finding diagnostic characteristics,

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lack of information in the morphological variation makes the difficulty in considering a morphological difference as intra- or inter-species variation. This problem mainly comes from the lack of reliable ways of collecting adult horsehair worms and their relatively short life span in the free-living adult stage (Bolek *et al.*, 2015). In addition, damages to the cuticle from the environment and different developmental condition in the definitive host are two possible factors to cause morphological variation among conspecific individuals. In our previous study (Chiu *et al.*, 2011), the 39 adult horsehair worms collected inside *Hierodula* mantids showed the slightly difference in their COi sequences which indicates the conspecific status among these samples. However, the characteristics are various among these examined individuals. The flat ornamentations or smooth cuticle on the head might be resulted from the damage from the environment or covering of the larval cuticle (Chiu *et al.*, 2011; Schmidt-Rhaesa, 2012). Other morphological differences including the short, unbranched or thin bristles in the bristlefields, the lack of thorn areole (Chiu *et al.*, 2011), and the lack of bristlefields and extreme small crowned areoles in the samples from novel hosts (Appendix 4), might respectively separate the samples into several different species.

The use of molecular data, as the application of SEM, is now improving the understanding of horsehair worm's phylogenetic relationship, despite more sequence data from each described and unknown species are necessary and it has not yet seen the technique to amplify the DNA sequence from the cysts inside the paratenic hosts. It also influences my strategy in the taxonomic study that in the following context, I basically judge the conspecific status of the samples collected from Taiwan, and describe their morphologies to compare their phylogenetic relationship with the described species and the possible morphological variation.

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3.2 Five known species of the horsehair worm in Taiwan

The main island of Taiwan is composed of two biogeographic realms: The Indomalayan realm in lowland and the Palearctic realm in high mountain (Amorosi, 1989). Fourteen genera of the freshwater horsehair worms have ever been found in these two biogeographic realms including *Acutogordius* (both), *Beatogordius* (Palearctic), *Chordodes* (both), *Digordius* (Palearctic), *Euchordodes* (both), *Gordionus* (both), *Gordius* (both), *Lanochordodes* (Palearctic), *Parachordodes* (Palearctic), *Paragordionus* (Palearctic), *Paragordius* (both), *Progordius* (Palearctic), *Semigordionus* (Palearctic), *Spinochordodes* (Palearctic) (Schmidt-Rhaesa, 2012). According to our collection, at least five species of freshwater horsehair worms live in Taiwan. They are *Chordodes formosanus*, *Acutogordius formosanus* n. sp., and *Gordius* sp. collected by the free living adult stage and one unknown species collected by only the cysts in aquatic hosts.

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Chordodes formosanus Chiu, 2011

Distribution: **Taiwan:** Xindian, Shimen (New Taipei City), Taipei Zoo (Taipei City), Lujiaokeng Ecological Protected Area in Yangmingshan National Park, (Taipei City), Jiaushi (Yilian County), Taroko National Park (Hualien County), (Hsinchu City, Taiwan), Lyudao (Taitung County); **Japan:** Sakado (Saitama), Kijo, Miyazaki (Miyazaki) (Chiu *et al.*, 2011; Chiu *et al.*, 2016a; Appendix 4); Other locality where the worms have ever been seen: Shihtoushan (Miaoli County, Taiwan), Hengchun Township, Neipu (Pingtung County, Taiwan), the Kobe University (Kobe City, Japan).

Host: *Hierodula formosana*, *Hier. patellifera* (Mantidae: Mantodea) (Chiu *et al.*, 2011, 2016); *Acromantis japonica* (Hymenopodidae: Mantodea), *Holochlora japonica*, *Leptoteratura* sp. (Tettigoniidae: Orthoptera) (Appendix 4).

Adult morphology (Fig. 1): Body length of *C. formosanus* 43–440 mm, 0.7–1.5 mm in wide (widest); male generally longer than female. Cuticle usually dark-brown (but one white individual has ever been seen) with bright lengthwise regions on both dorsal and ventral sides; a darkly pigmented line on ventral side in most specimens. Some females brown in color with dark patches.

Anterior end tapered, same color as body with a white tip. Anterior end round with moderately flat areoles and short bristles on surface. Anterior surface smooth in some individuals.

Posterior end not lobed, slightly narrowed in male (Fig. 1E) but round and slightly swollen in female (Fig. 1D). Cloacal opening oval, located subterminal with circumcloacal spines in male (Fig. 1E) and on terminal end of female (Fig. 1D). Male tail structured by a pair of oval regions without areoles posterior to cloacal opening, and paired oval bristlefields bearing bristles on the lateral side of cloacal opening; only few bristles scattered on around the cloacal opening (Fig. 1E). Bristles

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in the bristlefields generally branched or unbranched in most individuals; shorter or thinner unbranched bristles also found in few individuals; one sample from host, *A. japonica*, without paired oval bristlefields.

Entire body covered by six types of areoles (simple, tubercle, thorn, circumcluster, and two types of crowned areoles) (Fig. 1A, B). Simple areoles generally smooth on the surface but some with dots, grooves, or short bristles; most abundant covering most of body surface except anterior end and ventral side of posterior end. Some of the simple areoles significantly elevated in clusters of two to ten, and darker under light microscopy. Tubercle areoles and thorn areoles scattered among simple areoles, similar in shape but with a tubercle or a solid thorn, respectively, on latter or top of areoles. Thorn areoles much less abundant than tubercle areoles and absent in some samples. Solid thorn in the male thorn areoles longer than that in female. Crowned areoles (Fig. 1A, B, C, E, F) clustered in pair with a central tubercle in between and surrounded by 12–20 circumcluster areoles with short filaments on the apical surface. Clusters of crowned areoles and circumcluster areoles scattered over trunk except anterior and posterior ends. Each crowned areole structured by filaments originating from apical center and sidelong to edges. The apical filaments generally around 10–15 μm in length (short-crowned areoles), but crowned areoles arranged in two lines on ventral and dorsal midlines of the female structured with significantly longer apical filaments which usually longer than 200 μm (65.57–392.25 μm) (long-crowned areoles) (Fig. 1C). Long-crowned areoles bright in color in the alive female and makes two white lines on ventral and dorsal surface. Abnormally smaller crowned areoles found on one male sample (Fig. 1G) from the host, *A. japonica*, extreme smaller than crowned areoles on other individuals (Fig. 1F).

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Immature stage (Fig. 2, 3): Egg strings stuck onto substrate or drifting on bottom. Eggs nearly circular, around 30 μm in diameter. Egg strings white when laid and becoming light-brown within one day, turning dark-gray just before hatching. Egg strings collected in field stuck onto a rock (Fig. 3G), mostly brown to gray, or white as those just laid in laboratory.

Larvae remained near egg strings after hatching, not active. Some larvae leave the eggs and accumulated on the water bottom. Larva nearly 50 μm in length which composed of preseptum, postseptum, and ectodermal septum between them (Fig. 2A). Length of the preseptum and the postseptum nearly the same. A proboscis in the preseptum around 11 μm in length; movable when the larva is alive. A V-shaped pseudointestines in the postseptum visible under the light microscope. Under SEM, larvae superficially annulated. Three sets of hooks arranged in three rings on anterior preseptum. The outer and second ring relatively large; visible under the light microscope. Outer ring contains five single hook and one ventral double hook; six hooks on second ring located between each outer hook; inner ring contains at least three inner spines, but real number unknown. A proboscis appears inside the preseptum, ornamented with two sets of spines: nine spines on dorsal and ventral sides of the proboscis, and five small lateral papillae on left side. A pair of anterior and posterior terminal spines on posterior of the postseptum. Pseudointestine exterior opening centrally located between anterior terminal spines on the ventral body.

Cysts morphologically similar to the larva reared in the laboratory found in the field collected aquatic insects including larval chironomids, adult caddies flies (*Chimarra formosana*), and stoneflies (*Kamimuria* sp.). Larvae encysted almost the same shape with that of free-living stage except V-shaped pseudointestines not found (Fig. 2B). Cyst wall only found in few individuals (Fig. 2B); some larvae dark in

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color (Fig. 2C).

Diagnosis: The conspecific status of the adult described above are suggested by their low genetic distances (Chiu *et al.*, 2011, 2016a). The genetic distances among all the 39 samples in Chiu *et al.* (2011), 5 samples in Chiu *et al.* (2016a), and five samples from the "novel hosts" (see below) are from 0 to 0.019, which is significantly smaller than 0.168, the genetic distances between *C. formosanus* and its morphological similar species, *C. japonensis* (Chiu *et al.*, 2011). The morphology of *C. formosanus* and *C. japonensis* are similar that both of them are structured by the same five types of areoles, but distinct by the sexual dimorphism in *C. formosanus* since the long-crowned areole is present only in the female while that is present in both sexes of *C. japonensis* (Baek, 1993; Schmidt-Rhaesa, 2004; Chiu *et al.*, 2011). This slight difference might makes some *C. formosanus* samples to be ever considered as *C. japonensis* (Schmidt-Rhaesa, 2004).

The intra-species morphological variation of *C. formosanus* are first described in Chiu *et al.* (2011) by their head surface, morphology of bristles in the bristlefields, and the present of thorn areoles. In the samples collected from the non-*Hierodula* hosts showed additional differences included the absence, or smaller size of the bristlefields and the smaller size of the paired crowned areoles which makes them similar to their surrounded circumcluster areoles (Appendix 4). I believe these two morphological differences are obvious enough to separate these samples into several species but it is conflicted to the result of molecular data. Despite the phylogeny restructured by molecular data can be also misled (e.g. biased model or method (Yang and Rannala, 2012)), I still tend to accept the result suggested by the molecular data since there are still a few studies mentioned about the morphological variation of horsehair worms (Schmidt-Rhaesa and Geraci, 2006; Chiu *et al.*, 2011).

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The immature stages of *C. formosanus* are morphological similar to the previous description of *Chordodes* including the egg strings stuck on substances, the similar length of the preseptum and the postseptum, the V-shaped pseudointestine, and the unfolded encysted larva (Hanelt and Janovy, 2002; Szmygiel *et al.*, 2014), but the cyst wall is usually absent in the encysted larvae of *C. formosanus*. The cysts collected from the field were judged as *C. formosanus* by the similar morphology with larvae reared in the laboratory and the adult *C. formosanus* collected near the habitat of the infected paratenic hosts. Since there is no technique to amply the DNA sequence from the cyst inside paratenic hosts, the species status of these cysts are not supported by the molecular data. With the lack of morphological and molecular information, the species status of the cysts are also referred to the adult horsehair worms collected in the same river (Poinar *et al.*, 2004). It might be some cryptic species in the cysts with similar morphology, but the survey of the cysts can still provide the valuable field information of the horsehair worms (see below).

Collecting experience (Fig. 3): *Chordodes formosanus* is the horsehair worm most frequently seen in the low altitude in Taiwan. Before it is scientifically described, local residents in Taiwan have already know the animal emerging from the "large green mantid". The "large green mantid" means its main host, *H. formosana* (Fig. 3B, D, E, F) and *H. patellifera* (Fig. 3A). Among these two main hosts, *H. formosana* is much more frequently met due to its significantly larger population than *H. patellifera*. *Hierodula formosana* is endemic to Taiwan whose adult always emerge in shrubs during early summer (Chiu and Wu, 2008). The dead infected mantids are usually found to be killed on the road during the emerge season (Fig. 3B). Mature worms in the alive host is usually visible by the heads slightly emerging around the host anus (Fig. 3A). The free-living adult *C. formosanus* are usually found in the pool which is

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active in the night (Fig. 3C). *Hierodula patellifera* is the additional host of *C. formosanus* in Taiwan and the only known host in Japan. Adults of *H. patellifera* emerge about two months later than *H. formosana* in the both countries. They are also frequently found to be killed on the road during the emerge season but the population is much smaller than that of *H. formosana*.

The adult *C. formosanus* emerging from *A. japonica*, *H. japonica*, *Leptoteratura* sp., and nymphal *H. formosana* are rarely found. These horsehair worms are generally much smaller than that emerging from adults of *Hierodula* hosts. The hosts other than *Hierodula* mantids might be accidently infected since *Chordodes* are usually the parasite of mantids (Schmidt-Rhaesa and Ehrmann, 2001) and the infection in the aquatic paratenic hosts are not significantly increased after the emerge season of these hosts (Chiu *et al.*, 2016a).

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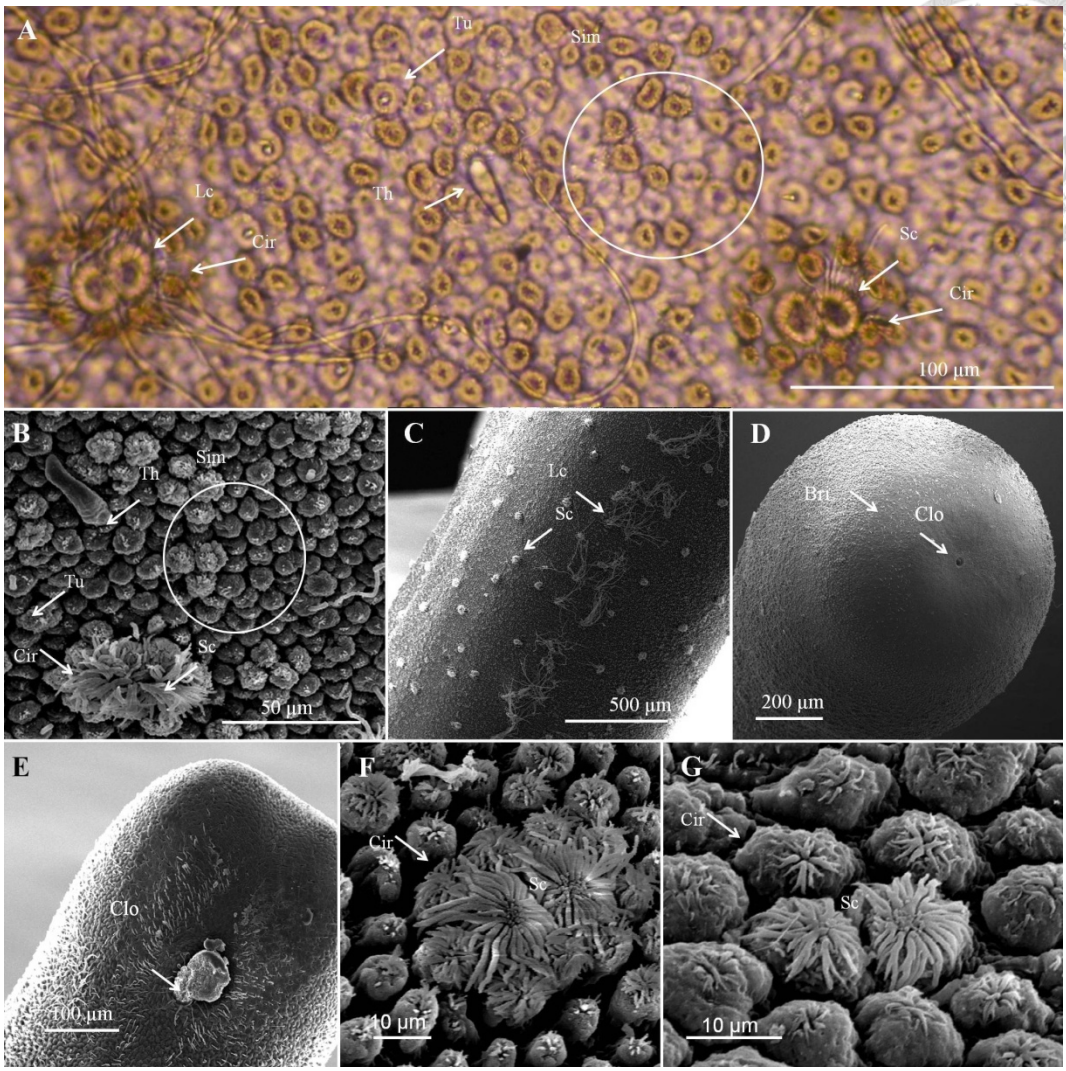

Fig. 1. Adult of *Chordodes formosanus*. (A) Cuticular surface (female) with six types of areole. (B) Cuticular surface (male) with five types of areole under SEM. (C) Ventral side of female body with both types of the crowned areoles. (D) Female tail. (E) Male tail. (F) Close view of the short-crowned areole. (G) Close view of the abnormal crowned areole on an extreme small adult male. Bri, bristle; Cir, circumcluster areole; Clo, cloacal opening; Lc, long-crowned areole; Sc, short-crowned areole; Sim, simple areoles; T, central tubercle; Th, thorn areole; Tu, tubercle areole. (Modified from Chiu *et al.*, 2011)

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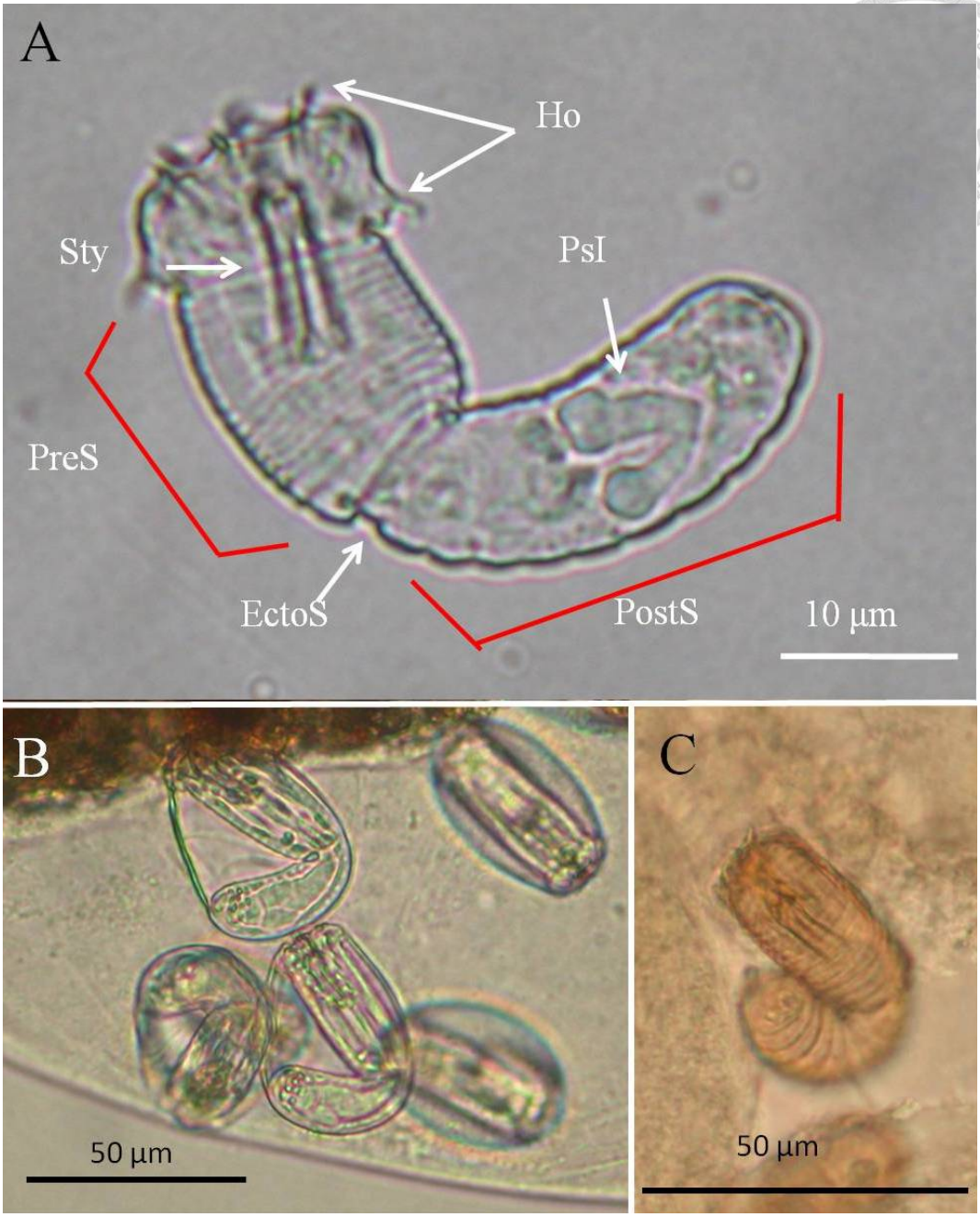

Fig. 2. Larvae and cysts of *Chordodes formosanus*. (A) Newly hatched free-living larva. (B-C) Cysts in the paratenic hosts (larval chironomids) with the general appearance (B) and the cyst attacked by the host immune response (C). EctoS, ectodermal septum; Ho, hooklet; PostS, postseptum; PreS, preseptum; PsI, pseudointestine gland; Rc, residual cuticle; Sty, stylet. (Modified from Chiu *et al.*, 2011 and Chiu *et al.*, 2016a)

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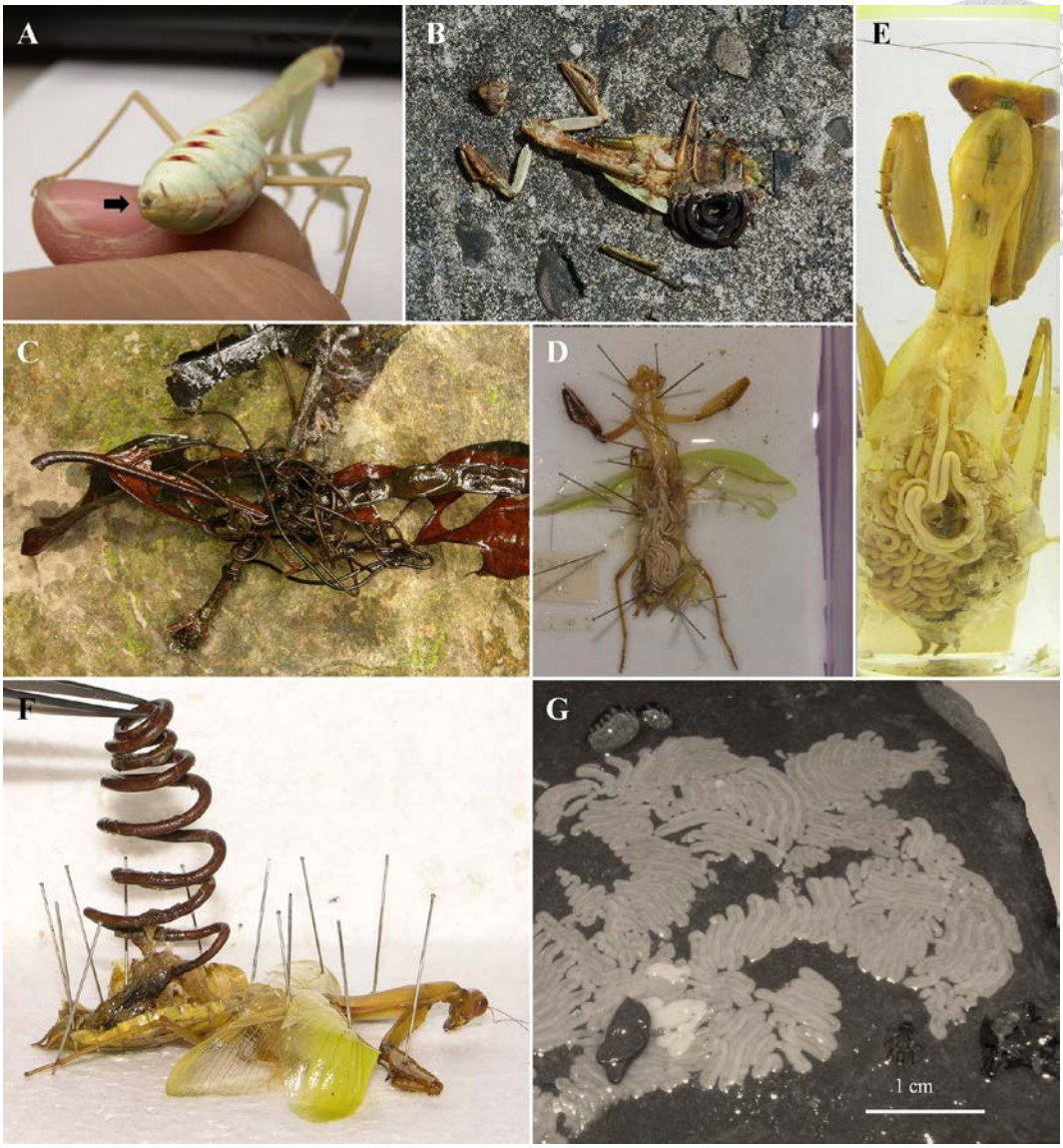

Fig. 3. *Chordodes formosanus* in the field. (A) Mature worm inside a male host, *Hierodula patellifera*, with the visible head (arrow). (B) Dead host, *H. formosana*, and worm on the road which are commonly seen during the reproductive season. (C) The free-living adult males collected from the water. (D-E) Juvenile worms in the body cavities of the host, *H. formosana*. (F) Mature worm inside the host dying on the road. (G) Worm egg strings collected from a rock in the stream. (Modified from Chiu *et al.*, 2011 and Chiu *et al.*, 2015)

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***Acutogordius formosanus* n. sp.**

Distribution: Taiwan: Xindian (New Taipei City), Fushan botanical garden, Jiaushi (Yilian County) (Appendix 4); Lujiaokeng Ecological Protected Area in Yangmingshan National Park, (Taipei City) (Chiu *et al.*, 2016a); Other locality where the worms have ever been seen: Xindian (New Taipei City), Taroko National Park (Hualien County).

Host: *Eugryllacris* sp., *Neanias magnus* (Orthoptera: Gryllacrididae). *Deflorita apicalis*, *Elimaea* sp., *Hexacentrus japonicus*, *H. unicolor*, *Isopsera* sp., *Kuzicus* sp., *Mecopoda elongata*, *Phaulula* sp., *Pyrgocorypha formosana*, *Sinochlora longifissa* (Orthoptera: Tettigoniidae) (Appendix 4).

Adult morphology (Fig. 4): Body length of *A. formosanus* 133–428 mm, 0.4–1.1 mm in wide (widest). All individuals had ever been seen are light-brown (Fig. 4A, B). The body surface is smooth. Cone-like bristles scatter on some samples are visible under SEM (Fig. 4G).

Anterior end columned and slightly narrowed at the tip (Fig. 4B, C); anterior tip white (white cap) and followed by a dark brown collar (Fig. 4B); white spots scatter on the brown collar of few samples. Anterior surface various among individuals under SEM as wrinkled, smooth (Fig. 4C), or smooth but wrinkled on the tip; short bristles scatter on some samples; boundary between white cap and dark brown collar unobvious (Fig. 4C).

Posterior end is divided into two tail lobes in the male (Fig. 4A, F) and columned and round in the female (Fig. 4H). Cloacal opening is oval, located subterminal anterior to the postclocal crescent in the male (Fig. 4D) and on terminal end of the female (Fig. 4H); circumcloacal spines not found. Male tail lobes tapered on tip (Fig. 4A, F, E); tip wrinkled or covered by moderately flat areoles with short spines among

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areoles (Fig. 4E); inner side tail lobe smooth (Fig. 4D); cone-shaped spines or flat areoles scattered on base of some tail lobe behind postclocal crescent. Postclocal crescent located ventrally on base of tail lobes (Fig. 4A, D, F). Postclocal crescent shape various among individuals as slightly curved, almost angled (Fig. 4D, F), and semicircular (Fig. 4A); Semicircular postclocal crescent usually slender than curved and angled ones (Fig. 4A); Postclocal crescents in few samples extend into the tail lobes while most of them are anterior to the starting point of bifurcation of tail lobes. Tiny bristles randomly scatter over ventral side of the male posterior end in most individuals, while that in few males scatter only anterior of postclocal crescent or over the ventral posterior end but concentrative on tail lobes (Fig. 4F).

Immature stage (Fig. 5): Egg strings are generally irregular in shape and deposited as short pieces (4.94–19.13 mm in length) without sticking onto substrate (Fig. 5B); they are white or light yellow in color. Eggs are oval-shaped with 28.79–34.67 μm in length and 24.04–27.71 μm in width.

The newly hatched larvae near eggs preformed "worm form" (Fig. 5A) or "cyst form" as encysted larva in paratenic hosts (Fig. 5C). Worm form larvae (Fig. 5A) around 110 μm in length, postseptum tapering on tip and around 2.5 folds longer than preseptum. Proboscis in the preseptum around 11 μm in length. Pseudointestine in postseptum unequally subdivided oval which around two-thirds length and nearly the same width of the postseptum. Cyst form larvae folded their postseptum forming as oval shape which around 25 μm in length and 17 μm in width; proboscis obvious in worm form larvae with the similar length.

Under SEM (worm form larvae), larvae surface superficially annulated. Ectodermal septum not distinguishable under both SEM and light microscope. Hooks arranged in three rings on anterior preseptum: outer ring contains five single hooks

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and two ventral double hook; middle and inner rings contains six hooks and six inner spines located between each outer hook, respectively. Proboscis inside a sheath appearing inside the preseptum, ornamented with two sets of spines: seven larger spines on each of later sides arranged into two lines except the largest terminal spine; seven smaller spines on dorsal side; no spine found on ventral proboscis. One large single posterior spine located on end of postseptum. Pseudointestine exterior opening on base of large single.

Cysts morphologically similar to the larva reared in the laboratory found in the field collected aquatic insects (larval chironomids (Chiu *et al.*, 2016a), mayflies (*Paraleptophlebia* sp.), and stoneflies (*Kamimuria* sp.)) and snails (*Physa acuta*) (Fig. 5C, D, E); encysted larvae usually folded (Fig. 5C, E) with clear cyst walls (Fig. 5C) or unfolded like the worm form larva (Fig. 5D). Unfolded encysted larvae are characterized by the long postseptum and the tapering posterior tip (Fig. 5D). Folded encysted larvae fold twice in the cyst wall; folding invisible after treated by KOH solution (Fig. 5C); proboscis visible in folded encysted larvae (Fig. 5C, E). Pseudointestine not found in both folded and unfolded encysted larvae.

Diagnosis: The low genetic distances suggest the conspecific status among the 27 examined *Acutogordius* samples. The genetic distance among them was ranged from 0 to 0.0112 (Appendix 4), which is similar to the intraspecific pairwise distances of *Gordius* cf. *robustus* (0.64–2.63%) (Hanelt *et al.*, 2015) and *C. formosanus* (0–1.92%) (Chiu *et al.*, 2011), and lower than the interspecific pairwise distances among species of genus *Gordius* (8.0–24.3%) (Hanelt *et al.*, 2015) and *Chordodes* (16.84%) (Chiu *et al.*, 2011).

Acutogordius formosanus n. sp. is morphologically similar to *A. protectus* Schmidt-Rhaesa and Geracu, 2006 by (1) the distributive pattern of tiny bristles on

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ventral posterior end, (2) moderately flat areoles (rounded elevation) covering tail tips, and (3) cone-shaped spines scatter on the base of tail lobes of the male (Schmidt-Rhaesa and Geraci, 2006), but distinct by small ornamentations on the mid-body of *Acutogordius formosanus* n. sp. The relative location of the postclocal crescent and the tail lobes in the male are traditionally applied to be the diagnostic characteristic in the spices level of *Acutogordius*, but it is various in *Acutogordius formosanus* n. sp. while both postclocal crescent extended into the tail lobes (similar to *A. acuminatus*, *A. feae*, *A. obesus*, and *A. sulawensis*) and that is anterior to the starting point of bifurcation of tail lobes (similar to *A. americanus*, *A. australiensis*, *A. doriae*, *A. incertus*, and *A. protectus*) (Schmidt-Rhaesa and Geraci, 2006) are found. The shape of the postclocal crescent is also various in *Acutogordius formosanus* n. sp., but it might be not only caused by the genetic variation, but also the mechanical factor from environment since most of the males reared in the water for reproduction show the angled postclocal crescent.

The cyst of *Acutogordius formosanus* n. sp. is usually coexisted with that of *C. formosanus* in the low altitude aquatic environment but they are easy to be distinguished. The encysted larvae of *Acutogordius formosanus* n. sp. fold twice in the clear cyst wall have never been seen in the *C. formosanus* and the unfolded encysted larvae are obviously different by the long postseptum and the tapering posterior tip. The cyst of *Gordius* horsehair worm is resembled to that of *Acutogordius* (Hanelt and Janovy, 2002; Szmygiel *et al.*, 2014). Nevertheless, it is still distinguishable in Taiwan since the *Gordius* is only found in the high mountain (the altitude higher than 1200 m) and its larva is much larger than that of *Acutogordius* (see below).

Collecting experience (Fig. 5): The *Acutogordius* horsehair worm is the common species in the low altitude in Taiwan. Comparing with *C. fomosanus*, it is

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less noted, although its population might be similar or even larger than that of *C. fomosanus*. Adult *Acutogordius formosanus* n. sp. is frequently seen in its definitive hosts from mid May to late October, which is much longer than *C. formosanus*, but its population might be sensitive to the human disturbance of the environment (Chiu *et al.*, 2016a) and its definitive hosts are generally nocturnal which are not easy to be encountered. The range of the definitive hosts is wide which might potentially covers most orthopteran insects especially the family Gryllacrididae and Tettigoniidae, but it might be higher specific to the predators such as the raspy crickets. As the *C. formosanus*, heads of adult *Acutogordius formosanus* n. sp. can be found from the posterior tips of the hosts (Fig. 4F) and the juvenile worms are sometimes visible from the translucent abdomen cuticle (Fig. 4G).

In the environment with less human disturbance, cyst of *Acutogordius* is easy to be found in the aquatic insects. In the aquatic insects collected from Taroko National Park (Hualien, Taiwan) in August, 2011, the stoneflies (*Kamimuria* sp.) showed nearly 100% of the infection rate in both naiads (13/13) and adults (28/30), while in the adult mayflies the infection rate is significantly lower (11/140). In the same samples, cysts of *Chordodes* is only occasionally found in the stoneflies and never appeared in the mayflies.

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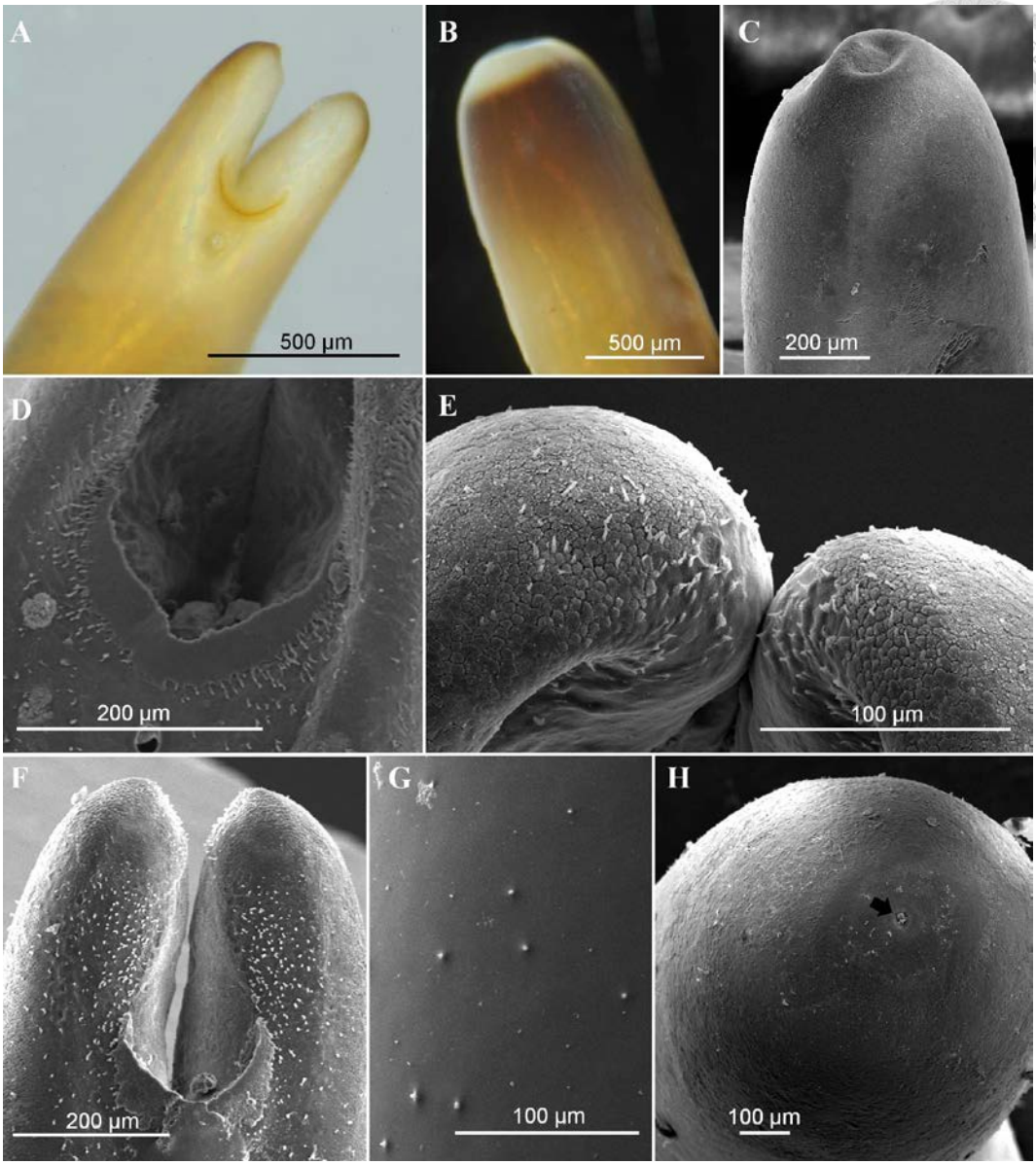

Fig. 4. Adult of *Acutogordius formosanus* n. sp. (A) Ventral view of the male tail. (B-C) Anterior end examined by the stereomicroscope (B) and SEM (C). (D) Close view of the postclocal crescent. (E) Moderately flat areoles on the tip of male tail lobes. (F) Close view of the ventral male tail. (G) Surface of the mid-body with cone-shaped spines. (H) Female tail.

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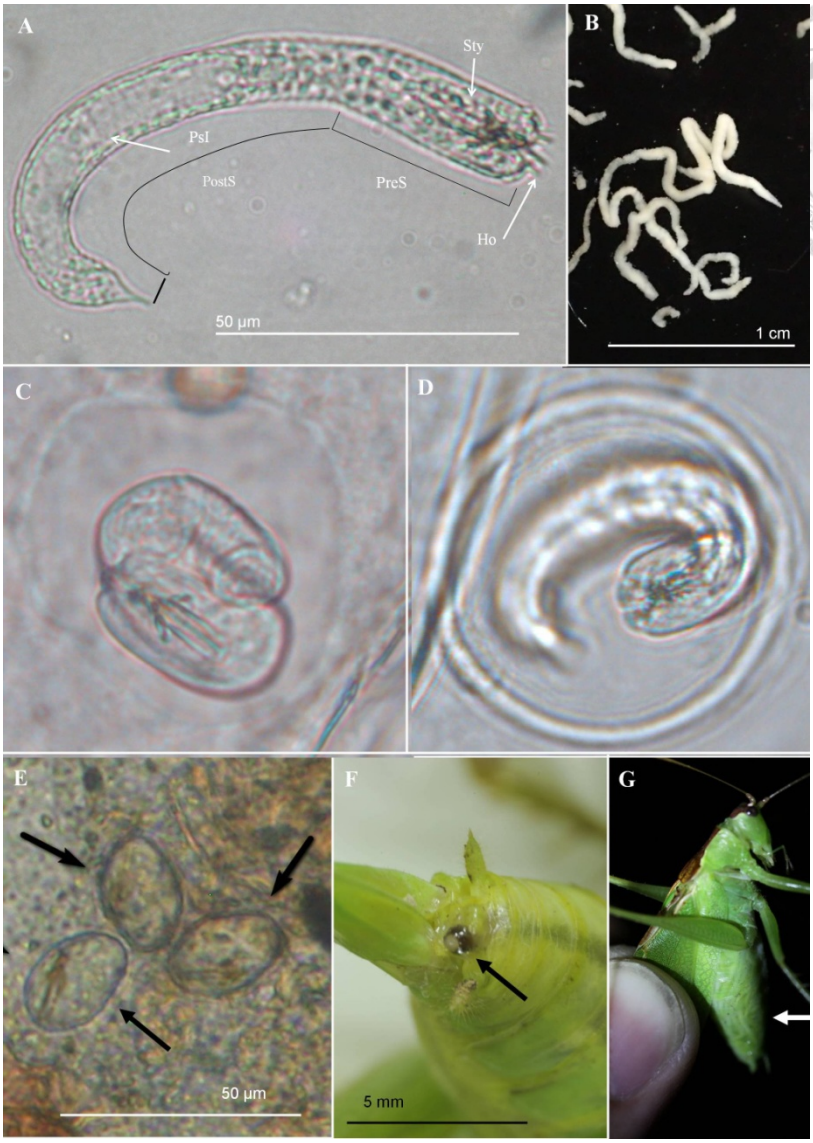

Fig. 5. Immature stage of *Acutogordius formosanus* n. sp. (A) Newly hatched free-living larva. (B) Egg strings. (C-E) Cysts from the field-collected mayflies (C-D) and a snail (E). (F) Mature worm inside a female host, *Hexacentrus japonicus*, with the visible head. (G) Juvenile worms inside a male host, *H. japonicus*, which are visible from the translucent abdomen cuticle. EctoS, ectodermal septum; Ho, hooklet; PostS, postseptum; PreS, preseptum; PsI, pseudointestine gland; Rc, residual cuticle; Sty, stylet.

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***Gordius* sp.**

Distribution: Taiwan: Shizhuo (altitude 1380-1400 msl), Dinghu (altitude 1680-1737 msl), Fenqihu (altitude 1405 msl) (Chiayi County); Xitou forest recreational area (altitude 1150 msl) (Nantou County); Hongshih forest road (altitude 1100-1300 msl) (Taitung County).

Host: Unknown.

Adult morphology (Fig. 6): Body length of *Gordius* sp. usually longer than 600 mm, 0.8-1.5 mm in wide (widest). Most samples dark brown or black in color (Fig. 6H, I); few individuals light-brown cuticle. Body surface smooth and mucous before fixed by alcohol. Cuticle surface scattered by white spots (Fig. 6G) but not found under SEM (Fig. 6E). Cuticle surface generally smooth with short bristles scattering on some samples (Fig. 6E, F).

Anterior end columned and round at the tip (Fig. 6D, H). Anterior tip white (white cap); a dark brown collar presents posterior anterior tip following with a vertical white stripe on the ventral (Fig. 6H). Under SEM, anterior end smooth with short bristles scattering on surface except the tip; boundaries of white cap, dark brown, and vertical white stripe are unobvious (Fig. 6D).

Posterior end divided into two tail lobes in the male (Fig. 6A) and columned, round in the female (Fig. 6C). Cloacal opening round, located subterminal anterior to postclocal crescent in male (Fig. 6A) and on terminal end of female (Fig. 6C); surface of cloacal opening covered by areoles in male (Fig. 6B); Circumcloacal spines not found. Male tail lobes round on tip (Fig. 6A, I); surface smooth with bristles scattering posterior the postclocal crescent (Fig. 6A). Postclocal crescent semicircular, located ventrally on male tail and extends into tail lobes (Fig. 6A, I).

Immature stage (Fig. 7): Egg strings white in color, irregular in shape and

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deposited as short pieces (2.12–13.76 mm in length) without sticking onto substrate (Fig. 7F). Eggs are round with 47.80–58.23 μm in diameter (Fig. 7A–D).

Newly hatched larvae nearly 170 μm in length. Postseptum tapered on tip and around 2.5 folds longer than preseptum. Proboscis in the preseptum around 13 μm in length. Pseudointestine in the postseptum unequally subdivided oval which around two-thirds length and nearly the same width of the postseptum (Fig. 7E).

Cysts morphologically similar to larva reared in the laboratory found in four of ten field collected mayflies, *Ephemera orientalis* (Fig. 7G, H). Larva encysted folds twice in the clear cyst wall; folding visible after treated by KOH solution (Fig. 7H). Proboscis visible in the folded encysted larvae.

Diagnosis: The low genetic distances of partial COI sequences suggest the conspecific status among the *Gordius* specimens collected from Taiwan. The genetic distance among the 5 samples was ranged from 0 to 0.0249, which is similar to the intraspecific pairwise distances of *Gordius* cf. *robustus* (0.64–2.63%), and lower than the interspecific pairwise distances among species of genus *Gordius* (8.0–24.3%) (Hanelt *et al.*, 2015). These samples of *Gordius* sp. display many unique properties. The areoles covering the surface of male cloacal opening has never been described in the known species (Schmidt-Rhaesa, 2012). The white spots which is likely to be the gland opening and the mucus on the body surface might be the first time to be described in the horsehair worm. *Gordius* sp. might be also one of the few horsehair worm able to survive and mate on the wet land instead of in the water, despite the females only laid eggs in the water during being reared in the laboratory. In addition, the horsehair worm reproduces in the winter is also rare among the species already described. These properties might suggest an undescribed species of *Gordius* with unique adaptation to the environment. However, as the less information on the

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morphology of known *Gordius* spices and the small sample size of the samples examined, here I would like to first introduce them to the science and judge the species status after more information is collected.

Collecting experience (Fig. 7, 8): *Gordius* sp. has never been found in the low-altitudes (< altitude 1000 msl) of Taiwan. Its adult emerges from November to February, which is the only known horsehair worm species mainly emerging in the winter. The free-living adult are found to be aggregated not only in the water as most horsehair worm species, but also usually mate on the wet ground after raining or fogging (Fig. 8B, C), and sometimes in the soil (Fig. 8D). The body surface is covered by mucus, which is visible as a misted layer when the live worm was treated by hot water (Fig. 8A). The host has not yet found (maybe millipedes, *Spirobolus* sp.), which is not frequently killed on the road as the host of *C. formosanus* and *Acutogordius formosanus* n. sp.

Egg strings of *Gordius* sp. are similar to that of *A. formosanus* but much wider. It might be caused by the larger body size of the female adult. Eggs are also two times longer in diameter than that of *A. formosanus*. The eggs are laid in the water (despite the adult worms are usually found to mate on the wet ground) and develop for around 60 days from being laid to hatch under 16°C (Fig. 7A–D). The hatched larvae are morphological similar to that of *A. formosanus* but much larger in both length and width. Cysts of *Gordius* sp. have ever been found in naiads of the mayfly, *Ephemera orientalis*. The mayflies are collected in Xitou forest recreational area (altitude 1150 msl), which might be the distribution boundary of *C. formosanus* and *Gordius* sp. since both of their cysts were found.

The field information of *Gordius* sp. in Taiwan is still lacked. Their ecological properties seems to be much different than that of the low-altitude species since its

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free-living adults are able to stay on the ground without being dehydrated. This ability promoted us to test if the adult can reproduce on the wet sphagnum but only mating were observed. More information is still necessary to understand the host and reproduction information of these high-altitude horsehair worms in Taiwan. Despite they might be ecological and phylogenetically closed to the horsehair worms from the Palearctic realm, they might still have several distinct differences in each other.

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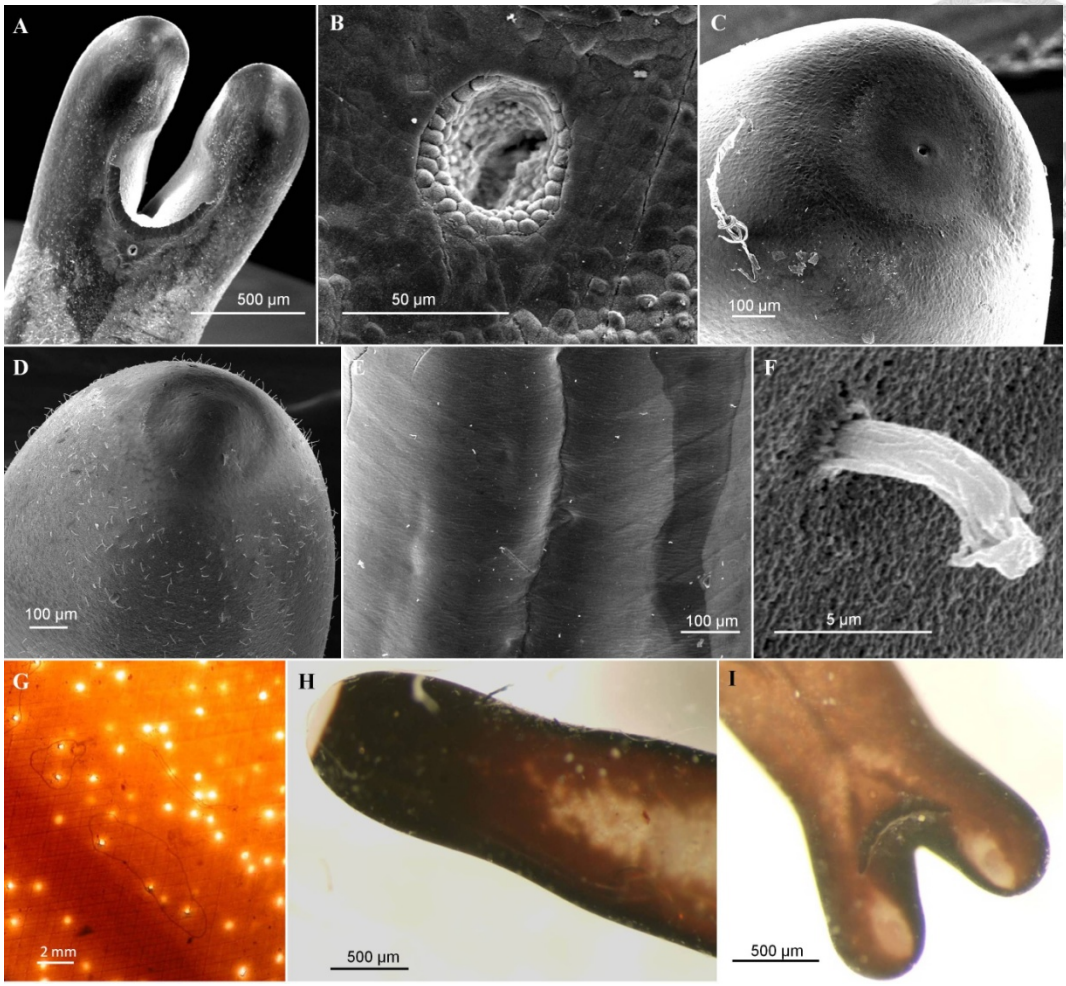

Fig. 6. Adult of *Gordius* sp. (A) Ventral view of the male tail. (B) Close view of the cloacal opening with areoles on the surface of the inner wall. (C) Female tail. (D) Anterior end. (E–G) Surface of the mid-body with small spines (F) and the white spots (G). (H) Anterior end under the stereomicroscope. (I) Ventral view of the male tail under the stereomicroscope.

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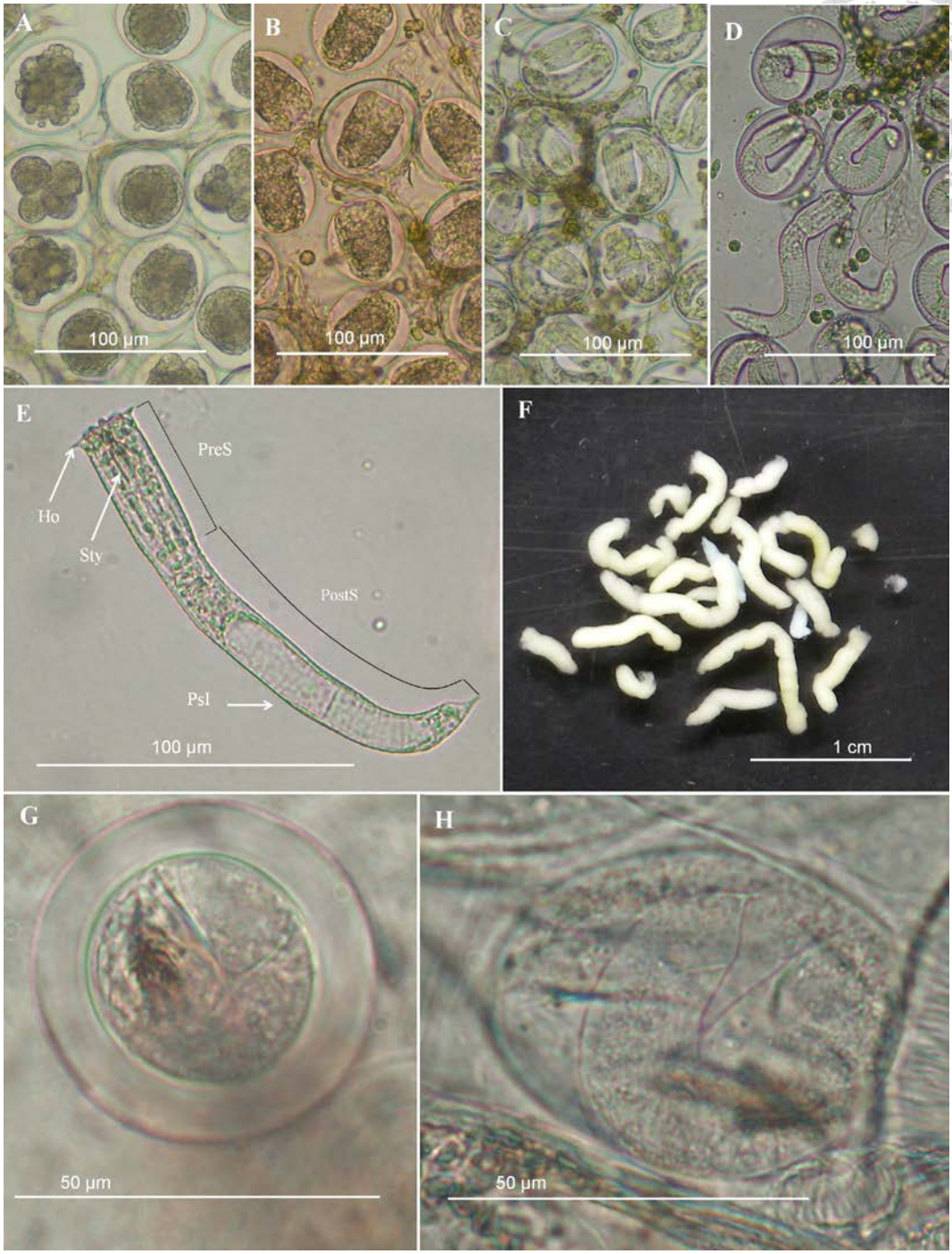

Fig. 7. Immature stage of *Gordius* sp. (A-D) Eggs developing under 16°C for 11 days (A) 18 days (B) 39 days (C), and 49 days (D). (E) Newly hatched free-living larva. (F) Egg strings. (G–H) a cyst inside a paratenic host, *Ephemera orientalis*, with the sample fixed by 70% EtOH (G) and the larva released from the cyst after 6 hr treatment in 5% KOH (H). EctoS, ectodermal septum; Ho, hooklet; PostS, postseptum; PreS, preseptum; PsI, pseudointestine gland; Rc, residual cuticle; Sty, stylet.

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Fig. 8. *Gordius* sp. in the field. (A) Adult worms killing in the hot water with the mucus detached from the body surface. (B) Newly collected adult worms mating on the wet tissue paper. (C) Dead worm on the road. (D) Live adult worm found in the soil of a dry river.

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Undetermined horsehair worm cyst

Distribution: Taiwan: Lujiaokeng Ecological Protected Area in Yangmingshan National Park (Taipei City) (Chiu *et al.*, 2016a)

Host: unknown.

Immature stage (Fig. 9): Six cysts found in five larval chironomids from October to next January. Morphology of these cysts similar to that of *C. fomosanus* but much larger in size. Three cysts with clear images measured length of preseptum (38.5–54.1 μm), postseptum (37.6–49.2 μm), and proboscis (17.5–21.5 μm); Pseudointestine not found.

Diagnosis: The current information of the cyst morphology is only enough to judge these six horsehair worm cysts to be different from the three species described above. Size of the larval horsehair worm has never been applied to be a diagnostic characteristic in distinguishing the horsehair worm species, but the significantly larger size of larval *Gordius* sp. comparing with *A. formosanus* makes it possible to suggest an unknown species according to these six cysts. The similar length of the preseptum to the postseptum, and the lack of clear proboscis might indicate the cysts are belonged to the genus *Chordodes* or *Neochordodes* (Poinar and Doelman, 1974; Hanelt and Janovy, 2002; Szmygiel *et al.*, 2014; Chiu *et al.*, 2016a), but this suggestion needs further examinations.

Collecting experience: Advantages in applying the cyst stage as an indicator for estimating the geographic distribution and species composition has been recently noted (Hanelt *et al.*, 2001; Bolek *et al.*, 2013b). However, the current information of the cyst morphology is not yet enough to be applied in the species identification. Bolek *et al.* (2013a) has successfully identified the cyst species by infecting the field collected cysts to reared crickets, but this techniques are limited by the definitive host

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specificity of the horsehair worm. The cysts appearing in the paratenic hosts collected in the late autumn and winter suggest the possible reproductive season in early autumn (Chiu *et al.*, 2016a). It is still necessary to search the adult worms in the water or definitive hosts during the reproductive season to restructure the detailed biodiversity and host use of the horsehair worm in Taiwan.

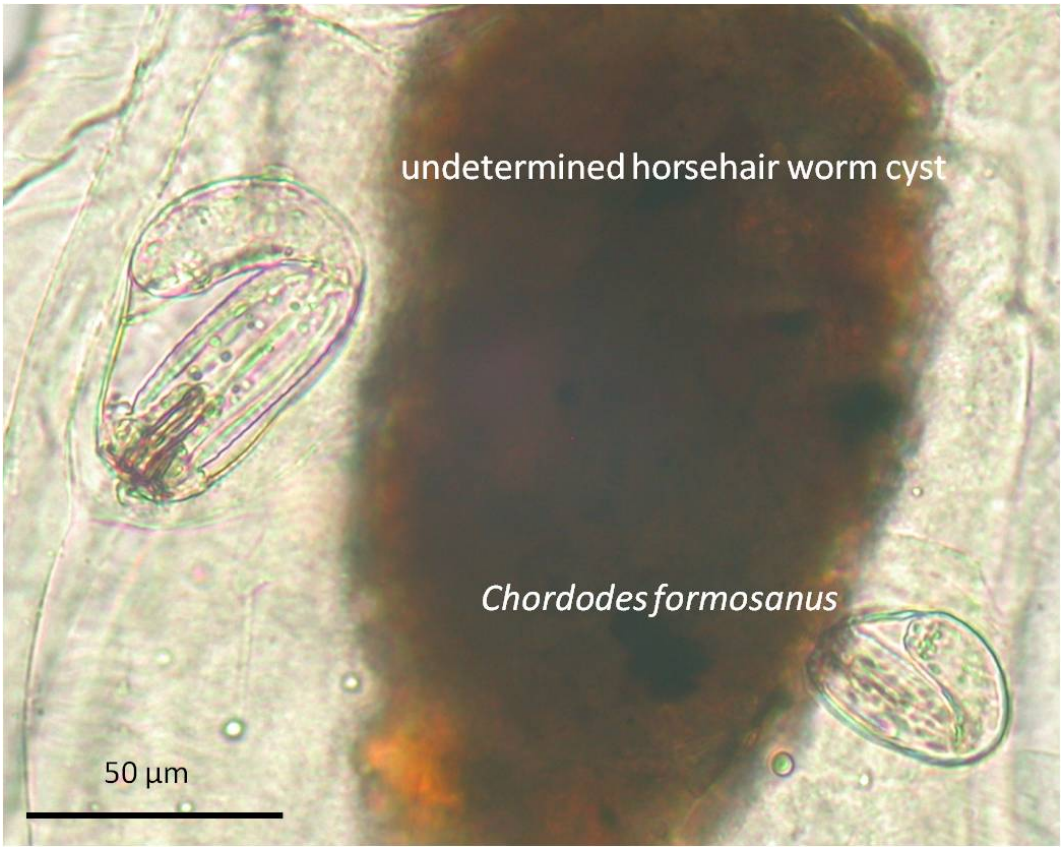

Fig. 9. Cysts of *Chordodes formosanus* and an undetermined nematomorph with larger size in the paratenic host (larval chironomid). (Modified from Chiu *et al.*, 2016a)

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Undetermined horsehair worm from a fresh water fish

Distribution: Taiwan: Wanan river (Pingtung County).

Host: Unknown.

Adult morphology (Fig. 10): Body 751 and 810 mm in length, 0.45 and 0.63 mm in wide (widest), color light brown. Body surface smooth, covered by transparent larval cuticle. Anterior end columned and a hole at the tip, white cap and dark brown collar not found. Posterior end round, cloacal opening not clear.

Immature stage: Unknown.

Diagnosis: The two horsehair worm samples show the similar color and posterior ends with the female *Acutogordius formosanus* n. sp. However, the white cap and dark brown collar were not found on the anterior end. The morphology of these two samples do not match the three known horsehair worms in Taiwan, but the current information is not enough for the species identification.

Collecting experience: The two horsehair worms were collected in a restaurant at Aug. 24th, 2014 instead of directly from the field. Partial of the worm body emerged from a sailfin molly (*Poecilia latipinna*, 37.8 mm in length) accidently fried with river shrimps. The collection information of the fish were informed by the manager of the restaurant. The sailfin molly might accidently ingest the horsehair worms by swallowing their definitive host which fell into the river. The transparent larval cuticle coving the whole worm body suggests the definitive host was digested before the worms emerged. The species of these two horsehair worms are not yet determined, but their small size suggests the definitive host is the small insect which emerges in summer.

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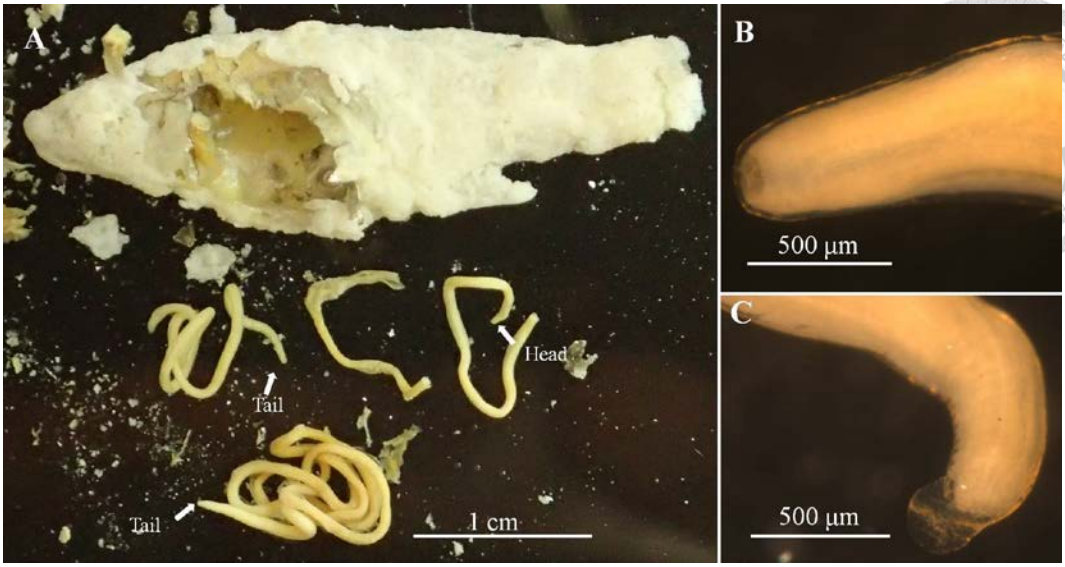

Fig. 10. Two horsehair worms in a fried sailfin molly (*Poecilia latipinna*). **(A)** Whole bodies of the two undetermined horsehair worms and the fried sailfin molly. **(B)** Anterior end of the horsehair worm **(C)** Posterior end of the horsehair worm.

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3.3 Key to the three known horsehair worm species in Taiwan

3.3.1 Adult stage

1 Cloacal opening ventral; posterior end narrowed or lobed……....2 (male)

1' Cloacal opening terminal; posterior end rounded.....4 (female)

2(1) Postcloacal crescent present; posterior end bilobed.....3 (Gordiidae)

2' Postcloacal crescent absent; posterior end not bilobed; cuticle structure complex with crowned areoles; body color usually dark brown; anterior tip white without dark collar ……..…….……..... (Chordodidae) .....……***Chordodes formosanus*** **(male adult)**

3(2) Tail lobes pointed; body color light brown; vertical white stripe absent on ventral side of anterior tip; usually found in the low-altitudes (< 1000 msl).....***Acutogordius formosanus*** **n. sp. (male adult)**

3' Tail lobes round; body color dark to light brown; vertical white stripe present on ventral side of anterior tip; usually found in the mid-altitudes (> 1000 msl).....***Gordius*** **sp. (male adult)**

4(1') Cuticle smooth; anterior tip white with a dark collar.....5

4' Cuticle with areoles; anterior tip white without a dark collar; body color light to dark brown.....***Chordodes formosanus*** **(female adult)**

5(4) Body color light brown; vertical white stripe absent on ventral side of anterior tip; usually found in the low-altitudes (< 1000 msl).....***Acutogordius formosanus*** **n. sp. (female adult)**

5' Body color dark to light brown; vertical white stripe present on ventral side of anterior tip; usually found in the mid-altitudes (> 1000 msl).....***Gordius*** **sp. (female adult)**

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3.3.2 Cyst stage in paratenic hosts

1 Unfolded larvae with postseptum twice longer than preseptum; postseptum tapered on tip; encysted larvae folded twice; cyst wall usually clear.....2

1' Unfolded larvae with postseptum shorter or same length with preseptum; postseptum round on tip; encysted larvae not fold; cyst wall usually not clear.....3

2(1) Proboscis shorter than 12 μm; unfolded larvae shorter than 140 μm.....***Acutogordius formosanus* n. sp.**

2' Proboscis longer than 12 μm; unfolded larvae longer than 140 μm.....***Gordius* sp.**

3(1') Proboscis shorter than 16 μm; unfolded larvae shorter than 70 μm.....***Chordodes formosanus***

3' Proboscis longer than 16 μm; unfolded larvae longer than 70 μm.....**Undetermined horsehair worm cysts ever found**

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3.4 Seasonality and host specificity of *Chordodes formosanus*

The field information is lacked in most of the horsehair worms due to their complex life cycles and difficulty in survey. Thus, species frequently encountered becomes the valuable material in building the model life history of the horsehair worms in the field. *Chordodes formosanus* is the most common horsehair worm species in Taiwan. Before the systematic scientific research, *C. formosanus* is generally known to be the parasite of *Hierodula* mantids which usually emerge in early summer. This legend was first confirmed by the annual survey of the mantids in Taipei Zoo in 2007–2008 (Chiu and Wu, 2008), in which seven mantid species were identified and one *Chordodes* species parasitizing with *H. formosana* and *H. patellifera*. The season of horsehair worm emergence is also confirmed since most of the adult worms were found in the adult *H. formosana* during its reproductive season in the early summer (Chiu and Wu, 2008). These horsehair worms were described as *C. formosanus* in Chiu *et al.* (2011).

The infection of *C. formosanus* only in *Hierodula* mantids suggested the highly host specificity among the sympatric mantids (Chiu and Wu, 2008). It is also the first evidence suggesting the diverged species status from its morphological closed species, *C. japonensis* (Chiu *et al.*, 2011). Comparing with the annual infection rate of the 16.67% and 20% in *H. formosana* and *H. patellifera*, respectively, no worm was found in the 101 samples of the Chinese mantids, *Tenodera sinensis*. Actually, horsehair worms emerging from *Tenodera* have never been found in Taiwan to date. However, the mantids *Tenodera* and *Hierodula* were both recorded to be the definitive hosts of *C. japonensis* (Inoue, 1952, 1955; Schmidt-Rhaesa, 2004). The further examination of the detailed morphology and the barcoding sequences of the horsehair worms in *Tenodera* and *Hierodula* from Taiwan and Japan isolated the new species *C.*

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formosanus from *C. japonensis*, and thus, suggested the definitive host specific to the *Hierodula* mantids in *C. formosanus*, and the *Tenodera* mantids in *C. japonensis* (Chiu *et al.*, 2011).

It has been a long time that I believe the definitive host range of *C. formosanus* is limited to the genus level, until five adult worms inside the mantid, *Acromantis japonica* and two katydids, *Leptoteratura* sp. and *Holochlora japonica*, were identified as *C. formosanus* by comparing the barcoding sequence (Appendix 4). The similar situation is also happened to *C. japonensis*, which has been once found to emerge from a katydid, *Hexacentrus japonicus japonicus* (Inoue, 1955). This result extends the host range of *C. formosanus* to cross different orders of Insecta, but passes some closely related taxa. The newly found hosts of *C. formosanus* are likely to be novel hosts, which are not the main host supporting the parasite population. A parasites does not equally parasitize with all the hosts it potentially can infect (van Klinken, 1999). The three main parasite metrics (prevalence, intensity, and abundance) are commonly applied to quantify the host suitability to a parasite, and use to compare the host specificity level of different parasites (Poulin, 2007). However, in the case of *C. formosanus*, these three parasite metrics among the known hosts are difficult to be measured due to the difficulty in sampling. In addition, the contribution of each host to *C. formosanus* might be not completely represented by these three parameters. One reason is the low or no chance to mate is possible to be caused by the low infection rate. Most species of the horsehair worms prolong their lineage by sexual reproduction (with only one known exception of *Paragordius obamai*) (Hanelt *et al.*, 2012), the horsehair worm cysts developing in the novel hosts might be finally not able to find the mate and have to pay for the cost in the loss of chance to enter the "main hosts" through paratenesis (Hanelt and Janovy, 2004a). Another possible reason

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is the size of the adult horsehair worms, which is highly determined by the host size and intensity, is the main factor to influence the fecundity of a female worm (Hanelt, 2009). The different among individuals can be higher than six folds (Hanelt, 2009) but is not considered in any of the three main infection parameters.

Other than the three parasite metrics commonly used, the seasonal infection of aquatic paratenic hosts provide an indicator in investigating the relative reproductive potential of the adult *C. formosanus* emerging from different hosts. The adult *C. formosanus* from different species of definitive hosts were collected in different seasons ((Chiu *et al.*, 2016b): *Hier. formosana* (late June to early August, and late January), *Hier. patellifera* (September) (Chiu and Wu, 2008; Chiu *et al.*, 2011, 2016a), *Acromantis japonica* (October, and February) (Appendix 4, personal observation), *Holochlora japonica* (November), and *Leptoteratura* sp. (March) (Appendix 4). Most of the adult *C. formosanus* were collected from the adult definitive hosts, except a pair of adult worms collected in late January comes from a nymphal *Hier. formosana*. The phenomenon that adult worms generally emerge from adult definitive hosts meets our artificial infection of *Hier. patellifera*. In our experiment, nine of the ten *Hier. patellifera* released male adult *C. formosanus* around one month after the last molting (32.8 ± 3.86 d (29–42 d)), despite some of the worm entered the host much earlier than others (two individuals developing for 103 and 143 days in the hosts which were infected earlier than the others 82.71 ± 7.52 d but they released the worms 32 and 35 days, respectively after the last molting). There is still one artificially infected host release an adult male worm one day after the last molting, but it is worth to note the possible developmental synchronization of the horsehair worms.

The horsehair worms emerging from these five hosts reproduce in the water and theoretically create intensive infection risks for their aquatic paratenic hosts in the

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different seasons. A regular survey was conducted in 2014 to early 2015 in Northern Taiwan (Chiu *et al.*, 2016a). The larval chironomids (Chironominae: Diptera) were collected and examined the number of *Chordodes* cysts in each host. The short life span of the larval chironomids in aquatic environments provides a good indicator to monitor the amount of the larval horsehair worms in the water. In total, 806 cysts were found during the 25 times sampling and showed a single infection peak in mid-September (Chiu *et al.*, 2016a). The horsehair worm larva is infectious for less than two weeks in the water (Hanelt and Janovy, 2004b). The single infection peak indicates there is one mass reproduction of *Chordodes*, despite the adult worms emerge several times from different definitive hosts during a year.

The seasonal infection in the paratenic host indicates the unequal contribution of the five definitive hosts, with a dominate one, to the parasite's reproduction. Egg of the horsehair worm goes through half to one month of embryogenesis before hatching (Poinar and Doelman, 1974; Zanca *et al.*, 2007; Bolek *et al.*, 2015) and spend 3-15 days to be encysted after enter paratenic hosts (Inoue, 1962; Hanelt and Janovy, 2004b). In the case of *C. formosanus*, the egg spends around 25 days on embryogenesis. In the artificial infection, 623.94 ± 102.12 eggs developing for different days under 28°C were feed to the newly hatched mosquito larvae (*Aedes albopictus*) (Method 5-2). The infection rate, and the mortality rate at 24 hours of the mosquito were almost zero in the eggs developing for less than 21 days, but increased rapidly in the eggs developing for 24 days which indicates the time when larval *C. formosanus* hatches and becomes infectious (Fig. 11). In summary, horsehair worms, after being laid as the egg strings, spend approximately 30 free-living days in the aquatic environment. The closest time is when the adult *C. formosanus* emerges from the adult mantid hosts, *H. formosana* in July, which is around 2–2.5 months before the

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infection peak (Chiu *et al.*, 2016a).

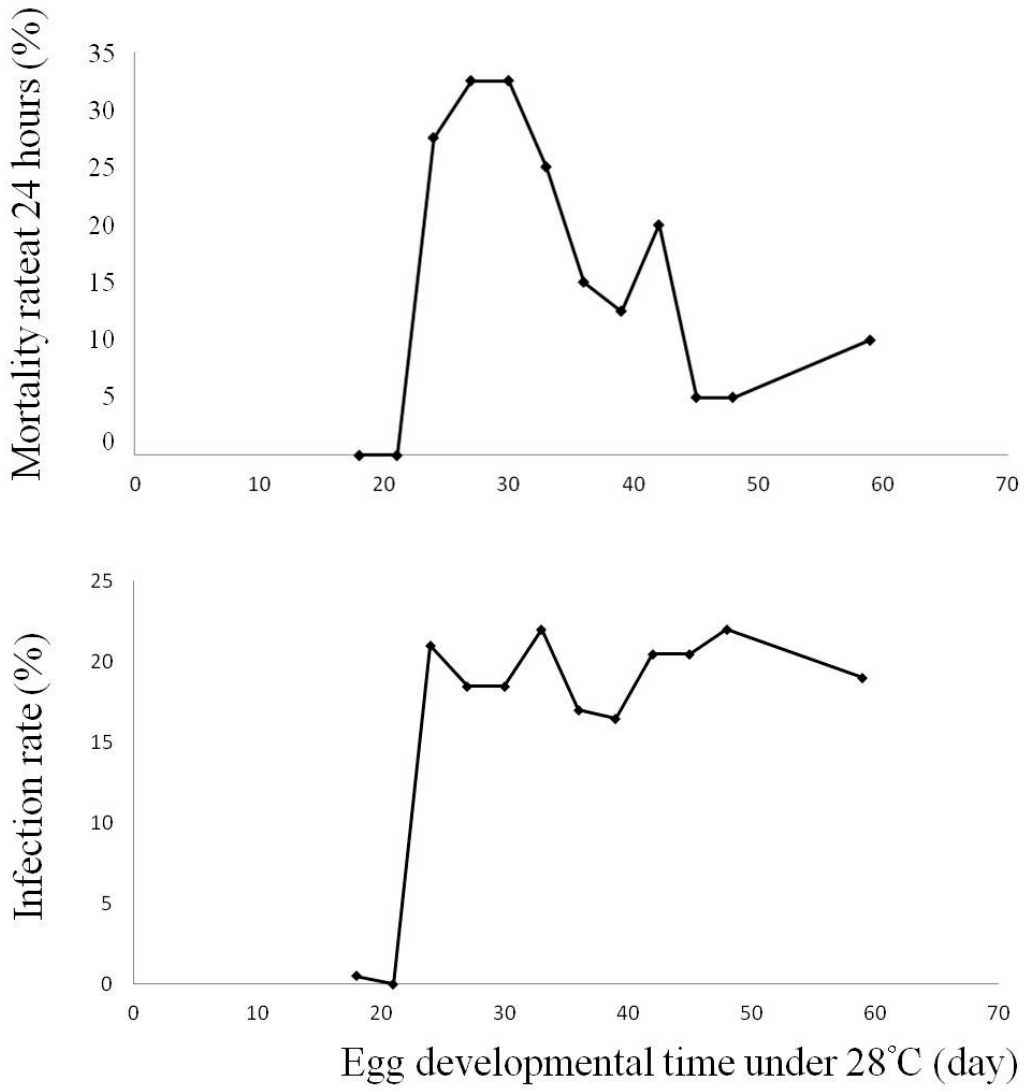

Fig. 11. Mortality rate and infection rate of the 20 newly hatched mosquitoes, *Aedes albopictus*, infected by the egg of *Chordodes formosanus* developing for different days.

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According to both of our collecting experience and the seasonal infection of the paratenic hosts, *H. formosana* is the dominant definitive host of *C. formosanus*. The annual survey of paratenic hosts not only provide the information of geographic distribution (Hanelt *et al.*, 2001) and species composition (Bolek *et al.*, 2013a), but also the seasonality of the horsehair worms (Chiu *et al.*, 2016a). The single mass reproduction of *C. formosanus* creates the high infection risk to the aquatic insects, and these infected aquatic insects will then bring the cysts to the definitive hosts. In Taiwan, *H. formosana* is the endemic species with one generation in each year. Its adults mate and reproduce in early summer and the eggs hatch in mid-August (Chiu and Wu, 2008). The newly hatched nymph will soon face to the highest infection risk of a year in autumn. It is plausible that nymphal mantids are infected by preying on infected aquatic insects, and they release the adult worms in the following summer. However, the real situation seems to be more complicated because the adult worms mature and emerge from nymphal *H. formosanain* spring (Chiu and Wu, 2008). During our laboratory rearing, we found that some horsehair worms parasitizing early-instar mantids matured before the host emerged (unpublished data). This might suggest the existence of 2 generations of a horsehair worm population per year. However, the scale of reproduction in spring is too small to cause the large number of larval worms produced in July. One possible scenario is that most of the cysts are not directly bring to the definitive hosts in autumn. They enter the food web of aquatic animals through paratenesis (Hanelt and Janovy, 2004b) and maintain their infectivity for several months until parasitizing with the definitive hosts (Bolek *et al.*, 2013b). Another reason of the smaller scale of reproduction in spring is the smaller size of the adult horsehair worms emerging from the nymphal host. Length of adult worms emerging from the nymphal *H. formosana* in spring is less than half of that emerging

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from the adult *H. formosana* in summer (Chiu *et al.*, 2011), which makes the millions of difference in the egg number produced by each adult female (Hanelt, 2009).

The mass reproduction and high fecundity of the large adult worms might be the benefit to promote high host specificity of *C. formosanus*. The clear specificity is also noted in *Gordius* sp. by the free-living adults frequently collected in the winter and the cysts found in the aquatic insects collected in March. In the case of *Acutogordius formosanus* n. sp., the seasonality is likely to be relatively unobvious. Adults of *Acutogordius formosanus* n. sp. are frequently found in several orthopteran insects which can be found from mid May to late October. The high infection risk of the aquatic insects might be continued during the whole year. This phenomenon is unfortunately not confirmed in our annual survey in 2014 due to the temporal extinction of *Acutogordius formosanus* n. sp. after the stream remediation (Chiu *et al.*, 2016a). Such temporal local extinction was also found after clear-cut logging in artificial forests (Sato *et al.*, 2014). While cysts of *C. formosanus* were still detected, *Acutogordius formosanus* n. sp. seems to be more sensitive to the human activity.

The life history model of *C. formosanus* is valuable to be the reference of other horsehair worms in Taiwan, and the investigation of the cyst stage provide an efficient tool and makes the large scale survey become possible. Although the identification of species is still relied on the adult stage, the seasonal dynamic of infection in aquatic insects provide the possible time period to collect the adult just emerging from the host. The application of the investigation in immature stage of the horsehair worms is likely to promote the understanding of the ecology of horsehair worms. Since the previous survey in Taiwan is mainly focused on the adult stage, the field information of more species with their horizontal and vertical distribution must to be structured soon by the newly developed method.

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4 Horsehair worm-induced host developmental manipulation

Among more than 350 described horsehair worm species, our understanding of the horsehair worms mainly comes from the studies of a few "model species". The current knowledge of host behavior manipulation mainly comes from the studies in *Paragordius tricuspidatus* and *P. varius* (Thomas *et al.*, 2002; Biron *et al.*, 2005b; Sánchez *et al.*, 2008a, b; Ponton *et al.*, 2011; Barquin *et al.*, 2015), host biochemical physiological change in *P. tricuspidatus* and *Spinochordodes tellinii* (Thomas *et al.*, 2003; Biron *et al.*, 2005a, 2006; Ponton *et al.*, 2006a), influence of the energy flow in riparian ecosystems in *Gordionus* spp. (Sato *et al.*, 2008, 2011), response to predator by *P. tricuspidatus* (Ponton *et al.*, 2006b; Sánchez *et al.*, 2008a), larval ecology in *C. morgani*, *C. nobilii*, *G. robustus*, *P. varius*, and *P. obamai* (Hanelt and Janovy, 2003, 2004a; de Villalobos *et al.*, 2003; Zanca *et al.*, 2007; Achiorno *et al.*, 2009; Bolek *et al.*, 2013b), and artificial infection in *P. varius* and *Chordodes* sp. (Inoue, 1962; Hanelt and Janovy, 2004b). These horsehair worm species are relative easy to be collected and reared in each area, and their influences on the hosts are obvious.

Chordodes formosanus is one of the most common species in Taiwan. The large population size and obvious seasonality makes it easy to be collected and provide a material to restructure the life history model in the field. In the study of the definitive host phase, despite the main definitive host, *Hierodula* mantids, is relative not easy to be cultured, the obvious morphological alternations frequently found on the infected *H. formosana* suggests the possible developmental manipulation induced by *C. formosanus*. In the very beginning of our studies from 2006, we already noted the abnormal wing shape in the infected male *H. formosana* during surveying. The morphological change is obvious to be directly applied in distinguishing the infected male individuals, which is seldom happened in other horsehair worm-infected insects,

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even in another main host, *H. patellifera*. Although this phenomenon has also been found in the mantids, *Tarachodella monticola* (Roy, 2003), this observation open a new series of studies in the parasite-induced host morphological change and the insect sexual differentiation.

4.1 The extended phenotype: Morphological alternation in the infected host

Parasite-infected hosts often display abnormal phenotypes which are not or rarely seen on the non-infected individuals. These phenotypical changes, which is expressed from parasite genotype but displays on the host (extended phenotype in Dawkins, 1982), visualizes the adaptation of the parasites by creating the new function on the manipulated structures or as the symptom showing the host physiological change (Poulin and Thomas, 1999). Both of the functional structures and the symptom are equally important to understand the living strategy of hosts and parasites. Only under the great interest in the new function created by the parasite's extended phenotype, significance of the symptoms are sometimes overlooked or mislinked to an imaginary functional adaptation (Poulin, 2000).

The morphological manipulation have previously been observed in the horsehair worm-infected hosts by the abnormal genitalia in katydids (Wülker, 1964), the extreme shorter wing in a male mantid (Roy, 2003), and the nymphal appearance in the adult female crickets (Biron *et al.*, 2005b). In our study, the field-collected mantids, *H. formosana*, infected by the horsehair worm, *C. formosanus*, displays the abnormal characteristics including the allometry in the legs and wings, and the intersexuality which found in the infected males (Fig. 12, 13) (Chiu *et al.*, 2015). The allometric morphology comes from the different ratio in the decrease of the walking legs and wings to the pronotum, which create the mantids with short legs and wings, but in the fore-leg (raptorial leg), the parasitic effect is not significant (Fig. 12). The

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intersexuality, which is defined as the change of sex characters in which clear signs of the opposite sex can be recognized (Wülker, 1964; Baudoin, 1975), happened on the fore-wing shape and distribution of the antennal sensilla of the infected adult males.

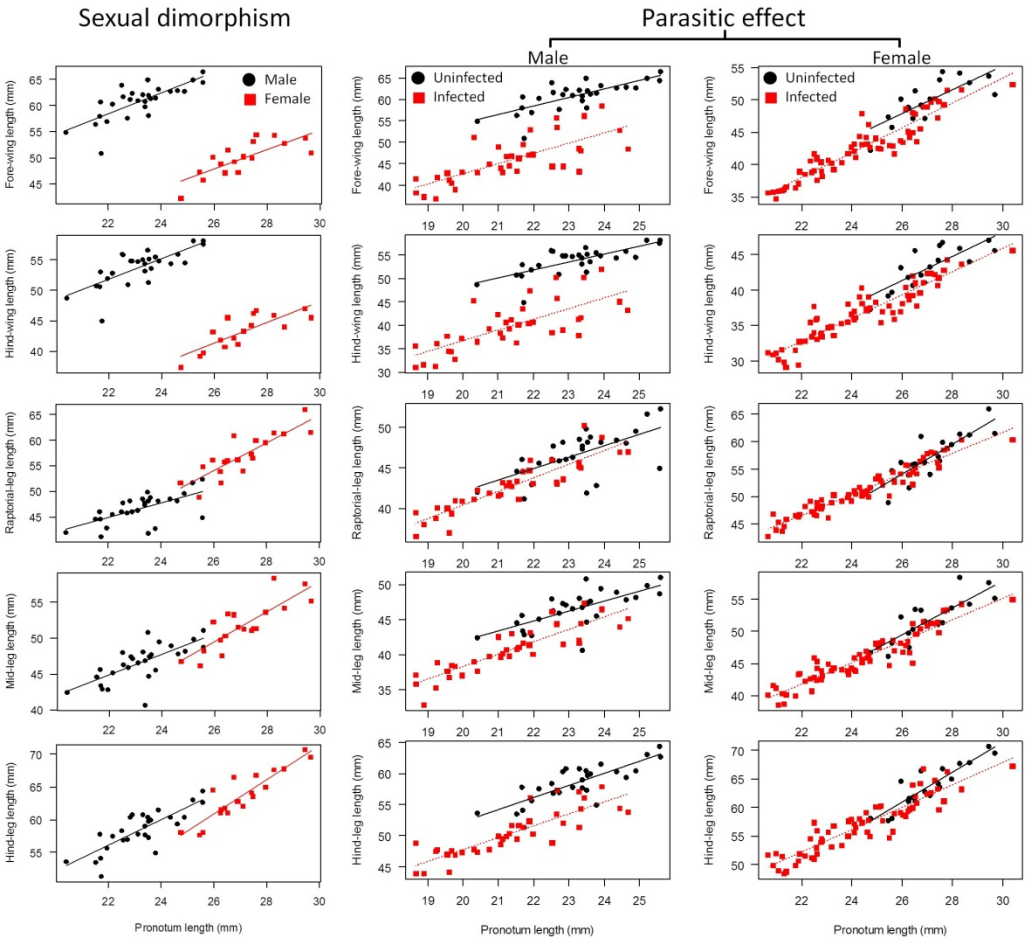

Fig. 12. Sexual dimorphism and parasitic effects of the horsehair worm (*Chordodes formosanus*) on the legs and wing lengths of infected mantids (*Hierodula formosana*). The lines inside the plots are linear regression lines generated using the data set of each plot. (Modified from Chiu *et al.*, 2015)

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These two characteristics suffering intersexuality are generally found to be sexually dimorphic represent the different roles in the courtship behavior. Female mantids release sex-pheromone to attract the male during the mantid calling behavior (Robinson and Robinson,1979; Perez, 2005). As a sex-pheromone receptor, the male mantid typically possesses stronger wings (Robinson and Robinson, 1979; Roy, 2003; Béthoux, 2010; Lombardo and Umbriaco, 2011), and denser grooved basiconic sensilla, which are hypothesized to be the sensory organ of the sex pheromone (Slifer, 1968; Hurd *et al.*, 2004; Holwell *et al.*, 2007; Allen *et al.*, 2012). In *H. formosana*, the higher ability of locomotor is not only expressed on the longer wings (Fig. 12), but also the higher ratio of the membranous area in the fore-wing. The fore-wing (tegmen) of a mantids is approximately separated into two parts by the radial vein with the leathery area (AR) above the radial vein for protecting the abdomen and the membranous area (BR) below the radial vein conductive to flying. The wing shape index (BR−AR)/(BR+AR) represents the different ratio of the membranous area to the leathery area while the higher value in the normal male indicates the more membranous wing than that of the female. The wing shape index is changed in the infected male by the decreased value falls between the value of the normal male and the normal female, whereas in the infected female, the shape index is similar to the non-infected one (Fig. 13) (Chiu *et al.*, 2015).

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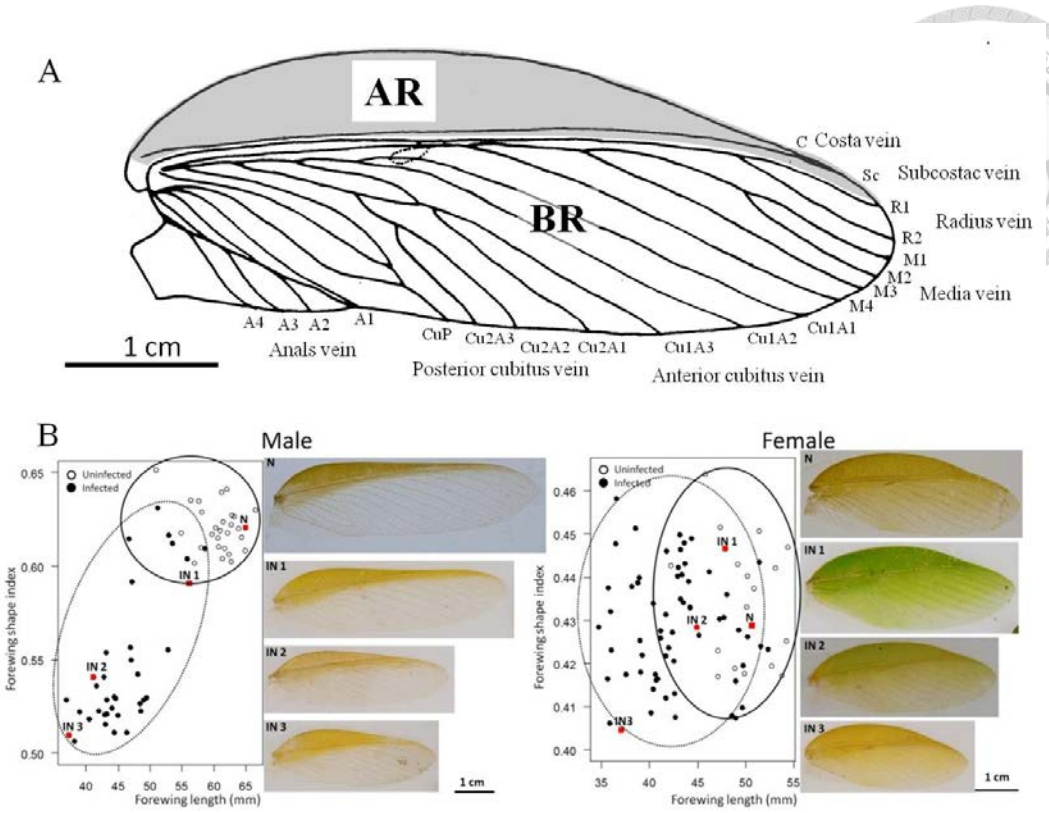

Fig. 13. Parasitic effects of the horsehair worm (*Chordodes formosanus*) on the forewing shapes of infected mantids (*Hierodula formosana*). **(A)** The forewing shape index was calculated to determine the difference between the area above (AR) and below (BR) the radial vein. **(B)** The formula for calculating the forewing shape index is (BR - AR) / (BR + AR). The black solid circles indicate the infected hosts, and the open circles indicate the uninfected hosts. The large circles surrounding each group (sold line for uninfected hosts, and dashed line for infected hosts) were determined subjectively to approximately include all data points of each group. The four red squares (N: uninfected hosts, IN1–3: infected hosts) in each graph are the trait values corresponding to the pictures of wings on the right side. (Modified from Chiu *et al.*, 2015, the wing vein is referred to Prete *et al.*, 1999)

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The antennae in the male *H. formosana* bear more grooved basiconic sensilla in two ways: the higher density of the sensilla in each flagellum segment (Fig. 14) and wider distribution of the sensilla toward to antennal base (Fig. 15A). The grooved basiconic sensilla is distributed in the male *H. formosana* from the 16th segment (16.42 ± 0.81 (16–18)) to the tip, while that in the female is from the 47th segment (47.10 ± 3.85 (38–55)). In these segments which grooved basiconic sensilla appears in both males and females, the density of the grooved basiconic sensilla is 1.74 to 25 folds in the male. In most of the infected males, both of the density and distribution of the sensilla were changed by the decrease of density in each segment and reduction of the distribution toward the tip of antennae. These two parasitic effects "feminize" the male antennae which makes these two antennal characteristics not significantly different from that of the females. But this does not mean the male antenna is totally feminized. The total number of the flagellum segment, despite it is also sexually dimorphic, in the infected male shows no significant difference to the non-infected ones (Fig. 15B). And again in the female, like the effect in the wing shape, no parasitic effects on the antennal sensilla were found (Chiu *et al.*, 2015).

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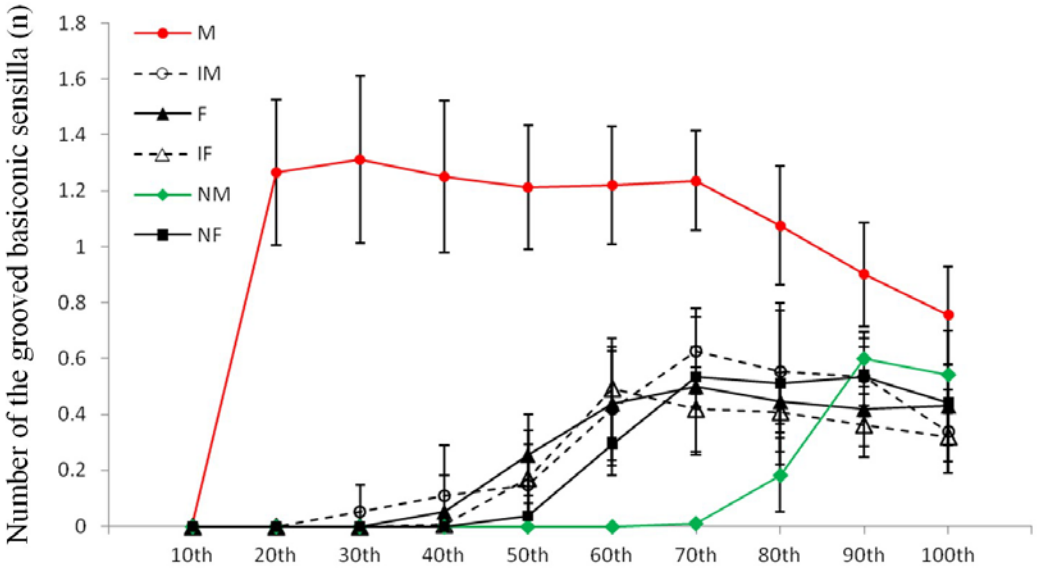

Fig. 14. Parasitic effect of the horsehair worm (*Chordodes formosanus*) on the mean (± standard deviation) number of grooved basiconic sensilla per 25 μm^2 on 10 selected segments of antennal flagella of infected and uninfected mantid adults (*Hierodula formosana*). M: uninfected male adult, IM: infected male adult, F: uninfected female adult, IF: infected female adult, NM: last-instar male nymph, NF: last-instar female nymph. (Modified from Chiu *et al.*, 2015)

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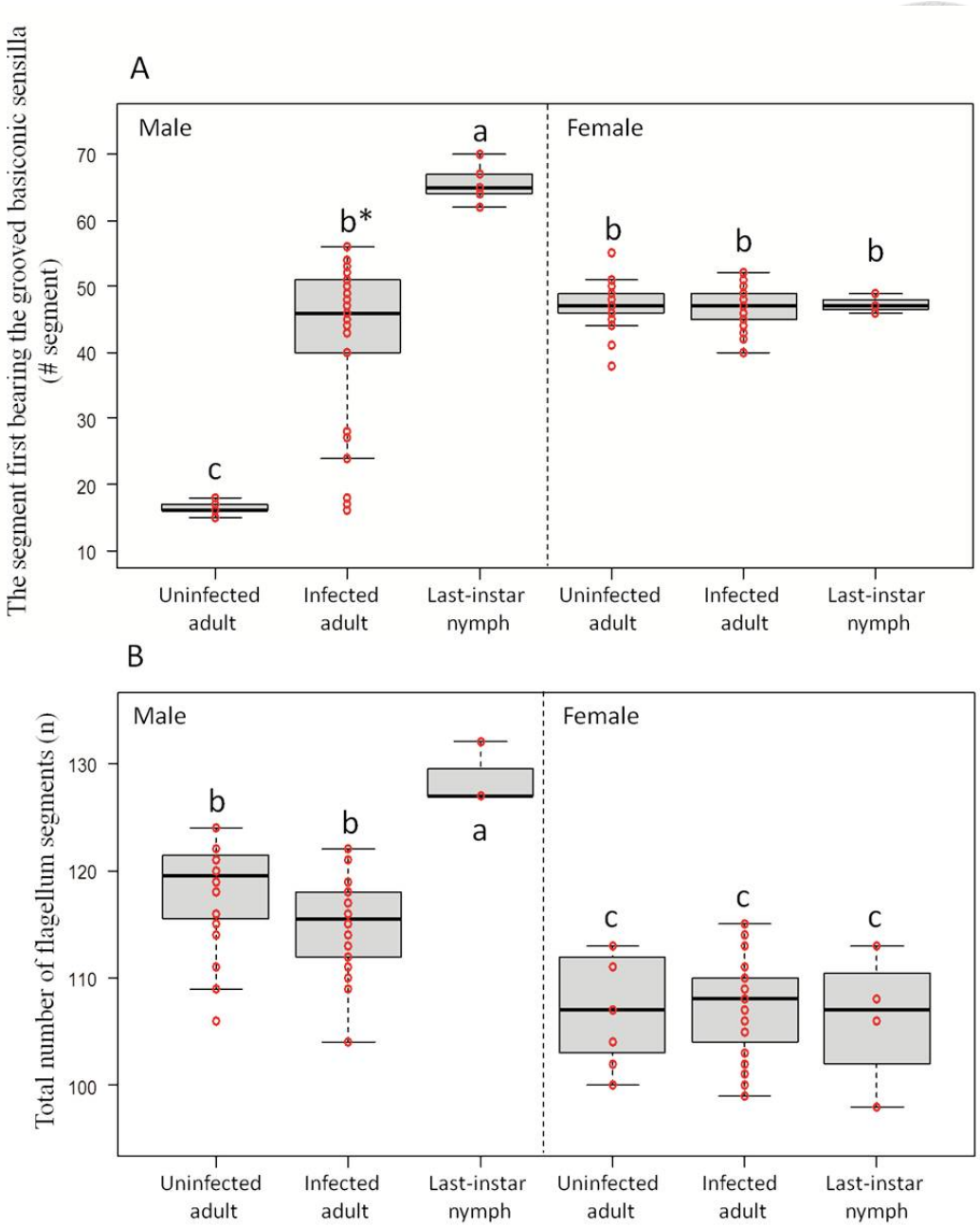

Fig. 15. Parasitic effect of the horsehair worm (*Chordodes formosanus*) on the antennal characteristics of the first flagellum segment bearing the grooved basiconic sensilla (A) and the total numbers of flagellum segments (B) of infected and uninfected mantids, *Hierodula formosana*. Letters indicate significant pairwise differences (multiple comparisons conducted using a Student's *t* test with Bonferroni correction, $P < 0.05$). Statistical analysis of the infected adult males (asterisks) in (A) was performed using the Mann-Whitney *U* test because of the violation of normality. (Modified from Chiu *et al.*, 2015)

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In summary, the parasitic effect of *C. formosanus* on *H. formosana* includes the morphological allometry and intersexuality. The reduced of the wing length has previously been found in the horsehair worm-infected mantids, *T. monticola*, and suggested as the case of intersexuality (Roy, 2003). Nevertheless, the extreme shorter wings described in Roy (2003) is more likely to be the general parasitic effect which is happened to both sexes of the infected host. In our report in Chiu *et al.* (2015), the "real intersexuality" was suggested which the changes are sex dependent instead of that happened on the sex characters. As the morphological changes can be resulted from the developmental manipulation induced by the parasite, the sex dependent change might indicates the different developmental processes undergoing in the opposite sex of the mantids.

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4.2 The symptoms resulted from reallocation in host energy budget

The horsehair worm-infected mantids display the abnormal morphology, which represent the manipulated development in the infected hosts. It is necessary to clarify that the "parasitic effect" is actually further confirmed by our artificial infection, in order to make sure these abnormal characteristics on the mantids are created by the infection (see detail below) instead of that the horsehair worm tends to parasitize the host with specific phenotype.

There are two possible evolutionary forces to trigger these new characteristics: to create the new function or to get the benefit from the manipulated host development. Horsehair worms, as the classical parasite manipulating host behavior, we did not eliminate the possibility that the abnormal morphologies create the new function of the structures which benefit to horsehair worms survival. However, the changes on the infected mantids is likely to be incompatible with the current understanding of the behavior changes since the locomotor ability of the infected crickets are found to be increased (Ponton *et al.*, 2011) but length of the host's walking legs and wings are both reduced in our study.

It is likely that the horsehair worms are benefited from the reallocation in host energy budget during host development, which increases the efficiency in energy exploitation without the lethal damage to the host. Intersexuality on the infected host is often accompanied by host fecundity reduction by means of parasite-induced gonad destruction (Baudoin, 1975) or energy reallocation (Lafferty and Kuris, 2009), which is thought to be evolved under the pressure of the virulence tradeoff (Hurd, 2001; Lafferty and Kuris, 2009). The parasite virulence tradeoff comes from the unavoidable host mortality during infection, which is increased with parasite's exploitation and consequently cause parasite's low transmission ability or high risk in

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premature death (Jensen *et al.*, 2006). With the side effect of host survival damage, unlimited increase in exploiting is unallowable, but it is unrestrained in those who intercept the resource in host reproduction. This hypothesis has been earlier noted according to the symptoms such as alteration of host gonad tissue (Wülker, 1964; Baudoin, 1975) and supported by mathematical models (O'Keefe and Antonovics, 2002; Hall *et al.*, 2007).

As many hosts go through parasitic intersexuality, the internal sex organs in the infected male *H. formosana* are also deformed. In the infected male mantids with the internal sex organs examined, most of them had no or extreme smaller visible testes (Fig. 16A) and the extreme smaller seminal vesicle (Fig. 16B). In the infected female, as the non-changed external morphology, the number of ovarioles was similar to the non-infected females (Fig. 16C), but none (0/10) of them has mature eggs whereas the five non-infected females collected from the field all harbored mature eggs (Chiu *et al.*, 2015). These phenomena support the horsehair worms re-allocate the energy of their hosts and inhibit the resource invested in other than the host survival. The energy re-allocation induced by the horsehair worm could also explain the allometric walking legs and wings in the infected mantids which sacrifices partial investment in locomotor to support the host survival. In the study of the giant water bug (*Lethocerus deyrolli*), Ohba *et al.* (2006) found the individuals provided with limited food displayed the similar phenomenon by the shorter body, mid-, and hind-legs (femura length), but there were no significant difference in the femura of raptorial leg. The similarity of the infected host and the starved individual might suggests the insects reduce the investment less important for survival under the limitation of resource. It also suggests the energy re-allocation can be triggered by the host itself (Poulin and Thomas, 1999), instead of the parasite, as a strategy

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to survival with limited resource.

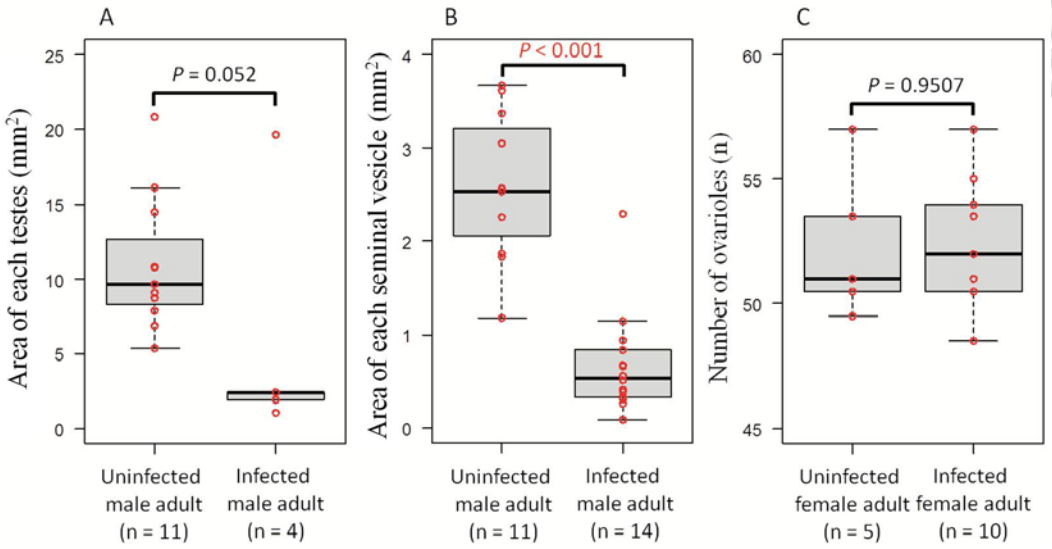

Fig. 16. Parasitic effect of the horsehair worm (*Chordodes formosanus*) on the size and number of internal sex organs (testes (A), seminal vesicles (B), and ovarioles (C)) of infected and uninfected mantid adults (*Hierodula formosana*). Whiskers indicate the maximal and minimal data values, except for outliers. *P* values indicate the results of testing the differences in median between the uninfected and infected individuals by using the Mann-Whitney *U* test. (Modified from Chiu *et al.*, 2015)

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It is logical that the horsehair worm reallocates host energy budget and consequently cause the altered morphological development, but the morphological intersexuality is only happened to the male mantids. Despite the infected female also displayed the reduction of fecundity in the egg production, the parasitic effect is not significant on the external sexual dimorphic characteristics. The opposite sexes of the bisexual organism play the different roles in the reproduction. Generally the male tend to evolve structures related to courtship and mating behavior, while the female maintains the energy to produce the larger gamete, known as eggs (Hurd, 2009). It is reasonable that the male will suffer intensive morphological change when the investment of resource in developing reproductive structures are inhibited by the parasite and consequently cause feminization on the structures. The horsehair worm's effects is now known to be related to the different developmental processes in the opposite sexes of the mantids, which is known as the sexual differentiation creating the sexually dimorphic characteristics on an organism. The mantid has never been the model in the studies about the development of sexual differentiation since its difficulty in the mass rearing. However, the study of the horsehair worms might give a new light in the current understanding of the regulation in insect sexual differentiation.

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4.3 The mechanism of parasitic intersexuality

4.3.1 Sexual differentiation in *Hierodula patellifera*

The intersexuality in *H. formosana* induced by *C. formosanus* makes the sexually dimorphic structures on the infected male to resemble that on the female (Chiu *et al.*, 2015). Most of the sexually dimorphic structures are evolved under the sexual selection which are developed for reproduction (Shine, 1989; but see González-Solís *et al.*, 2000). Since the sexually dimorphic structures is often related to host reproduction, many of these characters are identical in the early stage and differentiate during the development (Negri and Pellecchia, 2012).

In our examination of the developmental trajectories of the sexually dimorphic characteristics, the alternative *Hierodula* host, *H. patellifera*, were used since its smaller size and relatively shorter development time (there are generally 7–9 instars before the last molting in *H. patellifera* and 14–15 instars in *H. formosana*). To examine the morphological change in each instar, three sexually dimorphic characteristics were chosen since they can be examined the morphology without killing the mantids (except the early instars which are too small to be examined alive). The three sexually dimorphic characteristics are: 1) antennal sensilla, 2) red pigments on 5^{th}–7^{th} tergum of the female, and 3) morphology of the last three sternum (6^{th}–8^{th}) forming the genitalia. It is interesting that they displayed the different parasitic effects under the infection of *C. formosanus*.

The morphology of the 6^{th}–8^{th} sternum is the main characteristic frequently applied in distinguishing the sex of mantids (Prete *et al.*, 1999) and is also applied in the horsehair worm-infected mantids (Chiu *et al.*, 2015). There are six visible segments of sternum in the female adult and eight segments in the male adult (Prete *et al.*, 1999), but in the ten 1^{st} and 2^{nd} instar nymphal *H. patellifera* examined

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respectively, all the individuals are showed the eight-segment abdomen (Fig. 17A, B). The differentiation in the morphology begins to be detected from the 3rd instar. In the 3rd instar female, posterior edge of the 6th sternum slightly depress (Fig. 17C, E) and a pair of appendages respectively appear at posterior edge of the 7th (Fig. 17C, F) and 8th (Fig. 17C, D) sternum. In the 4th instar, both of the depression and the appendages become distinct and color of the appendages turns to red (Fig. 18A). In the 5th instar, the 6th sternum with depressed edge becomes wide and almost covers the 7th and 8th sternum with the slender appendages (Fig. 18B). The 6th sternum finally covers the 7th and 8th sternum and become the last visible sternum and keep this morphology until the last molting (Fig. 18C). The appendages on the 7th and 8th sternum are finally sclerotized and become the valvula of the ovipositor (Fig. 19). By contrast, the last three sterna of the male are almost not changed in the shape in all the development (Fig. 17G, 18D–E), only the last sternum (8th sternum) become moderately elongated in the last instar (Fig. 18F). Development of the sternum seems to be not influenced by the infection since the *H. patellifera* artificially infected by *Chordodes* shows the characteristic described above, only the shape of the last sternum is slighter smaller in some infected male (Fig. 21D). The character applied in distinguishing the insect sex has ever been found to be altered by parasites and causes the morphological sex reversal (Vance, 1996). The consistent development which free from the parasitic effect supports the correctness of the sex identification in Chiu *et al.* (2015), which, honestly, was ignored during the original field survey.

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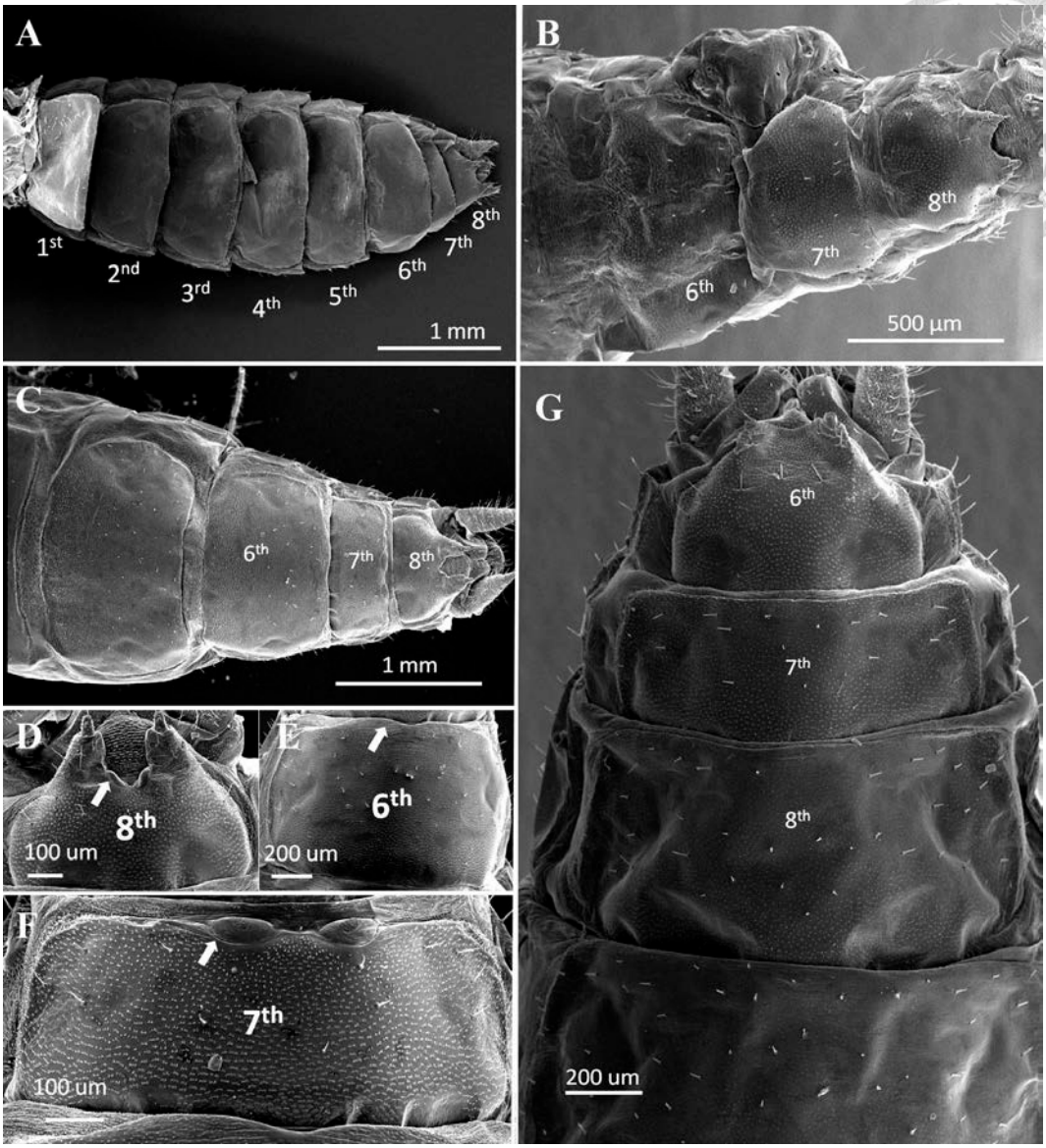

Fig. 17. Ventral view of sterna on the 1st to 3rd instar nymphs of *Hierodula patellifera* by SEM. (A–B) Sterna of the 1st and 2nd instar nymph. The sex is unable to be identified. (C–F) Sterna of the 3rd instar female nymph. The posterior edge is slightly depressed on the posterior edge of 6th (E), and the paired appendages respectively appear at posterior edge of the 7th (F) and 8th (D) sternum. (G) Sterna of the 3rd instar male nymph. The morphology is generally similar to that of the the 1st and 2nd instar nymph.

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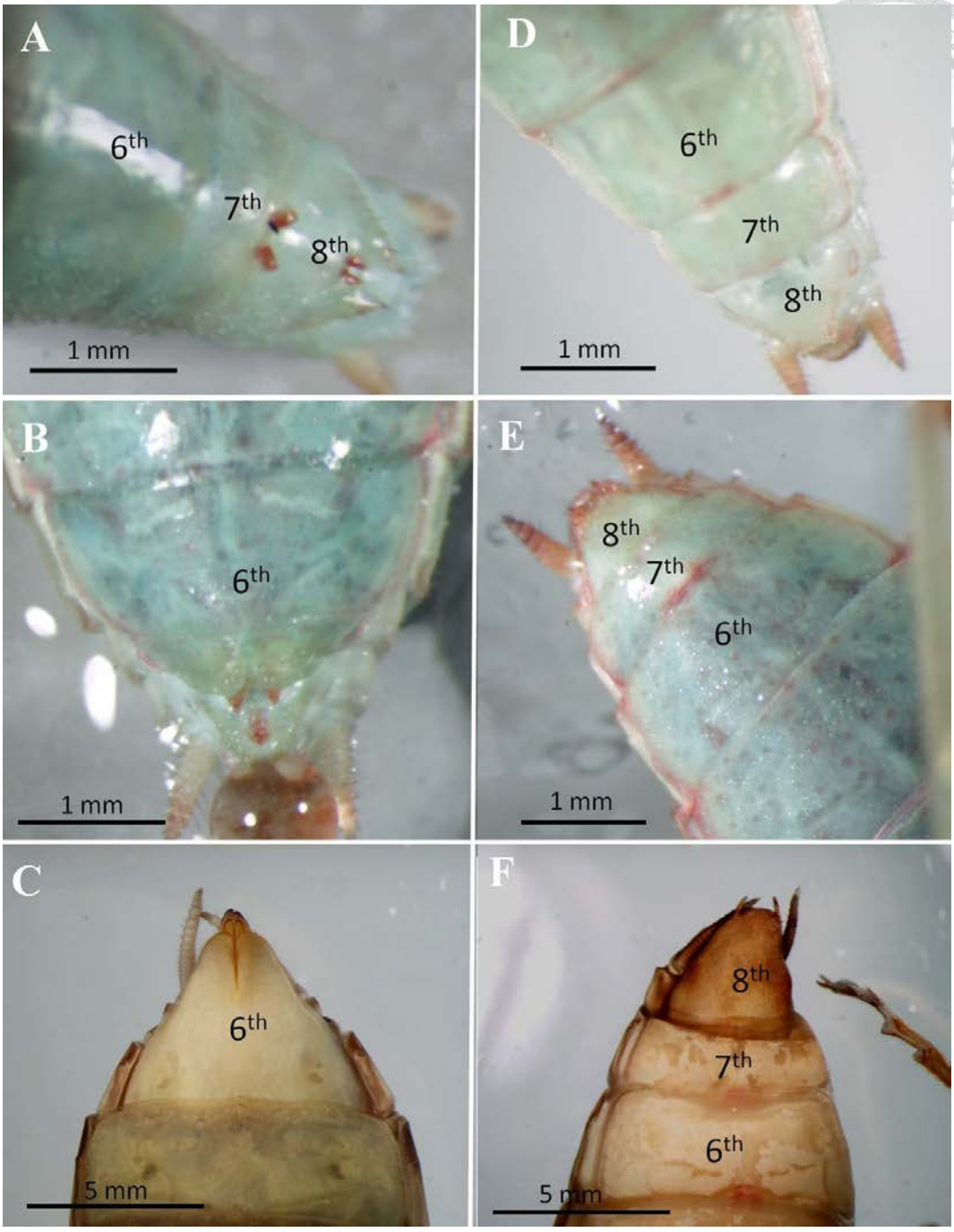

Fig. 18. Ventral view of sterna on the 4th, 5th, and 9th (the last) instar nymphs of *Hierodula patellifera*. (A–C) Sterna of the female instar nymph. The paired appendages become distinct and red in color from the 4th instar (A) and are covered by the 6th sternum from the 5th (B) to the last instar (C). (D–F) Sterna of the male instar nymph. The morphology is not obviously changed in the 4th (D) and 5th (E) instars. Only the last sternum (8th sternum) become moderately elongated in the last instar (F).

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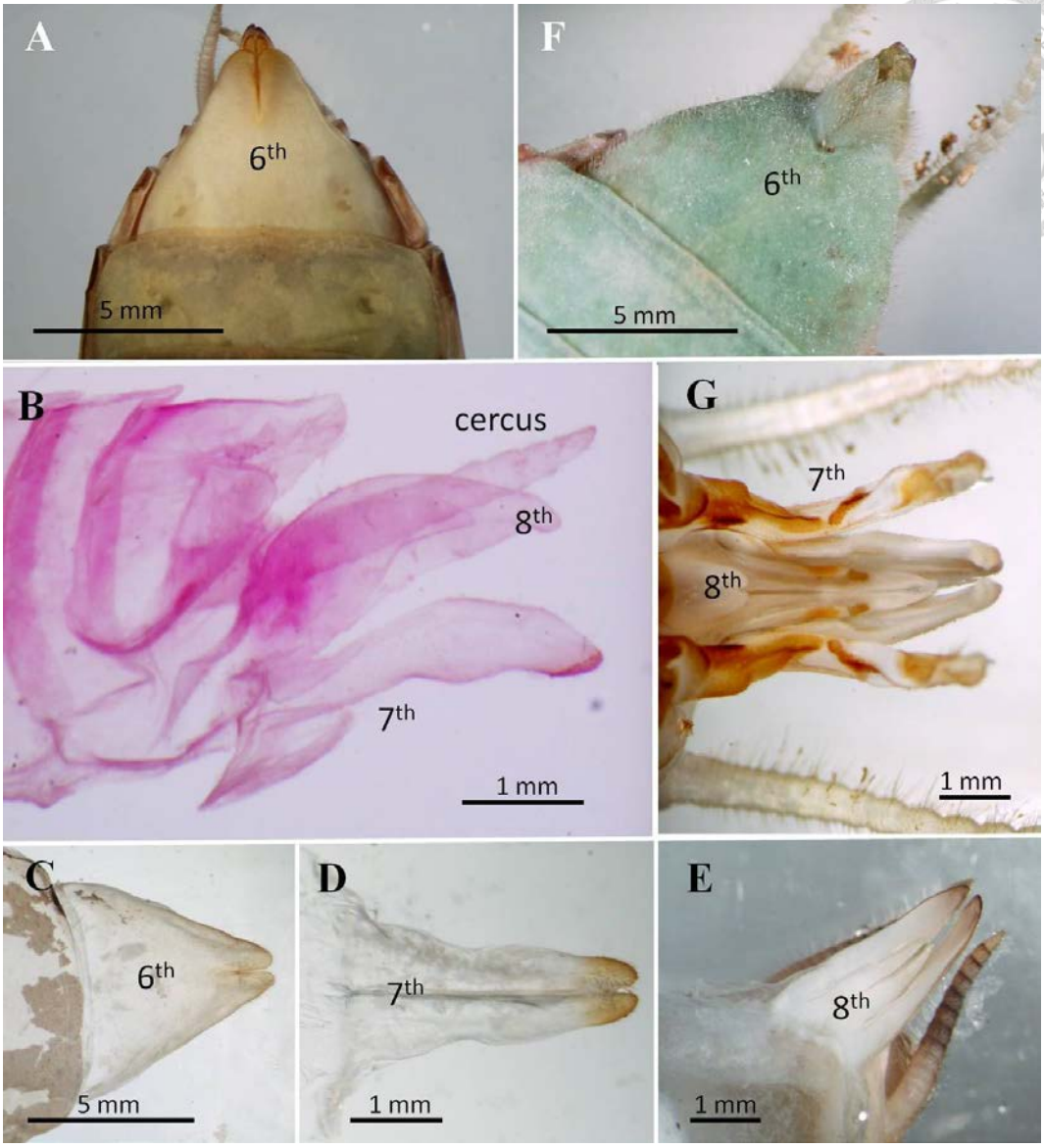

Fig. 19. Detailed comparison of the 6th-8th sternum in the female *Hierodula patellifera* of the last instar nymph and the adult. (A–E) The sterna of the last instar nymph with the ventral view of abdominal tip (A), the lateral view of the 7th-8th sternum (B), the ventral view of 6th (C) 7th (D), and 8th (E) sternum. (F–G) The sterna of the adult with the ventral view of abdominal tip (F), and ventral view of the 7th and 8th sternum (G).

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The red pigments on 5th-7th tergum of the adult female (Fig. 20A, B) is another characteristic differentiated from 3rd instar (Fig. 20C). It is believed that the gland which produces the sex pheromone is opened at these three segments of female mantids since they are usually covered by the wings and showed when the female mantid bends the abdomen to release the sex pheromone (Robinson and Robinson, 1979). We now have no evidence to suggest any relationship of the red pigments on 5th-7th tergum and the gland open, but know this characteristic is only appeared in the female *H. patellifera*. The examination of the ten 1st and 2nd instar nymphs suggests the red pigments are not displayed in the both sexes and can be slightly seen under the stereomicroscope from the 3rd insatr (Fig. 20C). The red pigments become clear and visible from the 4th instar (Fig. 20D) to the adult. The red pigment was found in some infected male *H. patellifera* artificially infected by *C. formosanus* (Fig. 21A) or *Chordodes* sp. (Fig. 21B, C) even all the mantids were fed with the horsehair worm cysts after 3rd instar. Although not all the infected males showed this characteristic, this result suggests the appearance of the red pigment can be induced by the parasite after the time of sexual differentiation. The different responses of the sexually dimorphic characteristics to the horsehair worm infection has been previously found in the field-collected *H. formosana* since the number of antennal segment is higher in the male than that in the female, but there was not significantly different in the non-infected males and the infected males (Chiu *et al.*, 2015). These characteristics not affected by the infection might be caused by the insensitivity of the factor which the horsehair worm applied to regulate the host development, or due to development of the characteristic is passed and determined (Rempel, 1940). The adult midges infected by the nematodes show the different level of morphological alterations with the different timing of infection, since the nematode is not able to manipulate the

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characteristics which are already differentiated during the ontogeny (Rempel, 1940). It might be one possible reason to explain development of the sternum since all the mantids were infected after 3rd instar, when the sexual differentiation has already proceed. However, the hypothesis of "ontogeny steps" might not explain the parasitic effect on the red pigment since this characteristic already pass the process of sexual differentiation but is still revisable by the parasitic effect.

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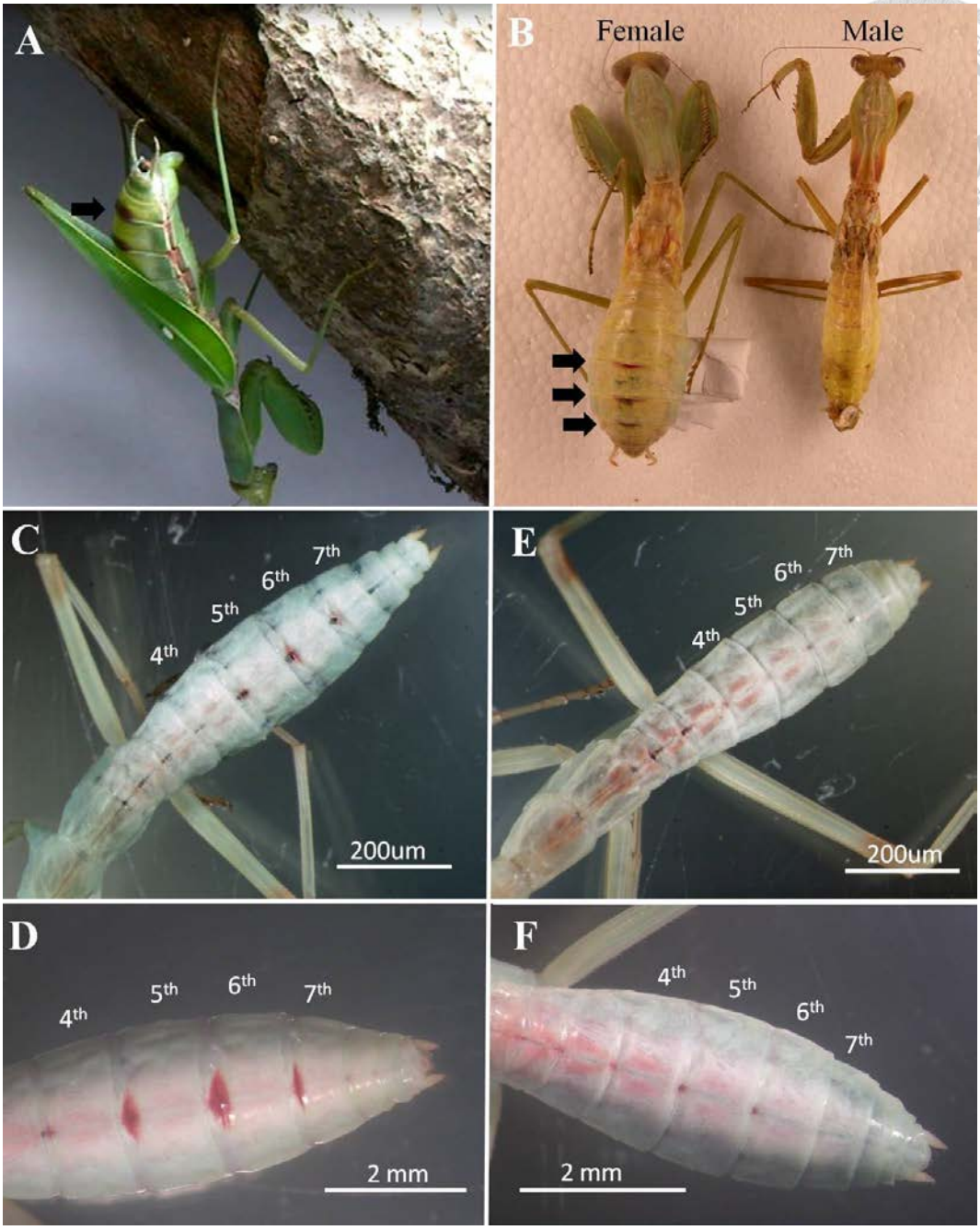

Fig. 20. Red pigments on 5th–7th tergum of the mantid, *Hierodula patellifera*. (A) Live female adult releasing sex pheromone and showing the red pigments on the abdomen (arrow). (B) The red pigments appearing in the female adult (arrows) but not in the male adult. (C–D) The red pigments are visible in the 3rd instar female (C) and distinct in the 4th instar female (D). (E–F) The red pigments are not found in the nymphal male of the 3rd (E) and the 4th (F) instar nymph.

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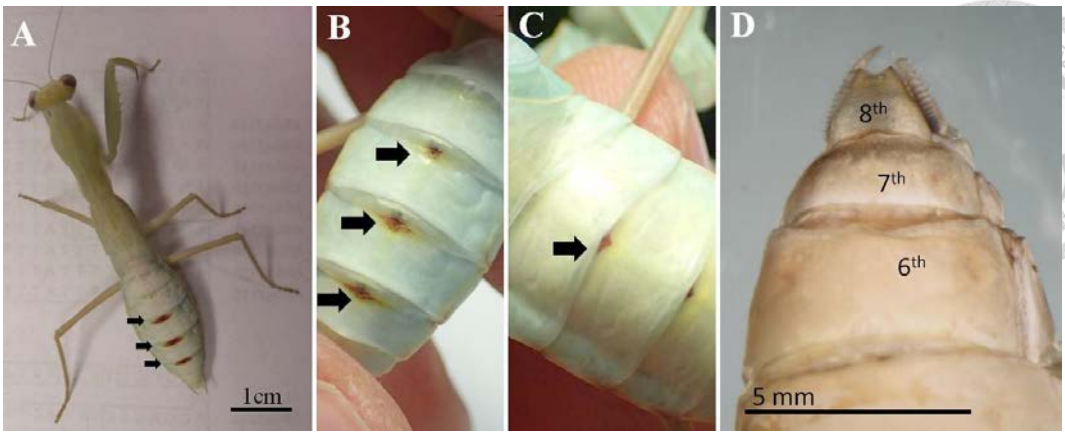

Fig. 21. Nymphal male mantids, *Hierodula patellifera*, infected by *Chordodes formosanus* and *Chordodes* sp. (A–C) Nymphal males infected by *C. formosanus* (A) and *Chordodes* sp. (B–C) displaying the red pigments which are only appeared in the female. (D) Ventral view of sterna of the nymphal male infected by *C. formosanus* (same individual with (A)).

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Development of the red pigments is not applied in the further experiment since its parasitic effect was not happened to all the individuals and the method to examine is not yet stable enough. The advanced experiment in the mechanism of parasitic intersexuality are conducted by the examination of the differentiation of sensillum distribution on antennae. As that happened on *H. formosana*, the sensilla amount is also significantly higher in the male adult of *H. patellifera* (Table 1, 2; Fig. 22A). Among the three types of the sensilla identified by their morphology (large trichoid sensilla, small trichoid sensilla, and groove basiconic sensilla) (Fig. 22), number of both large trichoid sensilla and the groove basiconic sensilla are significantly larger in the adult of the male than the female, especially the number of groove basiconic sensilla which is nearly three-folds larger in the male. Whereas there is no significant difference in the small trichoid sensilla between male and female. Since the density of seneilla on the insect cuticle cannot been unlimitedly increased (Wigglesworth, 1940), the male has wider surface to bear the sensilla by three means: higher number of the flagellum segment (Table 1, 2), larger antennal segment with wider surface area (Table 1, 2; Fig. 23A), and distribution of the groove basiconic sensilla which is anterior to the base of the antenna (the first flagellum segment bearing the grooved basiconic sensilla in Chiu *et al.*, 2015) (Table 1, 2; Fig. 24). These three sexually dimorphic characteristics of antennae respectively contribute to 5.02%, 54.42%, and 40.56% of the different amount of the grooved basiconic sensilla in the adult *H. patellifera* (Table 2). The time of sexual differentiation is different among these three characteristics. The total segment is that first differentiate from the penultimate molting at the last instar (Table 1). However, the sensilla number does not significantly differentiate at this time since the flagellum segment bearing the groove basiconic sensilla is similar between the male and the female (Table 1, Fig. 22B).

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Both of the antennal surface area and distribution of the groove basiconic sensilla, which mainly cause the higher among of sensilla in the male adult, are sexually differentiated from the last molting. It is worth to note that the distribution of the groove basiconic sensilla is sexually differentiated in both adults of *H. patellifera* and *H. formosana* which the male has the wider distribution of sensilla toward the anterior of the antenna. However, in *H. patellifera*, the distribution of female does not maintain the nymphal type of the last instar. In both the male and the female, the distribution of the groove basiconic sensilla spreads toward the antennal base at the last molting. Thus, the sexual dimorphism in the sensillum distribution is not caused by the maintaining of the nymphal character in the female but by the different degree of the distribution extending (Table 1).

Other than the sexual differentiation during the last molting, the examination of the antenna in each instar nymph reveals the change of sensillum distribution in every instars. The first flagellum segment bearing the grooved basiconic sensilla changes toward the posterior end of the antenna in each nymphal instar from around 25th segment to posterior than 50th segment in the last instar (Table 1, Fig. 24). This development is almost the same in both males and females until the 9th instar. At the 9th instar, the first flagellum segment bearing the grooved basiconic sensilla is significantly posterior in the male nymph than that of the female (Table 1). However, this difference is likely to be caused by the sexual differentiation of the antennal segment number while the male nymph has higher number of the segment in the 9th instar. In comparing the segment number bearing the groove basiconic sensilla, it is similar in the both sexes in all nymphal instars and diverged in only adult stage (Table 1). In it now known the distribution of the groove basiconic sensilla suffers the parasitic effect in the male adult (Chiu *et al.*, 2015). Since the female adult of *H.*

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formosana maintains the antennal characteristics as the last instar, it is believed that the intersexuality induced by the horsehair worm might be the result of juvenilization (Chiu *et al.*, 2015). The "juvenilization" and "interference in insect sexual differentiation" are two main hypothetical mechanisms in explaining the intersexuality. The development of the sensillum distribution includes both change in every instars and sexual differentiation in the last molting provide a suitable indicator to test these two exclusive hypotheses

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Table 1. Comparisons of sansillum amounts and antennal characteristics between male and female mantids, *Hierodula patellifera*

		1st instar		8th instar		9th instar (Last instar)		10th instar (Adult)	
LTS	Male	77.25 ± 4.03	*P* = 0.779	NA		153.00 ± 8.49	*P* = 0.230	190.17 ± 9.54	**P = 0.002**
(n)	Female	76.40 ± 4.72				146.80 ± 7.46		165.00 ± 9.72	
STS	Male	234.00 ± 6.78	*P* = 0.534	NA		702.67 ± 66.08	*P* = 0.724	1022.67 ± 37.32	*P* = 0.412
(n)	Female	237.40 ±8.82				722.20 ± 102.04		1058.60 ± 83.68	
GBS	Male	117.50 ± 15.10	*P* = 0.941	NA		540.17 ± 61.07	*P* = 0.756	3629.83 ± 110.97	**P < 0.001**
(n)	Female	117.00 ± 11.96				554.80 ± 175.77		1216.40 ± 997.97	
TSF	Male	51.00 ± 0.82	*P* = 0.162	91.60 ± 5.60	*P* = 0.230	102.44 ± 1.94	**P < 0.001**	101.67 ± 3.04	**P < 0.001**
(n)	Female	51.88 ± 1.13		88.71 ± 3.90		95.50 ± 2.88		93.67 ± 3.97	
ASA	Male	124.56 ± 3.14	*P* = 0.398	NA		589.64 ± 31.63	*P* = 0.397	1129.71 ± 60.56	**P < 0.001**
($1000\mu m^2$)	Female	126.83 ± 4.44				605.56 ± 27.75		920.31 ± 22.90	
FFG	Male	24.00 ± 2.31	*P* = 0.232	50.50 ± 8.86	*P* = 0.060	55.83 ± 3.65	**P < 0.001**	24.50 ± 8.68	**P < 0.001**
(#n)	Female	24.83 ± 1.59		45.67 ± 5.96		48.17 ± 6.19		34.17 ± 10.10	
HSF	Male	28.00 ± 0.82	*P* = 1	43.00 ± 2.83	*P* = 0.680	47.56 ± 1.51	*P* = 0.250	78.44 ± 4.75	**P < 0.001**
(n)	Female	28.00 ± 1.07		43.43 ± 1.27		48.63 ± 2.07		60.33 ± 2.69	

1. The *P* values conducted by Student *T* test indicate significant differences between sexes.
2. LTS: Total number of large trichoid sensilla, STS: Total number of small trichoid sensilla, GBS: Total number of groove basiconic sensilla, TFS: Total number of flagellum segment, ASA: Antennal surface area of the flagellum segment bearing the grooved basiconic sensilla, FFG: the first flagellum segment bearing the grooved basiconic sensilla, HFS: Total number of flagellum segment bearing the grooved basiconic sensilla.

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Table 2. Difference of the grooved basiconic sensilla in the three sexually dimorphic zones of adult *Hierodula patellifera*

Segment	Zone1 1st-43th	Zone2 44th-89th	Zone3 90th to the antennal tip
Male adult	1209.33 ± 635.80	2314.00 ± 1085.05	175.17 ± 89.84
Female adult	210.20 ± 143.13	973.40 ± 477.97	51.60 ± 56.97
P-value by *T*-test	***P* < 0.001**	***P* < 0.001**	***P* = 0.008**
Average difference	999.13	1340.60	123.57
Percentage to total difference	40.56%	54.42%	5.07%

1. The *P* values conducted by Student *T* test indicate significant differences between sexes.
2. Zone1: antennal segment anterior the female segment first bearing the grooved basiconic sensilla. Zone2: antennal segment between the female segment first bearing the grooved basiconic sensilla and the female total segment number. Zone3: antennal segment posterior the female total segment number. See Fig. 23A.

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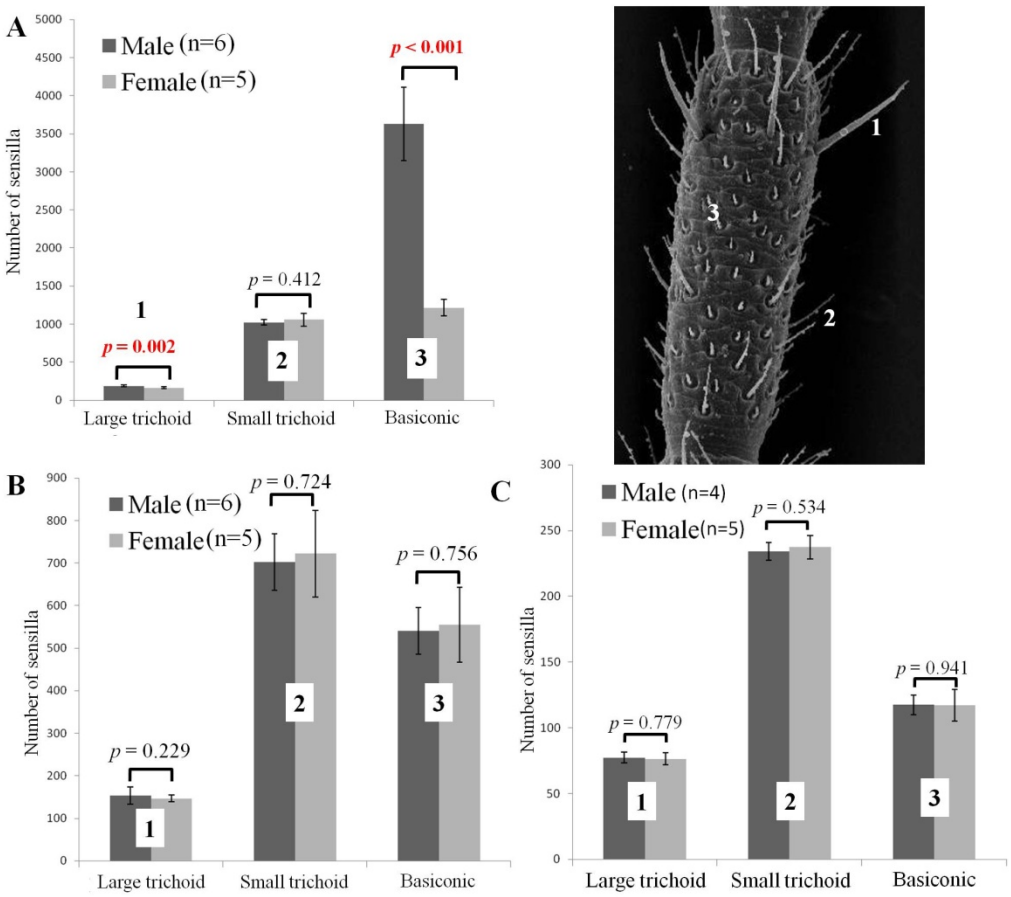

Fig. 22. Comparison of sensillum amounts between the male and the female *Hierodula patellifera* in the adult (A), the last (9^{th}) instar (B), and the first instar (C). The *P* values conducted by Student *T* test indicate significant differences between sexes.

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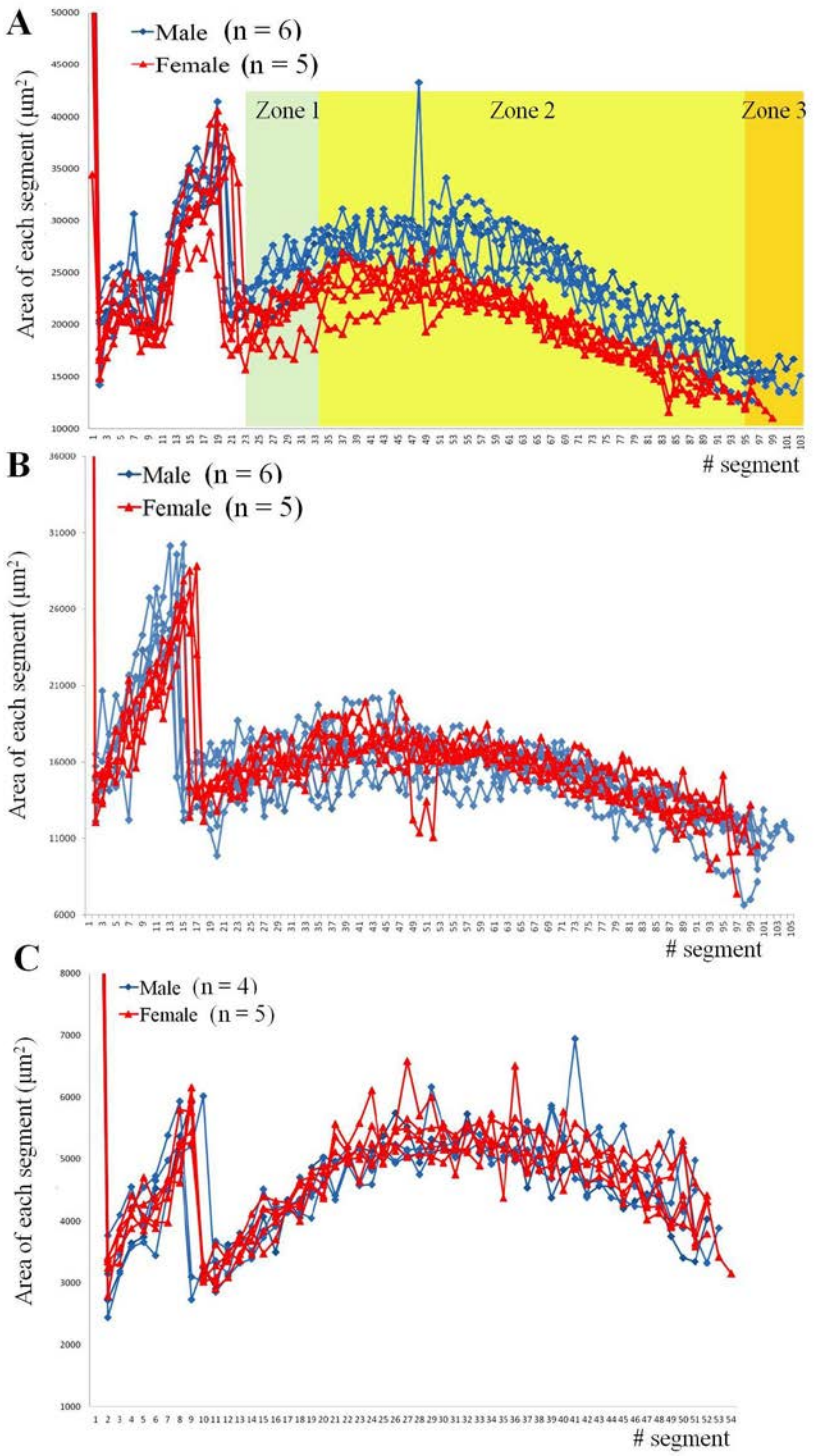

Fig. 23. Comparison of antennal size (surface area) between the male and the female *Hierodula patellifera* in the adult (A), the first instar (B), and the last (9^{th}) instar (C). Zone1-3 in (A) are separated by "the antennal segment of the female segment first bearing the grooved basiconic sensilla" and "the antennal segment of the female total segment number". See Table 2.

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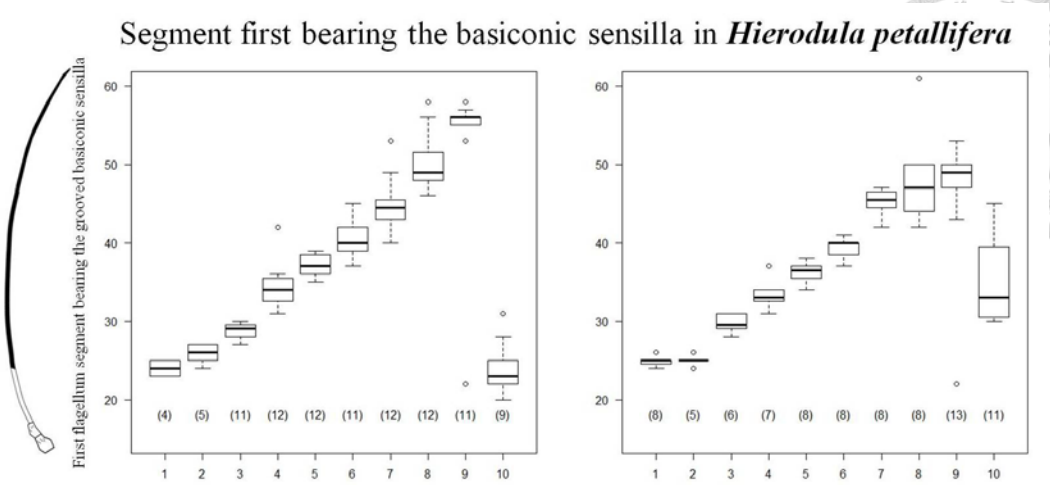

Fig. 24. Developmental trajectories of the sensillum distribution (the antennal segment first bearing the grooved basiconic sensilla) on mantid antennae, *Hierodula petallifera*. The 10th instar is the adult stage. Numbers in the brackets are numbers of samples examined.

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4.3.2 Interference in insect sexual differentiation or juvenilization

Mechanism of how a parasite manipulates the host phenotype is critical to further understand its adaptation (Poulin, 1995). It has long been known that horsehair worms and their ecological closed mermithids frequently cause the intersexuality in their hosts (*Metrioptera brachyptera*, *Pholidoptera* sp., *Pterostichus niger*, *Blaps mucronata*, *Vespa germanica* (Wülker, 1964), midges (Chironomidae) (Rempel, 1940; Wülker, 1985), biting midges (Ceratopogonidae) (McKeever *et al.*, 1997), grasshoppers (Acrididae) (Rowell, 2000), mayflies (Baetidae) (Vance, 1996), and mantids (Roy, 2003)), but it has almost never been mentioned how these parasites interfere in the insect sexual differentiation.

The common coexistence of the gonad destruction (castration) and the intersexuality (Baudoin, 1975) makes the sexually dimorphic characteristics in the infected host are believed to be altered due to the loss of stimulus produced by sex glands (e.g. Rempel, 1940). Such hypothesis is known as juvenilization, which keeping host characters in the early step of ontogeny (Baudoin, 1975; Hurd, 2009) and well explains the parasitic cases of the infected mammals (Vainio *et al.*, 1999) and infected crustaceans (Rodgers-Gray *et al.*, 2004). However, despite the "androgenic gland" has found in some arthropods (crustaceans) (Rodgers-Gray *et al.*, 2004), it is still under debate that if the insect sexual differentiation is the "cell-autonomous process" (Negri and Pellecchia, 2012). It is traditionally believed that every cell decides for itself what its sexual phenotype should be (DeFalco *et al.*, 2008). One of the evidence is the gynandromorph due to the incomplete cell division and consequently causes an insect individual mosaically contains both male and female characteristics (Negri and Pellecchia, 2012). The gynandromorph in a certain extent rejects the existence of sex hormone which has not detected in any insect species (de

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Loof and Huybrechts, 1998; Negri and Pellecchia, 2012). It is recently challenged by the finding of the avian gynandromorph (Bear and Monteiro, 2013) and the inter-cell regulating factor in the embryogenesis of *Drosophila* (DeFalco *et al.*, 2008). Nevertheless, it is still less evident of the factor regulating the insect sexual differentiation especially in the post embryonic development.

The lack of factors regulating insect sexual differentiation leads the "parasitic juvenilization" limited to its literal meaning. The juvenilization is still noted in the parasite-infected insect hosts by means of increasing the juvenile hormone titer and makes the juvenilized characteristics on the host (Fisher and Sanborn, 1962; Down *et al.*, 2008; Hurd, 2009). In some cases, the juvenilized characteristics cause the same phenomenon of the intersexuality since the sexual dimorphism in insects is sometimes resulted from development of the structures in one of the sex and maintenance of the juvenile form in alternative sex (de Loof and Huybrechts, 1998). The "general juvenilization" might be supported by the non-change in the female antenna of *H. formosana* since it maintain the same characteristic with the last instar nymph (Fig. 15A; Chiu *et al.*, 2015). However, this phenomenon does not exclude the possibility that the horsehair worm interferes in insect sexual differentiation to lead the "female developmental process" in the infected male. In addition, the antennal characteristic of the infected male *H. formosana* is not totally juvenilized which is morphologically different from its last instar nymph but similar to the female (Fig. 15A; Chiu *et al.*, 2015).

These two hypothesis (interference in insect sexual differentiation and general juvenilization) could cause the same phenomenon of intersexuality, but indicate different developmental processes that the horsehair worm might intervene during infection. Under the hypothesis of the general juvenilization, the antennal

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characteristic is not changed in the female *H. formosana* since it maintain the nymphal characteristic. Thus, there is two main points in the hypothesis testing: After the horsehair worm begin to manipulate host development, 1) if the infected females are still not affected (even they do not maintain the nymphal characteristics in the adult of *H. patellifera*), and 2) if the sensillum distribution of the infected male stop developing in each nymphal instar.

It should be first realized the beginning of the time when the horsehair worm start to manipulate host development after the cyst enters the mantids. The morphology of insect stop changing after the last molting. After this time point (the last molting), the horsehair worm effect on the host development will not be displayed on the host morphology. In other word, if the horsehair worm cyst enter the mantids "too late", the infected male will show the normal antennal instead of the feminized one. This phenomenon was found on the *H. patellifera* artificially infected by *Chordodes* sp., while the parasitic effect on the host antennae is significantly correlated with the span of time between the time of host last molting and ingestion of the horsehair worm cyst (Appendix 5). The infected mantids with longer developmental time spans before the last molting tend to have the manipulated antennae (Fig. 25, 26). With the estimation by the logistic regression model, the horsehair worm's effect begins at around 36 (95%CI: 32.24-40.53) days (90% of the host start to change the morphology) after the cysts are ingested (Fig. 26). In addition to the change in the antenna, few infected males (6 individuals) were also found to show the red pigments on abdomens 32 days after being infected (Fig. 21B, C). These results suggest the horsehair worms start to manipulate the host development at around 30 to 36 days, which is around the first one-third of their developmental period (the total developmental days of our artificial infected *C. formosanus* is 97 ±

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22 days (54-143 days, n = 18)).

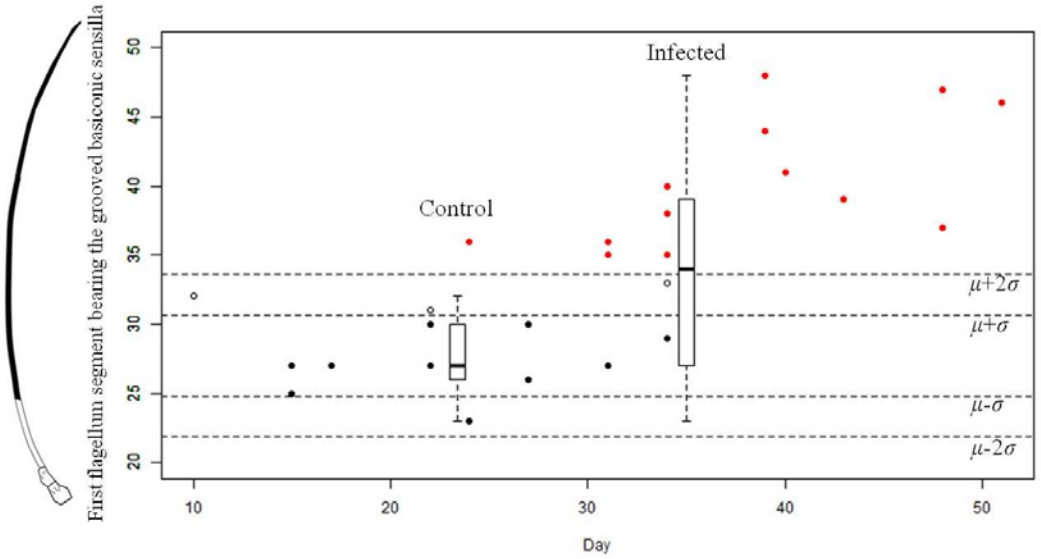

Fig. 25. Sensillum distributions (the antennal segment first bearing the grooved basiconic sensilla) in the adult male mantid, *Hierodula patellifera*, of the control mantids and that infected by the horsehair worm, *Chordodes* sp. Y-axis is the first flagellum segment bearing the grooved basiconic sensilla. X-axis is developmental time between the date of infection and the last molting. The box-plots are the values of the control mantids (left, n = 11) and the infected mantids (right, n = 26). The dots are values the infected mantids against the developmental time. The dash lines indicated the first and second standard deviations of the control value, which separates the infected hosts into non-manipulated group (black dots), manipulated group (red dots), and three individuals which are removed from analysis conducted by the logistic regression model (hollow dots).

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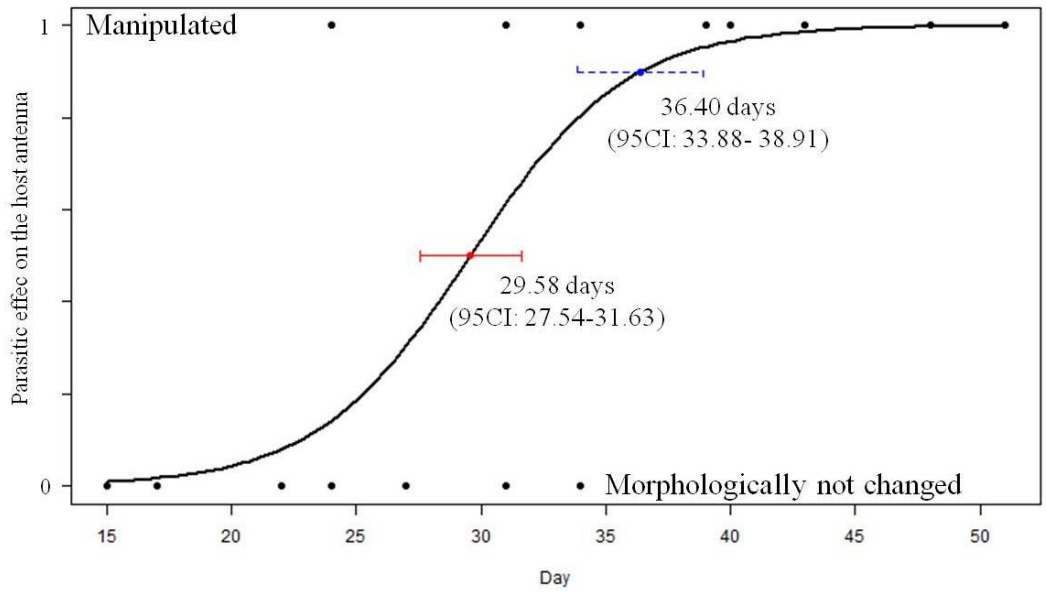

Fig. 26. The begin time when the horsehair worm, *Chordodes* sp., start to manipulate the development of the mantid, *Hierodula patellifera*. Y-axis is the appearance of parasitic effect on the host antennae against the time span between the date of infection and the last molting (X-axis). The black line is the regression line estimated by logistic regression model. Red dot and black dot are 50% and 90% individuals been manipulated, respectively, estimated by the logistic regression model with one standard deviation.

The estimation of logistic regression model suggests more than 90% of the infected host will show the manipulated morphology 36 days after being infected. In the artificial infection of *H. patellifera* and *Chordodes* sp., 12 of 14 infected male developed for more than 30 days and all the seven infected male developed for more than 36 days after being infected displayed the manipulated antennae. Under the hypothesis of general juvenilization, the male nymph and the female adult are expected to also show the juvenilized antennae 36 days after being infected. Unfortunately, in this dissertation we not yet have a very clear conclusion supported by enough samples, but the few samples make me to believe the horsehair worms interfere in the host sexual differentiation instead of the juvenilization. In the same artificial infection experiment, five infected males developing for more than 30 days

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(three of them are more than 36 days) after the infection but all showed the normal antennae (Fig. 27). The similar situation is also happened in the infected female adult. In the samples we have in these few years, five infected females also showed the normal antennae which are not juvenilized by the infection since their significantly different sensillum distribution from that of the last instar nymph (Fig. 28).

Now the samples is still not enough to support a very clear conclusion. Nevertheless, these few samples makes it is worth to keep testing if the horsehair worms interfere in the host sexual differentiation. In addition, these samples also suggest the much more complex parasitic effect than our previous thinking. In the artificial infection, the "manipulated host" shows the significantly increased level of the parasitic effect which makes the distribution of the sensilla shifts toward the posterior of antennae with the increasing developmental time (Linear regression model: β = 0.37, t = 2.63, P = 0.02) (Fig. 25, red points). This increasing parasitic effect implies the gradually diverged process in the host sexual differentiation. However, the differentiation of the sensilla distribution of *H. patellifera* is only happened during the last molting instead of gradually diverge in each nymphal instars. The gradual antennal change might be caused by the accumulation of parasitic effect on the time point of the host sexual differentiation, but this explanation still needs more evidence to be confirmed.

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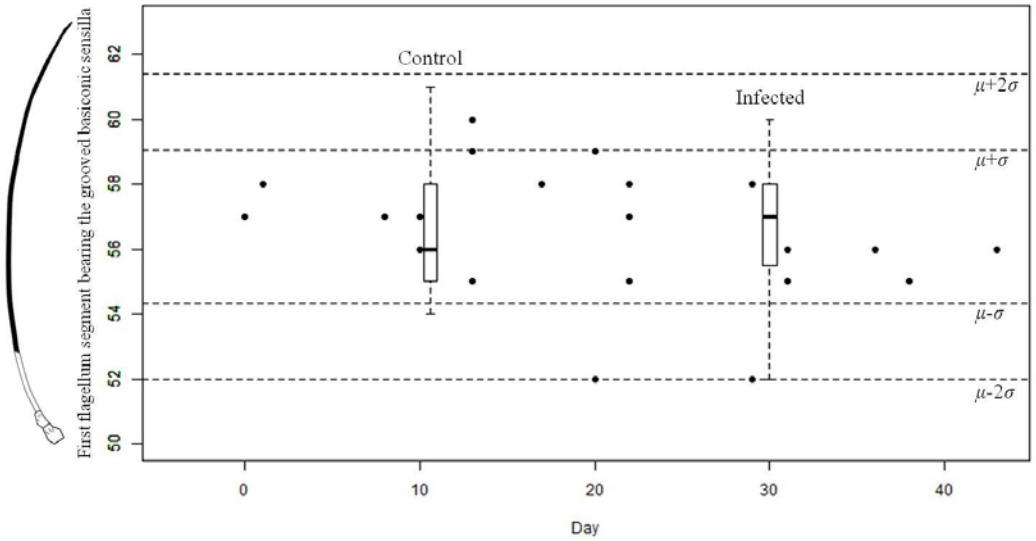

Fig. 27. Sensillum distributions (the antennal segment first bearing the grooved basiconic sensilla) in the last instar male mantid, *Hierodula patellifera*, of the control mantids and that infected by the horsehair worm, *Chordodes* sp. Y-axis is the first flagellum segment bearing the grooved basiconic sensilla. X-axis is developmental time between the date of infection and the penultimate molting. The box-plots are the values of the control mantids (left, n = 13) and the infected mantids (right, n = 23). The dots are values the infected mantids against the developmental time. The dash lines indicated the first and second standard deviations of the control value, while all the infected values fall inside the range of two standard deviations of the control mantids.

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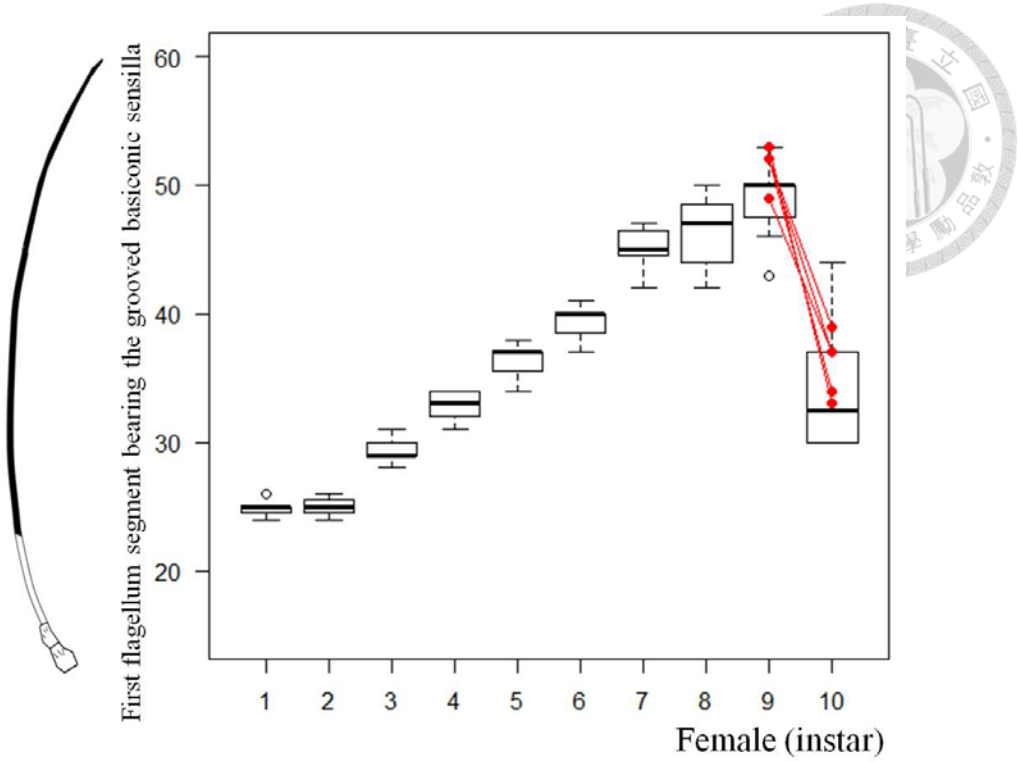

Fig. 28. Developmental trajectories of the sensillum distribution (the antennal segment first bearing the grooved basiconic sensilla) on mantid antennae, *Hierodula petallifera* and the female individuals (red dots, n = 5) infected by the horsehair worm, *Chordodes* sp. or *C. formosanus*. The 10th instar is the adult stage.

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5 Conclusion

The ten-year study in this dissertation is the first systematic investigation of the horsehair worm in Taiwan. Here we preliminarily reveal the five Taiwanese horsehair worm species. The two most common species *Chordodes formosanus* and *Acutogordius formosanus* n. sp., which are relatively easy to be collected and cultured, might be the model species in the advanced studies. The *Gordius* sp., despite its narrow distribution in mid-altitude mountains and unknown definitive host, might adopt the special adaptation which has never been revealed among the horsehair worm. Besides, several species are still not described in Taiwan.

The developmental manipulation in the mantid host infected by *C. formosanus* open the newly known host-parasite interaction in the horsehair worm's parasitism. Our current evidences suggest the *Chordodes* horsehair worms is likely to interfere in the sexual differentiation of its mantid host. This hypothesis provides a new evidence to challenge the traditional belief of the cell-autonomous process. In addition to collect more infected samples to test the hypothesis of the interference in insect sexual differentiation and general juvenilization, the regulating factor in the insect sexual differentiation and parasitic effect is the most attractive issue in the horsehair worm-mantid system. One possible factor is the mimetic Wnt proteins produced by the horsehair worm. From early 20th century, comparison of protein expression has been applied to reveal the possible mechanism in horsehair worm's manipulation. Biron *et al.* (2005b, 2006) found two Wnt proteins inside the horsehair worms were sequencely similar to the insects rather than their phylogenetic-related nematode, *Caenorhabditis elegans*. These similarity made them believed to be functional in physiological regulation of their host as that explaining in the concept of molecular mimicry which have been noted from 1960s (Damian, 1964). However, although

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evolutionary advantage of molecular mimicry can be easily understood, parasitoproteomics tells us only the difference of protein expression, whereas the consequences of mimicry on pathogenesis are still unclear (Salzet *et al.*, 2000). The mimetic Wnt proteins found inside the horsehair worms are originally strongly believed to be the factor manipulating the host behavior, but their function has not been confirmed to date (Biron and Loxdale, 2013). *Wnt* gene is the conserved genes appearing in metazoan animals and named after the *Drosophila* segment polarity gene, *wingless*, and the vertebrate homolog, *integrated*. Its expressed protein acts as extra-cellular signal stimulating internal-cellular signal transduction cascades and consequently regulates several aspects of developments including the cell fate determination (Komiya and Habas, 2008). In the recent study of embryogenesis in *Drosophila* gonad, the *Wnt2* gene was found to be the key factor in regulating the sexual differentiation of *Drosophila* gonads (DeFalco *et al.*, 2008). It is likely that the mimetic Wnt proteins produced by the horsehair worms cause the intersexuality in the infected host. It is worth to keep promoting the understanding in the mechanism of the horsehair worm-induced intersexuality before the technique is advanced enough to conduct the biochemical experiments.

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7 Appendixes

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Appendix 1.

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RESEARCH ARTICLE

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A new horsehair worm, *Chordodes formosanus* sp. n. (Nematomorpha, Gordiida) from *Hierodula* mantids of Taiwan and Japan with redescription of a closely related species, *Chordodes japonensis*

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Abstract

A new species of horsehair worm, *Chordodes formosanus* **sp. n.**, is described and compared to a closely related species, *C. japonensis*. Although both species possess the same six cuticular structures of areoles on the surface, the significantly longer filaments on the female crowned areoles can be used as diagnostic characters for the new species. The different taxonomic status of these two species was also confirmed after analyzing the partial cytochrome oxidase subunit I sequence, and the mantid hosts, which are respectively limited to the genus *Tenodera* for *C. japonensis* and *Hierodula* for *C. formosanus* **sp. n.** In addition, the immature stages of eggs and larvae of the new species are also described and discussed in detail.

Keywords

Nematomorpha, *Chordodes formosanus*, *C. japonensis*, new species, molecular analysis, immature stages, mantid hosts

Introduction

The host range of an organism is an important ecological character since it reflects the survival and reproduction of parasites (Onstad and McManus 1996). Parasite taxonomy can be clear only when the taxonomic status of both the parasites and their hosts are well understood. Horsehair worms are obligate parasites that pass through different hosts at various stages (Hanelt et al. 2005). Among the 350 described species of horsehair worms in 21 genera (Schmidt-Rhaesa and Lalramliana 2011), the genus *Chordodes* consists of about 90 species, and is one of the most diverse genera in the phylum Nematomorpha (Schmidt-Rhaesa et al. 2008). Members of *Chordodes* can be easily distinguished from those of other genera by their unique cuticular structures known as crowned areoles (Schmidt-Rhaesa 2002a). Nevertheless, the various structures of the areoles are not always clear at the species level.

Chordodes formosanus sp. n. which is morphologically similar to *C. japonensis* is hereby described as new to science. *Chordodes japonensis* was originally described by Inoue (1952) using light microscopy from specimens collected in Honsyu, Japan. At that time, this horsehair worm was known to parasitize the mantids *Tenodera sinensis* and *T. angustipennis*. Following that, Baek (1993) checked horsehair worms specimens deposited in Kon-Kuk University, Seoul, Korea (collected from Kyongsangbuk-do, Seoul, Chollabuk-do, and Kyunggi-do, Korea) and identified them as *C. japonensis* using scanning electron microscopy (SEM). Schmidt-Rhaesa (2004) checked horsehair worms (collected in Shiga, Japan) deposited in the Lake Biwa Museum, Shiga Prefecture, Japan. One female parasitized the mantid *Hierodula patellifera,* and one free-living male was considered to be *C. japonensis* after examining it with SEM. Although the descriptions in the previous three papers slightly differ (see Table 1 in Schmidt-Rhaesa 2004), those authors believed the similar morphologies still would not make them clearly separated species. In other words, *C. japonensis* was reported to be distributed in Japan and Korea and to have at least three mantid hosts, *T. sinensis, T. angustipennis,* and *H. patellifera*.

However, in April 2007 to February 2008, we conducted a field survey of horsehair worms in Taiwan and found that the mantids, *H. patellifera* and *H. formosana,* were infested by *C. japonensis*, but there was none in the 109 individuals of *T. sinensis* examined. This geographical difference in the host specificity of *C. japonensis* in Taiwan and Japan raised a question as to the taxonomic status of horsehair worms from the above three mantids. In order to answer this question, we examined morphological characters with both light and scanning electron microscopes, and the phylogeny was reconstructed using the mitochondrial (mt)DNA cytochrome oxidase subunit I (mtDNA-COI) gene of 40 adult horsehair worms collected from the three mantids, *T. sinensis*, *H. patellifera,* and *H. formosana,* in Taiwan and Japan. We believe that these horsehair worms actually consist of two distinct species: *Chordodes japonensis* Inoue, 1952 from *T. sinensis* in Japan, and a new species from *H. patellifera* and *H. formosana* in Japan and Taiwan. This paper deals with the new species of *C. formosanus* sp. n., and descriptions of its egg and larval morphologies are also provided.

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Materials and methods

In total, the morphologies of 40 adult horsehair worms (including two females which laid eggs in the laboratory) were examined, and these worms were used for a DNA analysis. The morphologies of larvae laid in the laboratory were examined by light microscopy. Eggs and larvae collected in 2010 were examined by an SEM, and their COI sequences were analyzed to determine their taxonomic status. After studying the specimens, the partial bodies of these 40 samples were preserved in the Department of Entomology, National Taiwan University, Taipei; National Museum of Natural Science, Taichung, Taiwan; and Lake Biwa Museum, Shiga, Japan.

Collection and preservation of horsehair worms

Mantids (*H. formosana*, *H. patellifera*, and *T. sinensis*) infected with horsehair worms were collected from trees, shrubs, and grasses near water in Taiwan and Japan. Most of the adult horsehair worms emerged from the mantids after the hosts' abdomens were immersed in water. Some individuals inside the mantids were only found after we had dissected the mantids. In total, 30 mantids with 40 horsehair worms inside them were examined (23 hosts with a single worm, five with two, one with three, and one with four, see Table 1). We first fixed the horsehair worms (except for two females which laid eggs (see below for detail)) and their hosts in a 75% alcohol solution for several days and then kept them in a 95% alcohol solution to preserve the DNA. Collection data are given in Table 1 including the locality, date, and collector.

Two pairs of adult horsehair worms (two males from Sindian, New Taipei City, and two females from Taipei Zoo) were collected on August 2, 2007. They were reared together in a plastic container (20 cm in diameter and 10.5 cm high) filled with 800 ml of aerated tap water, and maintained at 27 ± 1 °C. The females were kept in water to lay eggs for 1 month, then fixed in a 75% alcohol solution and preserved in a 95% alcohol solution. Egg strings were found after 5 days and had hatched to larvae by 1 month later. Larvae were kept alive until being observed under light microscopy. Egg strings stuck on rocks were collected from Wufengqi Waterfalls, Yilan County, on July 21, 2010. They were brought back to the laboratory and kept in a tank with 20 L of tap water under the same conditions as described above. Eggs hatched about 8 weeks later and were then fixed and preserved as described above.

Morphological examination

For adult specimens, the body surface was examined under light microscopy (Olympus BH-2, PM-10AD, Tokyo, Japan). For each specimen, a fragment of about 1 cm long of the mid-body was removed and cut longitudinally. Instead of using a scalpel to directly remove the internal tissues, we dipped the fragment into a 1% KOH solution for

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Table 1. Specimen information examined in the present study

Host					Horsehair worms			
Species	**Collecting date**	**Locality**	**Longitude and latitude**	**Collector**	**Species**	**Sex**	**GenBank no.**	**Deposition**
H. formosana[1]	10-VII-2008	Xindian, New Taipei City, Taiwan	24°56'58.62"N, 121°34'2.90"E	Ming-Chung Chiu	*C. formosanus*	Male	HM044112	NTU
"	"	"	"	"	"	Female	HM044113	NTU
"	"	"	"	"	"	Female	HM044114	NTU
"	"	"	"	"	"	Female	HM044115	NTU
H. formosana	12-VII-2008	Xindian, New Taipei City, Taiwan	24°56'58.62"N, 121°34'2.90"E	Ming-Chung Chiu	*C. formosanus*	Female	HM044119	LBM
H. formosana	20-VII-2008	Xindian, New Taipei City, Taiwan	24°56'58.62"N, 121°34'2.90"E	Ming-Chung Chiu	*C. formosanus*	Male	HM044116	NMNS
"	"	"	"	"	"	Male	HM044104	NMNS
"	"	"	"	"	"	Female[2]	**HM044105**	NMNS
H. formosana	10-VII-2009	Shimen, New Taipei City, Taiwan	NA	Chun-Kai Wang	*C. formosanus*	Male	HM044123	LBM
H. formosana	2-VIII-2007	Taipei Zoo, Taipei City, Taiwan	24°59'44.70"N, 121°34'49.49"E	Ming-Chung Chiu	*C. formosanus*	Female	HQ322115	NTU
"	"	"	"	"	"	Female	HQ322116	NTU
H. formosana[3]	29-I-2008	Taipei Zoo, Taipei City, Taiwan	24°59'44.70"N, 121°34'49.49"E	Ming-Chung Chiu	*C. formosanus*	Male	HM044122	NTU
"	"	"	"	"	"	Female	HM044121	NTU
H. formosana	23-VII-2008	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Male	HM044111	NTU
H. formosana	23-VII-2008	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Male	HM044118	LBM
"	"	"	"	"	"	Female	HM044108	LBM
H. formosana	24-VI-2009	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Male[3]	**HM044124**	NMNS

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Host					Horsehair worms			
Species	**Collecting date**	**Locality**	**Longitude and latitude**	**Collector**	**Species**	**Sex**	**GenBank no.**	**Deposition**
"	"	"	"	"	"	Male	HM044125	NMNS
H. formosana	16-VII-2009	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Male	HM044126	NTU
H. formosana	3-VIII-2009	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Male	HM044127	NMNS
H. formosana	16-VII-2009	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Male	HM044128	NMNS
H. formosana	23-VII-2008	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Female	HM044117	NMNS
H. formosana	10-VII-2008	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Female	HM044120	NTU
H. formosana	23-VII-2008	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Female	HM044106	NTU
H. formosana	23-VII-2008	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Female	HM044109	NTU
H. formosana	23-VII-2008	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	*C. formosanus*	Female	HM044110	NTU
H. formosana	5-VII-2008	Taroko National Park, Hualien, Taiwan	NA	Tsung-Hung Yang	*C. formosanus*	Male	HM044107	NTU
H. patellifera	30-IX-2006	Taipei Zoo, Taipei City, Taiwan	24°59'44.70"N, 121°34'49.49"E	Ming-Chung Chiu	*C. formosanus*	Male	JF808204	NTU
H. patellifera	X-2008	Hsinchu City, Taiwan	NA	Ju-Chun Hsu	*C. formosanus*	Female	JF808197	NTU
H. patellifera[3]	18-VII-2003	Lyudao, Taitung, Taiwan	NA	Hsing-Yu Chou	*C. formosanus*	Male	JF808203	NTU
"	"	"	"	"	"	Male	JF808205	NTU
H. patellifera	16-X-2010	Sakado, Saitama, Japan	35°96'44.87N, 139°40'27.39E	Etsuko Suzuki	*C. formosanus*	Female	JF808194	NTU
H. patellifera	1-XI-2010	Kijo, Miyazaki, Japan	32°13'30.59"N, 131°24'16.62"E	Yasukuni Ono	*C. formosanus*	Female	JF808198	LBM

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Host					Horsehair worms			
Species	**Collecting date**	**Locality**	**Longitude and latitude**	**Collector**	**Species**	**Sex**	**GenBank no.**	**Deposition**
H. patellifera	1-XI-2010	Kijo, Miyazaki, Japan	32°13'30.59"N, 131°24'16.62"E	Yasukuni Ono	*C. formosanus*	Female	JF808199	NTU
H. patellifera	10-XI-2010	Kijo, Miyazaki, Japan	32°14'57.82"N, 131°23'3.93"E	Yasukuni Ono	*C. formosanus*	Female	JF808202	NTU
H. patellifera	11-XI-2010	Miyazaki, Miyazaki, Japan	31°56'54.15"N, 131°16'22.71"E	Yasukuni Ono	*C. formosanus*	Female	JF808200	NTU
H. patellifera	26-XI-2010	Kijo, Miyazaki, Japan	32°10'21.37"N, 131°27'36.53"E	Yasukuni Ono	*C. formosanus*	Male	JF808196	NTU
H. patellifera	26-XI-2010	Kijo, Miyazaki, Japan	32°12'55.36"N, 131°24'52.13"E	Yasukuni Ono	*C. formosanus*	Female	JF808201	NTU
H. patellifera	16-X-2010	Sakado, Saitama, Japan	5°96'44.87N, 139°40'27.39E	Wataru Toki	*C. formosanus*	Female	JF808195	NTU
T. sinensis	5-XI-2010	Kijo, Miyazaki, Japan	32°10'21.50"N, 131°27'36.53"E	Yasukuni Ono	*C, japonensis*	Male	JF808206	NTU

LBM: Lake Biwa Museum; NMNS: National Museum of Natural Science; NTU: National Taiwan University.

[1] No host specimen preserved.

[2] Allotype.

[3] Holotype.

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2 h. The internal tissues became transparent and removable. The cuticles were placed on a microslide and observed under a microscope at a magnification of 40–200×. Eggs and newly hatched larvae were placed on the microslides, each with a drop of water and a cover glass. They were observed alive under the light microscope (at a magnification of 400×).

SEM was also used to examine adult and larval specimens. Its preparation protocol followed that of Schmidt-Rhaesa (2002b). Fragments of the anterior end, mid-body, and posterior end of preserved adult and larval specimens were dehydrated with a series of 75%, 95%, and 100% ethanol solutions and then replaced by acetone after using a series of alcohol/acetone mixtures of 2:1, 1:1, 1:2, and 0:1. Samples were then critical-point-dried, gold-sputter-coated, and examined under an SEM (JEOL JSM-5600, Tokyo, Japan) at a magnification of 100–15,000×.

The terminology of larvae follows that of Bohall et al. (1997), Hanelt and Janovy (2002), and Bolek et al. (2010).

Phylogenetic analysis

Genomic DNA was extracted from fragments of horsehair worms and whole larvae using an ALS Tissue Genomic DNA Extraction Kit (Kaohsiung, Taiwan). A partial COI sequence was amplified by a polymerase chain reaction (PCR) with a set of universal primers (LCO1490 and HC02198) (Folmer et al. 1994). The PCR was initiated at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 40 °C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were obtained by electrophoresis in 1.5% agarose gels and sequenced.

For the phylogenetic analysis, the COI sequence of *Paragordius* sp. (GenBank no. AY428843) was used as an outgroup. The 528 nucleotide base pairs of high quality were aligned using CLUSTALX 2.0.10 (Thompson et al. 1997). Pairwise genetic distances were calculated, and a phylogenic tree was reconstructed by the Neighbor-joining (NJ) method based on the Kimura 2-parameter model using MEGA 4.0.2 (Tamura et al. 2007). The support for the topology of the NJ tree was estimated by bootstrapping using 1000 replicates.

Results

***Chordodes formosanus* Chiu, 2011, sp. n.**
urn:lsid:zoobank.org:act:288E5D71-A694-4B65-BC15-164606F0DE4B
http://species-id.net/wiki/Chordodes_formosanus

Type locality. Wufengqi Waterfalls (24°49'55.62"N, 121°44'50.10"E), Jiaushi Township, Yilan County, Taiwan (Holotype). Dachijieu (24°56'59.21"N, 121°34'2.12"E), Sindian (New Taipei City) (allotypes). Paratypes collected from Taiwan and Japan:

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Taipei Zoo (Taipei City), Sindian (New Taipei City), Taroko National Park (Hualien County), Wufengqi Waterfalls (Yilan County), Taiwan and Miyazaki Prefecture and Sakado (Saitama Prefecture), Japan. For detailed data, see Table 1.

Type material. Partial bodies of holotype (male, 167 mm), and allotype (female, 282 mm) deposited at the Department of Entomology, National Taiwan University with the hosts. Paratypes deposited at the Department of Entomology, National Taiwan University, Taipei, and National Museum of Natural Science, Taichung, Taiwan and Lake Biwa Museum, Shiga, Japan. For detailed information, see Table 1.

Type-host. *Hierodula formosana* Giglio-Tos (Mantodea: Mantidae). *Hierodula formosana* endemic to Taiwan, and the adult always emerging from late June to early August. Hosts of some samples belonging to *H. patellifera* which distributed in both Taiwan and Japan. Their adults usually emerging in late autumn, about 2 months later than *H. formosana*.

Etymology. The specific name refers to Taiwan, the collection locality of the type specimens.

Description. (Figs 1–5)

Male adult (n = 17) (Figs 1, 2). Body length 74–277 mm, width (widest) 0.7–1 mm (after dehydration). In alcohol-preserved specimens, body rough and flat with dorsal and ventral grooves; dark-brown with bright lengthwise regions on both dorsal and ventral sides and darkly pigmented line on ventral side in most specimens (Fig. 1D).

Posterior end (Fig. 1C) not lobed, with short spines (ca. 5–12 μm) among areoles on margin. Cloacal opening subterminal, oval, 27–78 μm long and 17–63 μm wide. A pair of oval regions without areoles posterior to cloacal opening, each with scattered bristles extending as two rows of ventral strips (155–160 μm wide), structured by cord-like folds or flat areoles; flat areoles ornamented with short filaments in a cluster on top or scattered on cord-like folds, or absent. Paired oval bristlefields (70–77 μm wide and 145–243 μm long) bearing bristles on borders between flat areoles and normal areoles on lateral side of cloacal opening; bristles in bristlefields varying among individuals; some bearing only shorter or thinner unbranched bristles and some with both branched and unbranched bristles (Figs 2D–F). Anterior end tapered, same color as body, with white tip (white cap) but no dark collar under a stereomicroscope. Under SEM, anterior end round with moderately flat areoles and short bristles on surface; about 10 of them elevated and cone-like near anterior terminal; long thick bristles scattered among areoles, some between areoles and some penetrating areoles (Fig. 2C). Anterior end on one individual with residual larval cuticle tapered but flat terminally (Fig. 2A); also flat surrounding ornamentations and bristles (Fig. 2B). Mouth opens terminally in some individuals.

Entire body covered by areoles with cord-like folds in between. Areoles characterized into five types (simple, tubercle, thorn, circumcluster, and crowned areoles). Simple areoles (Fig. 1A), most abundant, covering most of body surface except anterior end and ventral side of posterior end; each 5–8 μm in diameter, more or less circular or oval, generally with a smooth surface but some with dots, grooves, or short bristles on surface. Simple areoles varying in height and some significantly elevated areoles in

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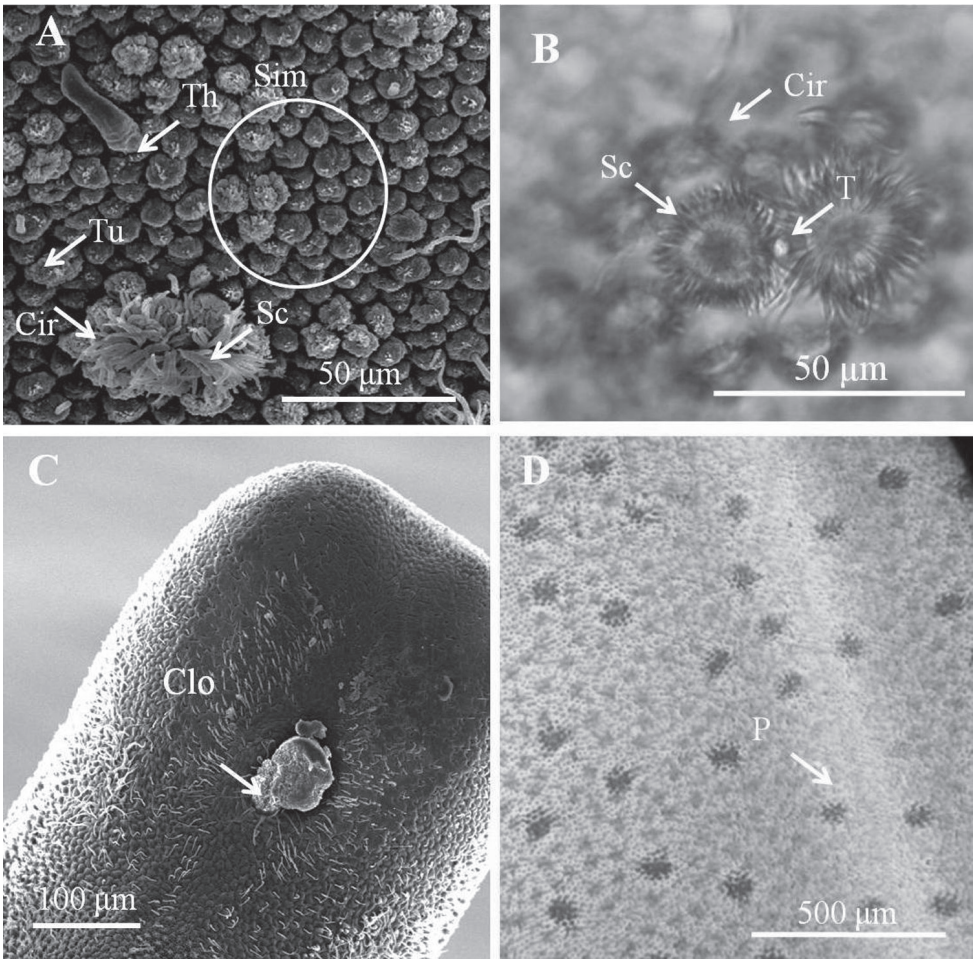

Figure 1. Male adult of *Chordodes formosanus* sp. n. **A** Cuticular surface with five types of areole **B** paired crowned areoles with a central tubercle **C** posterior end of the male **D** bright lengthwise regions with a darkly pigmented line on the ventral side of male body. Cir, circumcluster areole; Clo, cloacal opening; P, pigmented line; Sc, short-crowned areole; Sim, simple areoles; T, central tubercle; Th, thorn areole; Tu, tubercle areole.

clusters of two to ten, looking like bulging areoles as mentioned by Schmidt-Rhaesa et al. (2008); but darker under light microscopy (Fig. 1D). Tubercle areoles (Fig. 1A) scattered among simple areoles, each shaped similarly to simple areole but with a tubercle (6–9 µm long) on apically concave center. Thorn areoles (Fig. 1A) distributed slightly along dorsal and ventral middle lines, similar to tubercle areoles but with a long solid thorn (22–57 µm long) instead of a tubercle. Thorn areoles small or absent in two samples. Crowned areoles clustered in pair with a central tubercle in between and surrounded by 12–20 circumcluster areoles with short filaments on apical surface (short-crowned areoles) (Figs 1A, B); scattered over trunk except anterior and posterior ends; each with medium filaments (10–15 µm) originating from apical center and

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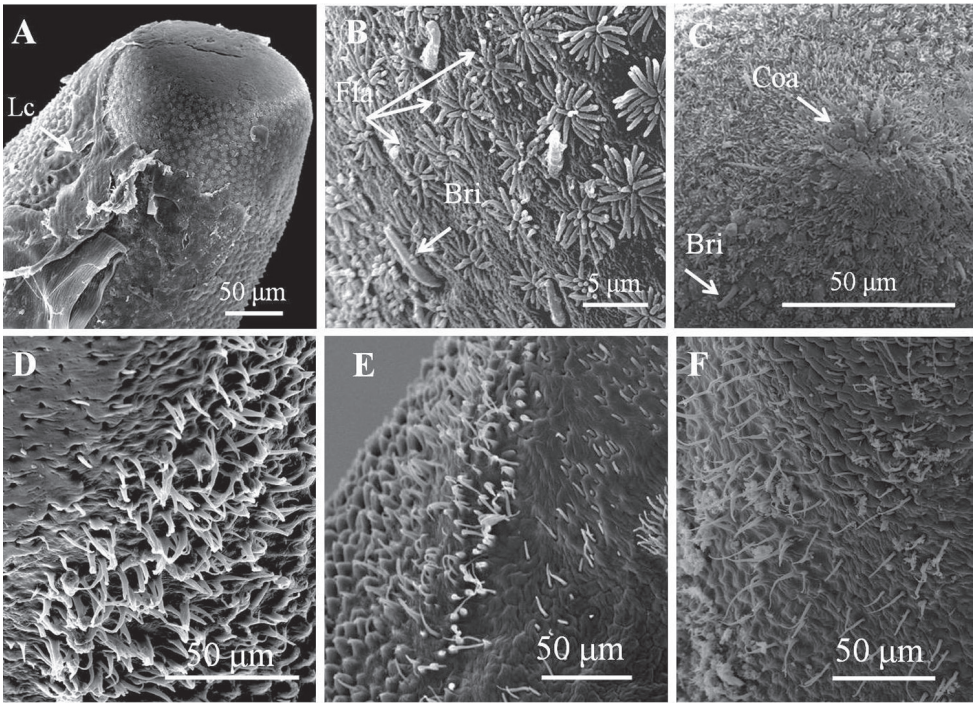

Figure 2. Details of ornamentations on anterior and posterior ends of male *Chordodes formosanus* sp. n. **A** Anterior end with larval cuticle **B** flat ornamentations and bristles on top of anterior end **C** cone-like areoles with bristles on top of anterior end **D–F** bristlefields with branched and unbranched bristles (D), short and unbranched bristles (E), or thin and unbranched bristles (F). Bri, bristle; Coa, cone-like areole; Lc, residual of larval cuticle; Fla, flat ornamentations.

sidelong to edges; only one male with a few crowned areoles containing a few filaments of around 100 µm.

Female adult (n = 14) (Fig. 3). Length 263.7 (78–440) mm; body width (widest) 1–1.5 mm (after dehydration); body rough, flattened, dorsal and ventral grooves present; light to dark-brown with lengthwise regions on both dorsal and ventral sides, and darkly pigmented line on ventral side in most specimens. Some individuals with dark patches on bodies.

Posterior end (Fig. 3B) rounded, slightly swollen, covered by moderately flat areoles with cord-like folds surrounding cloacal opening; short bristles (10–27 µm) scattered between borders of moderately flat areoles and cord-like folds. Cloacal opening on terminal end, circular, 18–33 µm in diameter, no circumcloacal spine.

Anterior end with similar structure and color to males except lower cone-like areoles; terminally flat anterior end also appearing in one individual. Pattern and distribution of areoles (Fig. 3A) also similar to those of males but much more crowded in most individuals. Thorns of areoles shorter than those of most males (11–30 µm) but small or absent in three females. Cord-like folds present between areoles. Crowned areoles

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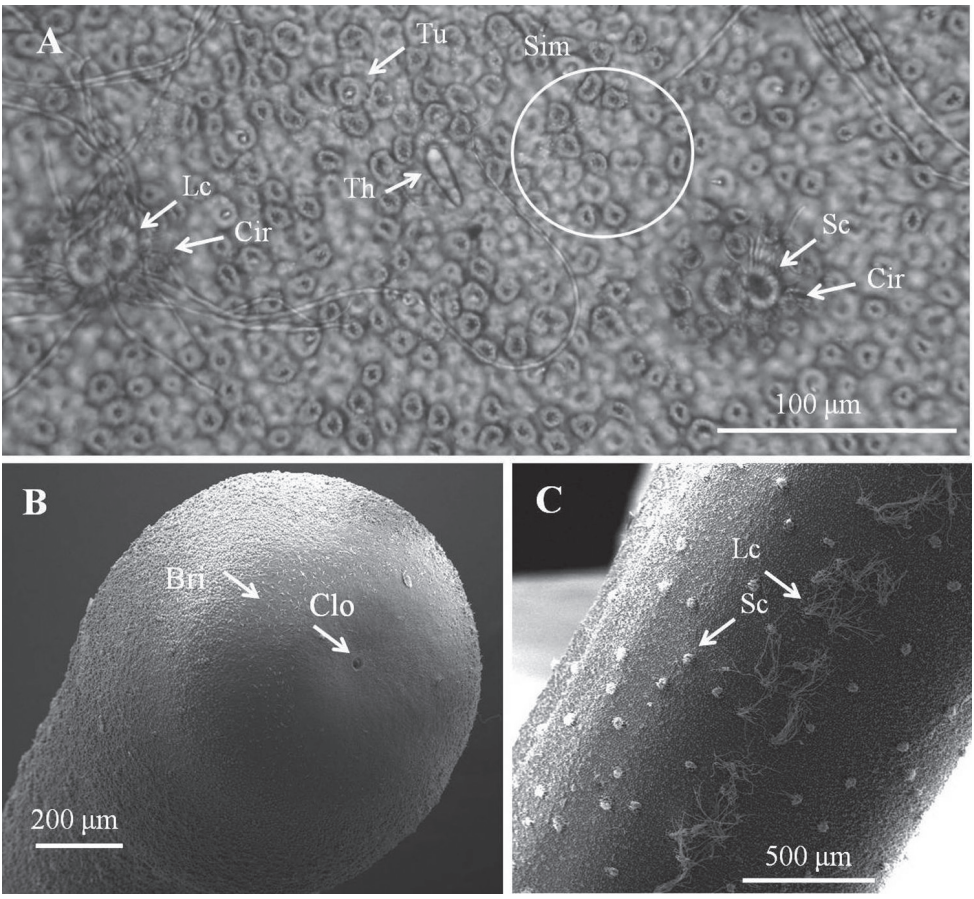

Figure 3. Female adult of *Chordodes formosanus* sp. n. **A** Cuticular surface with six types of areole **B** posterior end of female **C** ventral side of female body. Bri, bristle; Cir, circumcluster areole; Clo, cloacal opening; Lc, long crowned areole; Sc, short-crowned areole; Sim, simple areoles; Th, thorn areole; Tu, tubercle areole.

scattered over trunk as in males while roughly arranged in two lines on ventral and dorsal midlines, bearing significantly longer filaments (longest apical filaments ranging 65.57–392.25 μm (237.47 ± 66.22 μm, for details see "Diagnosis")) (Figs 3A, C).

Eggs (Fig. 4). In laboratory, egg strings stuck onto substrate or drifting on bottom. Eggs (6 days after being laid) (Fig. 4B) nearly circular, 30.39 ± 1.15 μm (n = 10) in diameter. Egg strings white when laid and becoming light-brown within 1 day, turning dark-gray just before hatching. Eggs collected in field (Fig. 4C) all stuck onto rocks; mostly brown to gray, but some light-brown as those just laid in laboratory.

Larvae (Figs 4, 5). Larvae remaining near egg strings after hatching, not active. Under light microscopy, larval preseptum (Fig. 4A) averaging 20.55 (16.32–24.78) μm long and 13.21 (10.93–16.34) μm wide; postseptum averaging 24.91 (22.52–27.44) μm long and 10.06 (9.25–11.49) μm wide, stylet averaging 11.04 (9.59–13.25) μm

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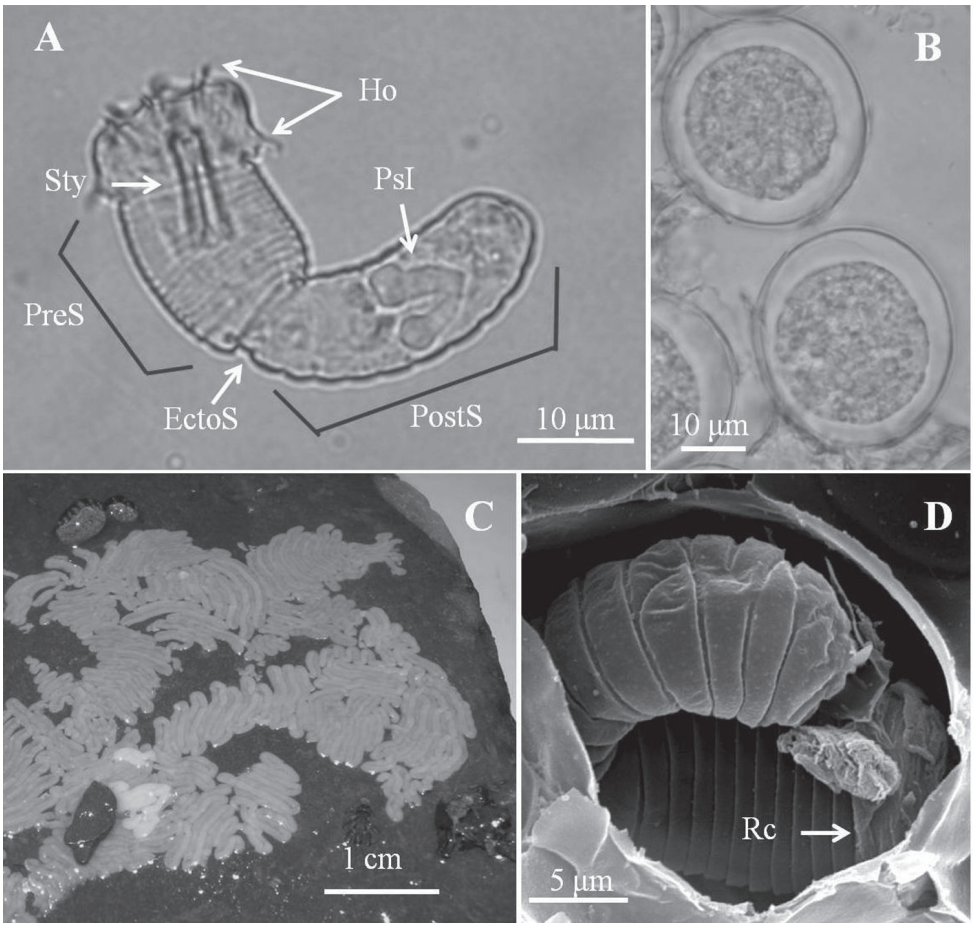

Figure 4. Eggs and larvae of *Chordodes formosanus* sp. n. **A** Live larva in water **B** eggs (6 days after being laid) **C** egg strings stuck onto a rock **D** larva in an egg with residual cuticle. EctoS, ectodermal septum; Ho, hooklet; PostS, postseptum; PreS, preseptum; PsI, pseudointestine gland; Rc, residual cuticle; Sty, stylet.

long and 3.36 (2.76–3.91) µm wide. Pseudointestines V-shaped (Fig. 4A) with one small and one large branch, both with a swelling on posterior ends. Large branch averaging 8.27 (7.28–9.82) µm, small branch averaging 6.70 (5.43–7.59) µm long. Under SEM, larvae superficially annulated with 13 segments on preseptum and 10 on postseptum, ectodermal septum as a single segment between them. Three sets of hooks arranged in three rings on anterior preseptum (Fig. 5A): outer ring containing seven hooks (outer hooks), two ventrally positioned and closely together on base (ventral double hook); six hooks on second ring located between each outer hook (middle hook); inner ring containing at least three inner spines, but real number unknown. A stylet (Figs 5A, C) appearing inside preseptum, ornamented with two sets of spines: nine spines on dorsal and ventral sides of stylet, five small lateral papillae on left side.

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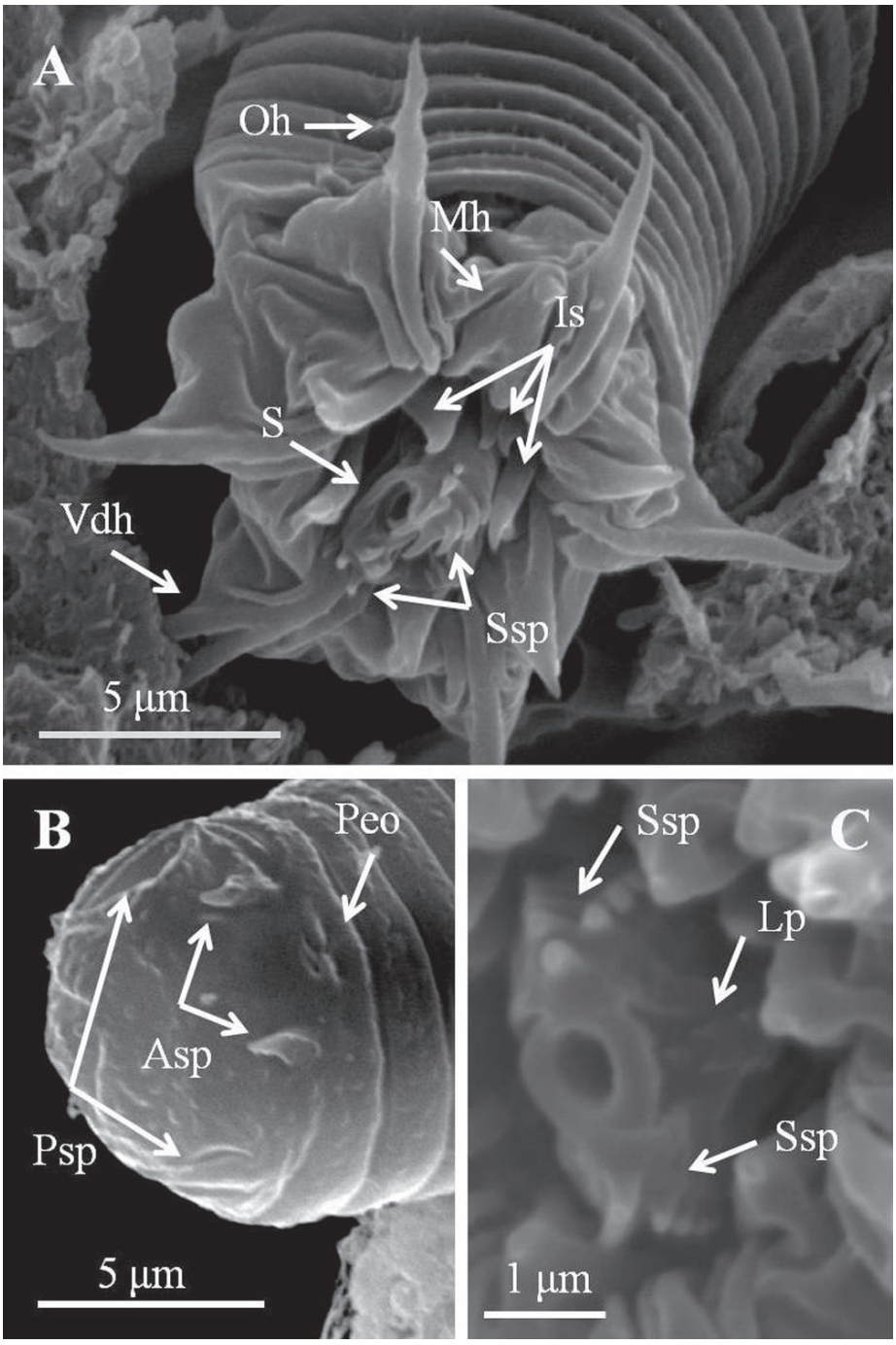

Figure 5. Detail of larvae of *Chordodes formosanus* sp. n. **A** Anterior view of a larva showing stylet and hook arrangement **B** posterior view of a larva **C** style with spines and lateral papillae. Asp, anterior terminal spine; Lp, lateral papillae; Is, inner spines; Mh, middle hook; Oh, outer hook; Peo, Pseudointestine exterior opening; Psp, posterior terminal spine; S, stylet; Ssp, stylet spines; Vdh, ventral double hook.

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A pair of anterior and posterior terminal spines (Fig. 5B) on posterior of postseptum. Pseudointestine exterior opening (Fig. 5B) centrally located between anterior terminal spines on ventral body. Several larvae covered by residual skin: one observed in broken egg suggesting that molting had occurred before emergence (Fig. 4D).

Diagnosis. Horsehair worms from the mantids *H. formosana* and *H. patellifera* were characterized by all six types of areoles, including simple, tubercle, thorn, circumcluster, short-crowned, and long-crowned areoles in the female. The same six areole types are similar to those of *C. japonensis* described by Inoue (1952) and Baek (1993). Nevertheless, the significantly longer filaments on female crowned areoles suggest they belong to a new species, *C. formosanus* sp. n. By the way, the absence of long-crowned areoles in our male sample of *C. formosanus* sp. n. probably implies their potential for distinguishing these two different species. However, since the dimorphism of male crowned areoles has not been mentioned in *C. japonensis*, more studies are needed to uncover this phenomenon.

The crowned areole is an autapomorphy of the genus *Chordodes*. In *C. formosanus* sp. n. and *C. japonensis*, it is composed of two major areoles ornamented with apical filaments and several surrounding circumcluster areoles. The dimorphic length of the apical filaments divides the crowned areoles into two types, short-crowned areoles with short ornamental filaments and long-crowned areoles with long ones. All samples we checked (both sexes of *C. formosanus* sp. n. and one male *C. japonensis*) had short-crowned areoles scattered all over the body trunk, with the long-crowned areoles only appearing on the ventral and dorsal midlines of the female *C. formosanus* sp. n. and male *C. japonensis,* but not the male *C. formosanus* sp. n. We did not personally observe the female *C. japonensis,* but these dimorphic crowned areoles must be present according to the descriptions of Inoue (1952) and Baek (1993). Additionally, the apical filament lengths of long-crowned areoles were significantly longer on *C. formosanus* sp. n. We randomly chose two to five sets of long-crowned areoles from our female samples and measured each of their longest apical filaments. In the 68 sets of long crowned areoles, the longest apical filaments ranged 65.57–392.25 (237.47 ± 66.22) μm. Fifty-one of these 68 (75%) sets of crowned areoles had apical filaments of > 200 μm. The longest apical filaments in our male *C. japonensis* were 92.03–139.70 μm. Significantly shorter filaments in *C. japonensis* were also described by Inoue (1952) and Baek (1993). *Chordodes japonensis* has long-crowned areoles with filaments of around 50 μm (Inoue 1952; fig. 1a) and filaments of < 200 μm in the description by Baek (1993). For other differences and detailed comparisons, see Table 2 and the "Discussion".

Chordodes japonensis Inoue, 1952

http://species-id.net/wiki/Chordodes_japonensis

Material examined. Examined male collected with its host from Miyazaki Prefecture, Japan (32°10'21.50"N, 131°27'36.53"E) by Yasukuni Ono on 5-XI-2010. Partial body of horsehair worm deposited with its host at Department of Entomology, Na-

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Table 2. Comparison of areolar types between *Chordodes formosanus* sp. n. (Schmidt-Rhaesa 2004, samples of which were considered to be *C. japonensis*), *C. japonensis* (Inoue 1952, Baek 1993) and in this investigation

Areolar type	*Chordodes formosanus* sp. n.		*Chordodes japonensis*		
	This study	**Schmidt-Rhaesa (2004)**	**This study**	**Inoue (1952)**	**Baek (1993)**
Sample size	17 ♂♂, 22 ♀♀	1 ♂**, 1 ♀	1 ♂	49 ♂♂, 37 ♀♀	17 ♂♂, 7 ♀♀
Collecting locality	Taiwan, Japan	Japan	Japan	Japan	Korea
Host	*Hierodula formosana* and *H. patellifera*	*H. patellifera* (unknown for the male worm)	*Tenodera sinensis*	Not mentioned	Not mentioned
Crowned areoles with short projections	+	+	+	-	+
Crowned areoles with long projections	+ (most areoles with filaments > 200 μm)	+ (> 200 μm)	+ (mostly around 100 μm, maximum 140 μm)	+ (around 50 μm in Fig. 1a)	+ (< 200 μm)
Sexual dimorphism in crowned areoles	+	-	? (Only males were investigated)	-	-
Circumcluster areoles	+ (12-20)	+ (at least 12 in Fig. 2C)	+ (7-14)	+ (10 in Fig. 1a)	+ (7-17)
Tubercle areoles	+	+	+	+	+
Thorn areoles (spine areoles)	+ (small or absent in some samples; 9/40)	-	+	+	-
Bulging areoles	* (some elevated simple areoles in clusters of 2-10)	- (simple areoles elevated differently in Fig. 2A and 2D)	* (some elevated simple areoles in clusters of 2-5)	- (paired elevated areoles)	- (simple areoles elevated differently in Fig1)
Simple areoles	+	+	+	+	+

Terminology based on Schmidt-Rhaesa (2004) and Schmidt-Rhaesa et al. (2008).

+, present; -, absent; *, difficult to determine; ?, unknown.

**, According to the description, we consider the specimen not to be *Chordodes formosanus* sp. n.

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tional Taiwan University, Taipei, Taiwan. Accession number of partial COI sequence in GenBank: JF808206.

Host. Chinese mantids, *Tenodera sinensis* (Mantodea: Mantidae), which are sometimes classified as *T. aridifolia*.

Redescription.(Fig. 6)

Male adult (*n* = 1). Body length 220 mm, width (widest) 0.94 mm (after dehydration). In alcohol-preserved specimens, body rough and flat with dorsal and ventral grooves, dark-brown with a darkly pigmented line on ventral side.

Posterior end (Fig. 6A) not lobed, with short spines (ca. 5–13 μm) among areoles on margin. Cloacal opening subterminal, oval 44 μm long and 25 μm wide, with circumcloacal spines. A pair of oval regions without areoles posterior to cloacal opening, each with scattered bristles extending as two rows of ventral strips (115–120 μm wide) structured by cord-like folds or flat areoles. Paired oval bristlefields (82 μm wide and 231 μm long) bearing numerous branched and unbranched bristles on borders between flat areoles and normal areoles on lateral side of cloacal opening. Anterior end tapered, same color as body, with white tip (white cap) but no dark collar under stereomicroscopy. Under SEM, anterior end (Fig. 6F) smooth with short, thick bristles and small spines; mouth open on cone at anterior extremity.

Entire body covered by areoles with slightly cord-like folds in between. Areoles characterized into six types (simple, tubercle, thorn, circumcluster, and two types of crowned areoles). Simple areoles (Fig. 6C), most abundant, covering entire body surface except anterior end and ventral side of posterior end; each 5–11 μm in diameter, more or less circular or oval, surface uneven, some areas with short bristles. Simple areoles varying in height and some significantly elevated areoles in clusters of two to five, appearing as bulging areoles but darker under light microscopy. Tubercle areoles (Fig. 6C) scattered among simple areoles, each similarly shaped to simple areole but with a tubercle (about 7 μm long) on apically concave center. Thorn areoles (Fig. 6E) similar to tubercle areoles but with a long solid thorn (15 μm long) instead of tubercle; number of thorn areoles much fewer than tubercle areoles. Crowned areoles (Figs 6B, D) clustered in pairs with a central tubercle in between and surrounded by 7–14 circumcluster areoles with short filaments on apical surface; scattered over trunk; each with medium filaments (12–20 μm) originating from apical center and sidelong to edges. Crowned areoles roughly arranged in two lines on ventral and dorsal midlines, bearing significantly longer filaments (most around 100 μm, none > 150 μm) (Figs 6B, C).

Phylogeny. A phylogenetic tree of the 40 samples of horsehair worms collected from three species of mantids *H. formosana*, *H. patellifera*, and *T. sinensis* from Taiwan and Japan with one outgroup (*Paragordius* sp.) is shown in Fig. 7.

Comparison of the 40 horsehair worm samples (39 *C. formosanus* and one *C. japonensis*) revealed there were 31 haplotypes with 432 invariable sites, 64 singletons, and 32 parsimoniously informative sites. Newly sequenced COI data were deposited in the GenBank database (see Table 1 for accession nos.). Samples from the host mantids of *Hierodula* and *Tenodera* (which are considered two species, *C. formosanus* sp. n. and *C. japonensis,* respectively) were significantly separated into two groups based

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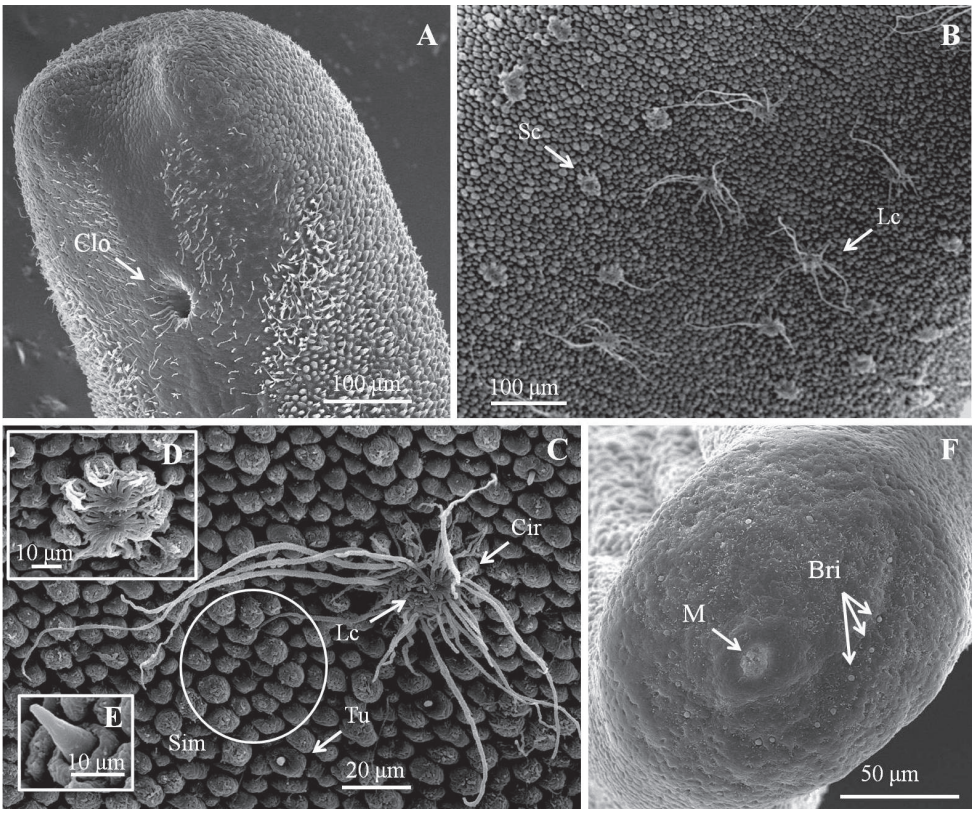

Figure 6. Male adult of *Chordodes japonensis*. **A** Posterior end of male **B** ventral side of male body **C** cuticular surface with four types of areole **D** short-crowned areoles **E** thorn areole **F** anterior end. Bri, bristle; Cir, circumcluster areole; Clo, cloacal opening; Lc, long crowned areole; M, mouth; Sc, short-crowned areole; Sim, simple areoles; Tu, tubercle areole.

on the phylogenetic tree restructured by the NJ method (Fig. 7). The genetic distance between these two groups was 0.16840. This result supports that they belong to different species as we suggested based on morphology. The phylogenetic tree also revealed a polytomic topology among the 39 horsehair worms parasitizing *Hierodula*. Although some clades were observed, they were not highly supported due to low bootstrap values and short genetic distances; the mean genetic distances among them was 0.00979 with a range of 0.000-0.01922. Since the genetic distance between the larvae collected in the field and *C. formosanus* was only 0.00759, we suggest that those larvae belong to the same species.

Discussion. In this article, a new species, *C. formosanus* sp. n., which parasitizes *H. formosana* and *H. patellifera,* was proposed and described including the morphology of the egg and larval stages. Because of a similar morphological description by Schmidt-Rhaesa (2004), we categorized the species in that study into *C. formosanus* sp. n. and also limited the mantid host range of *C. japonensis* to the genus *Tenodera*.

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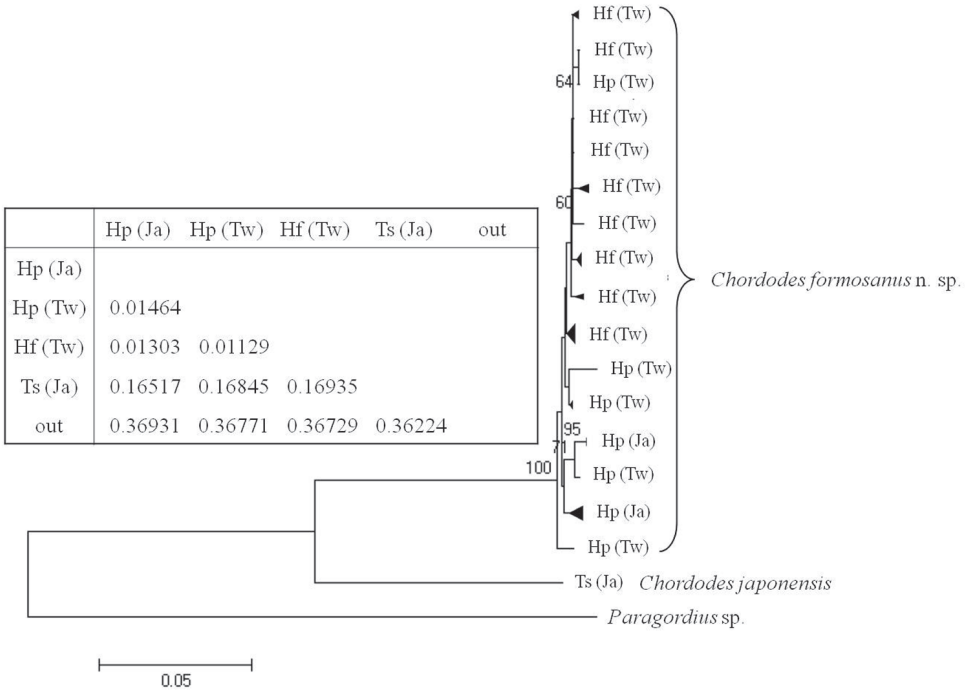

	Hp (Ja)	Hp (Tw)	Hf (Tw)	Ts (Ja)	out
Hp (Ja)					
Hp (Tw)	0.01464				
Hf (Tw)	0.01303	0.01129			
Ts (Ja)	0.16517	0.16845	0.16935		
out	0.36931	0.36771	0.36729	0.36224	

Figure 7. Neighbor-joining tree of *Chordodes formosanus* sp. n. and *C. japonensis* with genetic distances between each group. Abbreviations in the table indicate the horsehair worms' mantid hosts (Hf, *Hierodula formosana*; Hp, *H. patellifera*; Ts, *Tenodera sinensis*) and collecting localities (Tw, Taiwan; Ja, Japan). Numbers at the nodes represent the percentage of 1000 bootstrap values of > 70%. The outgroup (out) was a *Paragordius* sp.

Morphological comparison of *C. formosanus* sp. n. and *C. japonensis*

The two species, *C. formosanus* sp. n. and *C. japonensis*, can be distinguished by the presence of dimorphism in male crowned areoles and the length of long filaments on female crowned areoles. A comparison of areolar types in *C. formosanus* sp. n. and *C. japonensis* is given in Table 2. Both *C. formosanus* sp. n. and *C. japonensis* have two forms of crowned areoles on their cuticles, one with moderate attachments on the top (short-crowned areoles) and the other with significantly longer attachments (long-crowned areoles). Both types of crowned areoles were found on these two species except for the male *C. formosanus* sp. n. (the short-crowned areoles were not mentioned in the description of *C. japonensis* by Inoue (1952)), which indicates sexual dimorphism in *C. formosanus* sp. n. We believe the female worm which was previously considered to be *C. japonensis* by Schmidt-Rhaesa (2004) actually belongs to the species, *C. formosanus* n. sp, described here. The reason why the sexual dimorphism was not mentioned by Schmidt-Rhaesa (2004) is probably that the free-living male is not the same species as the female. In addition, the length of the filaments on the long-crowned areoles is also a significant character differentiating these two species; they are

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always > 200 μm in *C. formosanus* sp. n. (or > 6-fold that of paired crowned areoles) but < 200 μm in *C. japonensis* (or < 5-fold that of paired crowned areoles, and most of them are around 100 μm).

In addition to the crowned areoles, other minor differences in Table 2 (thorn areoles and bulging areoles) are much more difficult to use in discriminating these two species. Although the presence of thorn areoles is always questionable due to their small number, it is still considered a key character for distinguishing different species (Schmidt-Rhaesa et al. 2008). Thorn areoles were not reported by Schmidt-Rhaesa (2004) or Baek (1993) but appeared in the description by Inoue (1952). In our study, thorn areoles were not found in nine *C. formosanus* sp. n. but appeared in the other 31 *C. formosanus* sp. n. and one *C. japonensis*. Contrary to the thorn areoles which can be easily distinguished from other types of areoles, bulging areoles are much easier to be confused with simple areoles (Schmidt-Rhaesa et al. 2008). Although bulging areoles were consistently ignored in descriptions of *C. japonensis*, we observed various heights of simple areoles, and some of them clustered in groups similar to bulging areoles as described by Schmidt-Rhaesa et al. (2008). This renders the presence of bulging areoles in *C. japonensis* as well as *C. formosanus* sp. n. questionable.

Molting and environmental effects on morphology

About 90 species belong to the genus *Chordodes* which were proposed using only five characteristic types of areoles (Schmidt-Rhaesa et al. 2008). Unfortunately, most characters other than these areole types have seldom been mentioned. According to our observations, the complex ornamentations of the structure of the anterior end were previously considered to be smooth. A horsehair worm explores unknown environments with its head, and the head is also the first part which contacts outer environments when they emerge from a host. Schmidt-Rhaesa and Gerke (2006) suggested that the apical filaments of the crowned areoles may have sensory functions. It is rational that the anterior and posterior ends of horsehair worms with the complex ornamentations play important roles in exploration and mating. Therefore, the smooth anterior ends in some descriptions (e.g., de Villalobos and Zanca 2001) and some of our samples may have been caused by damage from the environment. In addition to the damage, some samples were found to have been covered by residual juvenile skins, indicating that the worms had just molted. Shapes of the ornamentations on the ends under the larval cuticle were flat. The molting of hairworms was observed (Schmidt-Rhaesa et al. 2003), and its potential effects on the morphology should be carefully considered in the future studies. Residual skin was also found on newly hatched larvae, and on a larva still inside an egg, indicating that molting occurred before it emerged from the egg. As a group of Ecdysozoa (Aguinaldo et al. 1997), horsehair worms molt between each instar. According to our observations of metamorphosis from cysts to wormlike juveniles and from juveniles to adults, we suspect those horsehair worms may proceed through at least three molts before maturing.

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Host specificity

Compared to the wide range of paratenic hosts, horsehair worms' definitive host range is limited to one or a few species. Because nematomorphs are sometimes found after they have emerged from their hosts, definitive information on hosts is unknown in some species. *Chordodes japonensis* was reported to be parasites of the mantids, *T. sinensis*, *T. angustipennis* (Inoue 1952), *H. patellifera* (Schmidt-Rhaesa 2004), and longhorn grasshoppers, *Hexacentrus japonicus japonicus* (Inoue 1955) in Japan. Since the hairworm described by Schmidt-Rhaesa (2004) was here considered to be *C. formosanus* sp. n., the mantid host of *C. japonensis* is now limited to two species of *Tenodera*. Until now, we have no evidence to exclude *Hex. japonicus japonicus* from being a potential host of *C. japonensis*. However, in our investigation, the host range of these two species seems to be limited to the generic level. Therefore, we still question the ability of *C. japonensis* to develop inside hosts other than mantids.

Molecular approach and perspectives

In the 40 samples from Taiwan and Japan we examined, the taxonomic status was supported not only by their morphologies, but also by the partial COI sequences. COI sequences were used to study inter- and intraspecific relationships due to the high mutation rate (Hebert et al. 2003). The low genetic divergence among our hairworm samples suggests their conspecific relationship, and our samples can also be separated from those hairworms that emerged from *Tenodera* by their significantly divergent sequences. Since species of immature stages can only be conjectured by adults in the same area (Winterbourn 2005), molecular data were herein proven to be a useful tool for identifying both adults and immatures. As molecular information for the phylum Nematomorpha is still limited, we believe more molecular data would be helpful and can be used to uncover uncertain relationships among horsehair worms in the near future.

Acknowledgments

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Appendix 2.

Morphological allometry and intersexuality in horsehair-worm-infected mantids, *Hierodula formosana* (Mantodea: Mantidae)

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SUMMARY

Parasitic castration is a strategy used by parasites to minimize damage to the host by consuming its reproductive system, which results in the morphological alteration of the host. We determined that the forewing shape and density of the antennal sensilla of field-collected adult male mantids (*Hierodula formosana*), infected by horsehair worms (*Chordodes formosanus*) was partially feminized (intersexuality), and both male and female mantids infected by horsehair worms exhibited allometric changes in their wings and walking legs. In addition, the testes of most infected male adults disappeared or reduced in size, whereas the number of ovarioles in infected female adults was unaffected. The infection mainly influenced the structures related to host reproduction and locomotion, suggesting unbalanced energy exploitation and the reduction of parasitic virulence. In addition, the intersexuality of infected male adults indicated that sexual differentiation in insects, which researchers have considered to be an autonomous process, was influenced by the infection. The similarity of the antennae of infected male adults with those of last-instar female nymphs suggested that parasitic juvenilization may cause such feminization, but the mechanism of parasitic influence on insect sex characteristics should be studied further.

Key words: allometry, intersexuality, parasitic castration, mantids, horsehair worms.

INTRODUCTION

Parasites must prevent host death to ensure the success of transmission and the support of resources. As unavoidable host mortality rises with increasing resource exploitation, virulence tradeoff becomes a critical concern (Alizon *et al*. 2009). One strategy for achieving virulence tradeoff is to reduce host fecundity to divert damage from the host (Hurd, 2001). Such parasites not only destroy or alter host gonad tissues (i.e. castration) (Baudoin, 1975), but also interfere in the host's development of sexually dimorphic structures contributing to reproduction and mating behaviours (i.e. intersexuality) (Wülker, 1964; Hurd, 2009). This behaviour resembles the virulence tradeoff caused by unbalanced energy exploitation (Hall *et al*. 2007), which occurs in numerous parasitic systems (Wülker, 1964; Baudoin, 1975; Hurd, 2009), provides an initial direction for the study of host–parasite interactions.

Horsehair worms (Phylum: Nematomorpha) are known as the castrators of terrestrial definitive hosts (Biron *et al*. 2005; Lafferty and Kuris, 2009). Larval horsehair worms live in aquatic environments and enter definitive hosts through aquatic paratenic hosts (Hanelt *et al*. 2005). Before parasitizing the definitive host, a larval worm is approximately 50 μm in length. The larva then grows larger to fill most of the host body cavity during development inside the definitive host. Despite the intense resource demands caused by the extreme size increase of the worm, the parasites generally do not kill the hosts before emerging. Instead, the parasites 'manipulate' the hosts' development and consequently cause the host to experience unique symptoms. The infected hosts are castrated and prevented from producing eggs (Biron *et al*. 2005). In addition, researchers have observed that hosts infected by horsehair worms and other ecologically close parasites, such as the mermithid, often exhibited intersexuality. Hosts including *Metrioptera brachyptera, Pholidoptera* sp., *Pterostichus niger, Blaps mucronata, Vespula germanica* (Wülker, 1964), midges (Chironomidae) (Rempel, 1940), biting midges (Ceratopogonidae) (McKeever *et al*. 1997), grasshoppers (Acrididae) (Rowell, 2000), mayflies (Baetidae) (Vance, 1996) and mantids (Roy, 2003) have been reported to exhibit parasite-induced intersexuality. These morphological alterations indicate that the parasite creates a unique developmental pathway in the host. However, few statistical comparisons in which a large sample size was used have been performed because of the difficulty of sample collection, particularly for parasites with variable life cycles (Hanelt *et al*. 2001) and those with a low infection rate (Looney *et al*. 2012). Therefore, despite the frequency of reported findings, most of these findings cannot provide a comprehensive understanding of the influence of such parasites.

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The objective of this study was to determine the influence of parasites on mantids of both sexes, which may include parasite-induced host resource reallocation in sexual characteristic development. Mantids are common hosts of horsehair worms in tropical and subtropical regions (Schmidt-Rhaesa and Ehrmann, 2001) and have been observed to undergo intersexuality after being infected. Roy (2003) discovered 1 horsehair-worm-infected male mantid, *Tarachodella monticola*, with extremely small wings that resembled those of female adults. A similar phenomenon was observed in the male holotype of *Parastagmatoptera abnormis*, which was suspected to be the 'parasite-induced' synonym of *Parastagmatoptera flavoguttata*, based on its feminized wing pigmentation, small genitalia, and small ocelli, the sizes of which were between those of healthy male and female *P. flavoguttata* (Lombardo and Umbriaco, 2011). The distinct sexual dimorphism of mantids is most likely derived from their diverse mating behaviours. Compared with the female mantid, the male mantid, as a sex-pheromone receptor (Robinson and Robinson, 1979), typically possesses stronger wings (Robinson and Robinson, 1979; Roy, 2003; Béthoux, 2010; Lombardo and Umbriaco, 2011), longer mid and hind legs (termed walking legs) (Prete *et al*. 2002), and denser grooved basiconic sensilla, which are hypothesized to be the sensory organ of the sex pheromone (Slifer, 1968; Hurd *et al*. 2004; Holwell *et al*. 2007; Allen *et al*. 2012). By contrast, the front legs (also called the raptorial legs), which are mainly involved in preying, of female mantids are longer than those of male mantids (Prete *et al*. 2002). In this study, we compared sexually dimorphic characteristics, including wing length, wing shape, leg length, density and distribution of antennal sensilla, number of antennal segments and the internal sex organs of field-collected mantids (*Hierodula formosana*) of both sexes and various infection statuses caused by parasitic horsehair worms (*Chordodes formosanus*). Researchers have typically used the juvenilization hypothesis (retention of juvenile characteristics) to explain the causation of intersexuality and reproductive inhibition (Baudoin, 1975; Hurd, 2009); therefore, we also examined the antennae of last-instar male and female mantids.

MATERIALS AND METHODS

Sample collection and preservation

A total of 197 mantids (*H. formosana*), including 188 adults (89 males and 99 females) and 9 nymphs (5 males and 4 females), were collected at 4 sites in Northern Taiwan between 2006 and 2013. These 4 sites were Xindian (New Taipei City) (24°89′ 20·21″N, 121°56″93·51″E), Taipei Zoo (Taipei City) (24°99″24·53″N, 121°58″93·66″E), Jiaushi (Yilan County) (24°83′23·16″N, 121°74′67·84″E) and Taroko National Park (Hualien County) (24°17′01·41″N, 121°61′04·99″E).

Forty adult mantids were prepared for the dissection of their internal sex organs. To separate horsehair worms (*C. formosanus*) from the host mantids, the hosts were first immersed in water for 1–5 min. Mantid samples were preserved at −80 °C until dissection.

The remaining 148 adults were used to measure the external morphology of the antennae, legs and wings. The parasites and hosts were separated using a similar method. The sexes of the mantid adults were determined by examining the external genitalia and the last abdominal sterna. The status of infection was determined by the presence or absence of worms. The number of worms per host was counted. Both the worms and hosts were preserved in 75% ethanol for the following examinations.

The sex of the horsehair worm was determined by examining the posterior end of the body, which appears round and slightly swollen in females but narrowed in males (Chiu *et al*. 2011).

Nine nymphal mantids (all uninfected) were reared in a walk-in incubator (28 ± 1 °C, 70–90% RH, 12L:12D photoperiod). Each mantid was confined in a plastic container (20 cm in diameter and 10·5 cm in height). When the wing pad became plump, the nymphs were anesthetized on ice and one of the antennae were removed and preserved in 75% ethanol for morphological examination. Since the antennal characters might change during each nymphal stage, all nymphs with 1 antenna cut were reared to adulthood to ensure that the cut antennae were from last-instar nymphs.

Dissection of internal reproductive organs. The reproductive organs of the 40 adult mantids were dissected under a stereomicroscope (Leica S8APO stereomicroscope, Wetzlar, Germany). The abdomen and the internal sex organs were removed in phosphate-buffered saline (PBS) buffer (KCl, 0·2 g; KH_2PO_4, 0·24 g; Na_2HPO_4, 1·44 g; NaCl, 8 g; in 800 mL of distilled water) and fixed in 75% ethanol for at least 5 min. The structural components of the internal sex organs were identified based on descriptions provided by Winnick *et al*. (2009). To study the effects of infection on the structure of reproductive organs, the number of ovarioles, size of testes, and size of seminal vesicles were recorded for comparison. The number of ovarioles was counted under a microscope. The testes and seminal vesicles were photographed using a camera (Nikon D700, Tokyo, Japan), and the sizes (areas) were measured using the polygon selection function in ImageJ 1.47 (Abràmoff *et al*. 2004).

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Measurement of external morphological traits. The morphological traits of the antennae of adults and nymphs, including (1) density of the grooved basiconic sensillum on antennae, (2) the first segment bearing grooved basiconic sensilla, and (3) the total number of antennal segments, were examined using a scanning electron microscope (SEM) (JEOL JSM-5600, Tokyo, Japan) or a light microscope (Olympus BH-2, PM-10AD, Tokyo, Japan). Other traits specific to adults, including (1) pronotum length, (2) raptorial leg length, (3) mid leg length, (4) hind leg length, (5) forewing length, (6) hindwing length and (7) forewing shape, were examined and photographed using a camera (Nikon COOLPIX P6000, Tokyo, Japan).

Measurement of antennal characteristics. A total of 54 antennae were examined using an SEM following the protocol described by Hurd *et al*. (2004) and Allen *et al*. (2012). The antennae were sequentially dehydrated in 75, 95 and 100% ethanol, and 2:1, 1:1, 1:2 and 0:1 ethanol–acetone mixtures, each for 15 min. Samples were then critical-point-dried, gold-sputter-coated and examined under an SEM at a magnification of 200–10 000. The remaining 107 antennae were examined using a light microscope.

To estimate the density of grooved basiconic sensilla on each antennal flagellum, we categorized the morphologies of the sensilla into 3 types (large trichoid sensilla, small trichoid sensilla and grooved basiconic sensilla (Fig. 1A)) according to the description provided by Hurd *et al*. (2004). The frequency distributions of their lengths were estimated by choosing 3 sensilla of each type from 24 samples (4 uninfected male adults, 4 uninfected female adults, 4 infected male adults, 4 infected female adults, 4 nymphal males and 4 nymphal females). Among the 3 types of sensilla, the grooved basiconic sensillum is considered to be the sex pheromone receptor and exhibits distinct sexual dimorphism (Slifer, 1968; Hurd *et al*. 2004). The density of grooved basiconic sensilla was calculated using the average number of sensilla per $25 \times 25\ \mu m^2$ on each selected antennal flagella. One antennal flagellum was selected for every 10 segments from the 10th to 100th segment; thus, 10 antennal segments were selected from each sample. The narrow median strip of each antennal segment was first divided into 16–96 cells of $25 \times 25\ \mu m^2$ (Fig. 1B). The total number of grooved basiconic sensilla in this narrow median strip was counted and then divided by the number of square areas. In addition to the sensillum density, the first segment bearing grooved basiconic sensilla and the total number of flagellum segments were also recorded (samples with incomplete or broken antennae were not included in the analysis of the total number of segments).

Measurement of wing, leg and pronotum characteristics. Wings, legs and pronota were removed from specimens and preserved in 75% ethanol solution. The wings were unfolded, flattened and covered by a transparent plastic slide. All dissected parts were photographed using a camera with a scale of 1 mm.

Pronotum and leg lengths were measured according to the description provided by Prete *et al*. (1990, 2002). The pronotum was measured from the rostral-most to the caudal-most midpoint of the prothoracic tergum. The leg was measured from the coxa to the end of the tarsus (the length of the tarsus was not included in the analysis of raptorial legs). The wings were measured according to sclerotized wing venation, as described by Roy (2005). The length of the forewing was measured from the base of the costal vein to the end of the second radial vein, which is the visually longest wing length. The length of the hindwing was measured from the base of the costal vein to the end of the radial posterior vein. The measurements were performed using the segmented line function in ImageJ 1.47, and calibrated spatially to the scale included in each picture.

Measurement of forewing shape characteristics. The forewing shape index was computed using the following formula:

$$(BR - AR)/(BR + AR)$$

where AR is the area above the radial vein and BR is the area below the radial vein. A high shape index indicates the relatively large area below the radial vein. The area of each part was measured using the polygon selection function in ImageJ 1.47.

Statistical analysis. Continuous data were expressed as mean ± standard deviation. P values less than 0·05 were considered statistically significant. The normality of data was tested using a Pearson's chi-square test. The homogeneity of variance in the analysis of covariance (ANCOVA) was tested using a Breusch–Pagan test.

The number of ovariole per ovary, area of the testes and area of the seminal vesicles were compared between the infected and uninfected hosts. When the data exhibited normal distribution, comparisons were performed using a 2-tailed Student's t-test with unequal variance. When data did not exhibit normal distribution, the Mann–Whitney U-test was used for comparison. Pronotum length and forewing shape index were also compared using a 2-tailed Student's t-test with unequal variance for each sex. Multiple comparisons of Student's t-test results with P values adjusted using Bonferroni correction were performed to compare antennal characteristics, including the density of the grooved basiconic sensilla, the first segment bearing grooved basiconic sensilla, and the total number of flagellum segments.

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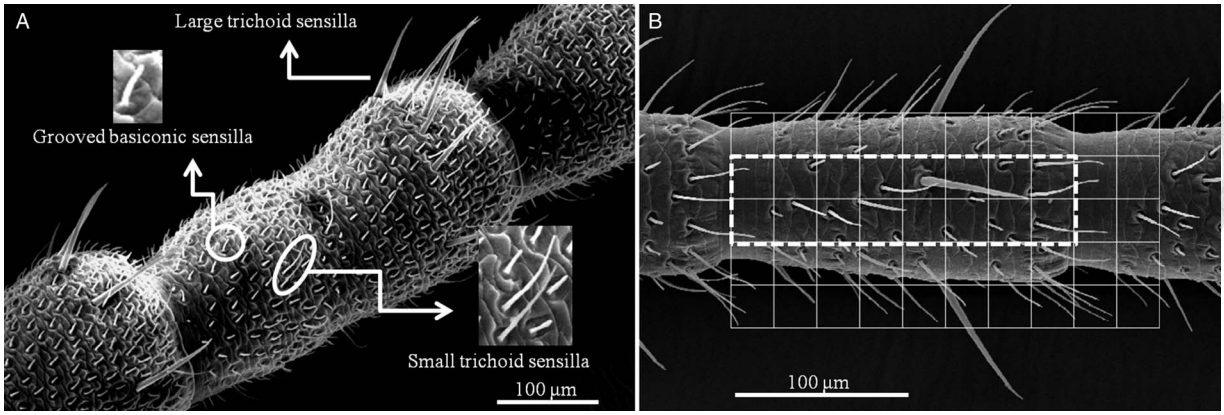

Fig. 1. Three types of antennal sensilla (large trichoid sensilla, small trichoid sensilla, and grooved basiconic sensilla) on the mantids (*Hierodula formosana*) (A), and the method used to calculate the density of the grooved basiconic sensilla (B). The narrow median strip (dotted line area) of each antennal segment was first divided into 16–96 cells of 25-μm^2 areas. The total number of grooved basiconic sensilla in this narrow median strip was counted and then divided by the number of square areas. In this example, 9 grooved basiconic sensilla were identified in the narrow median strip, which was divided into 16 square areas. Thus, the density was 0·5625.

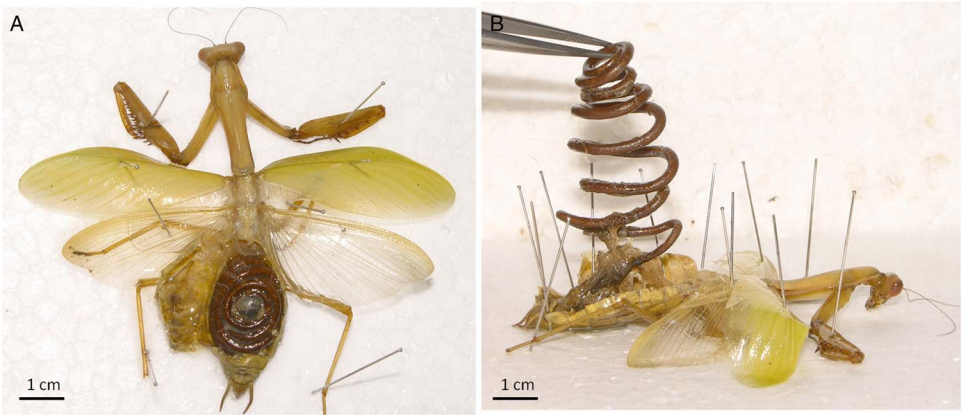

Fig. 2. Dorsal (A) and lateral (B) view of a female mantid, *Hierodula formosana*, harboring a female horsehair worm, *Chordodes formosanus*.

Data that were not normally distributed were also analysed using the Mann–Whitney *U*-test.

The leg length and wing length were rescaled based on body size. Body size was determined by the length of the pronotum rather than the full body length, which may vary if the soft abdomen contains a horsehair worm. The data compared in this study were first separated into 2 groups by the sex (uninfected male adult + infected male adult and uninfected female adult + infected female adult) and tested for variance homogeneity and slope ($\beta 1$) homogeneity in each group using a linear regression model (character length ~ infection + pronotum length + error). Data that were consistent with the assumption of homogeneity of variance were analysed using ANCOVA, with sex and infection status as independent variables. The effects of infection and sexual dimorphism on leg length and wing length were tested by comparing the vertical shift of the regression lines of each characteristic against the pronotum length. If the assumption of homogeneity of variance was violated, the trait lengths were rescaled by dividing the value by the pronotum length, and the ratios were compared using Student's *t*-test (normally distributed data) or the Mann–Whitney *U*-test (non-normally distributed data).

All statistical analyses were performed using R (version 3.1·0, R Development Core Team, 2014) with the base package, as well as the 'nortest' and 'car' packages downloaded from the R Web site (http://www.r-project.org/).

RESULTS

Basic infection parameters

A total of 194 horsehair worms were collected from the 50 infected male adult and 75 infected female adult hosts (Fig. 2). The infection rate is not displayed here because the locomotor activity of the infected mantid adults may have been altered (most of the infected mantids were collected during the daytime, and the uninfected mantids were mainly collected at night). The mean infection intensity of an infected male adult was 1·66 ± 1·36 (1–8)

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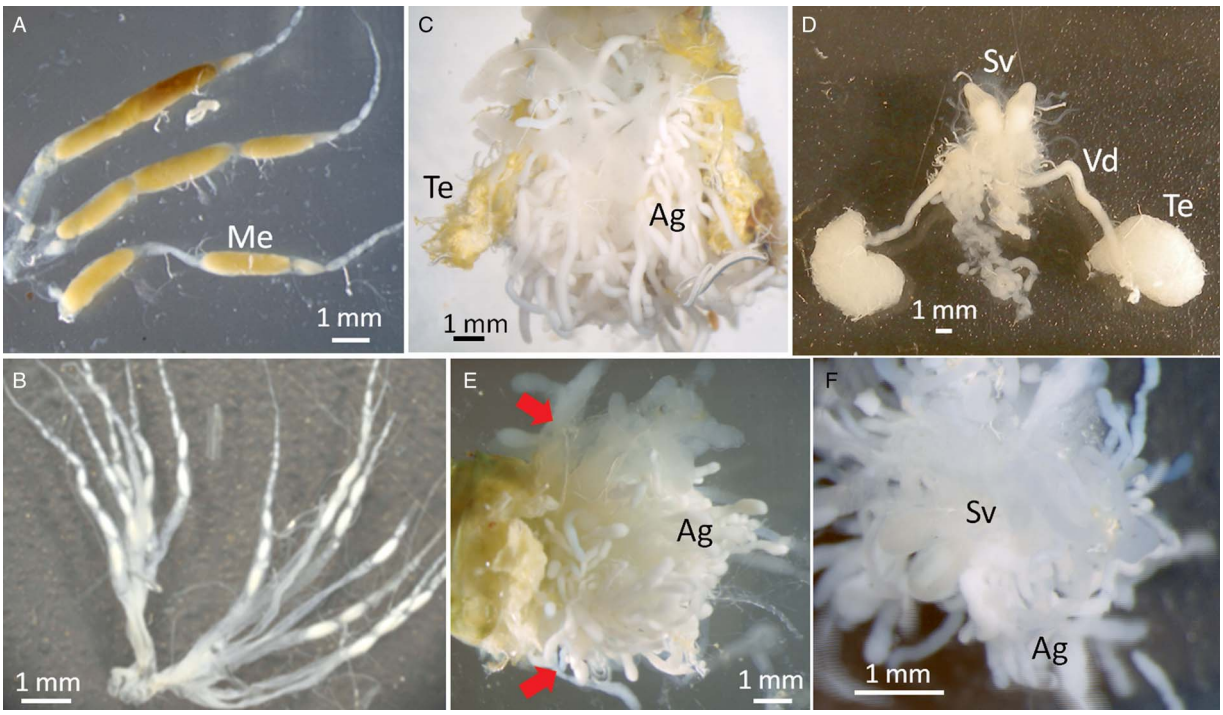

Fig. 3. Internal sex organs of adult mantids, *Hierodula formosana*. (A) ovarioles with mature eggs (yellow eggs) of an uninfected female adult. (B) Ovarioles of an infected female adult. (C–D) Internal sex organs of uninfected male adults without (C) or with (D) removing the accessory glands. (E–F) Dorsal (E) and ventral (F) view of internal sex organs of the infected male adults. The red arrows in (E) is where the testes should be in uninfected male adults but was disappeared in infected male adults. Ag: accessory glands, Me: mature eggs, Sv: paired seminal vesicles, Te: testis, Vd: vas deferens.

worms per host, and 1·48 ± 0·92 (1–6) worms in an infected female adult. The sex ratios (male:female) of the parasites were approximately 1:1 in hosts of either sex (in male hosts: 38:45, in female hosts: 55:56).

Internal reproductive organs (Figs 3 and 4). Uninfected female adults possessed a pair of ovaries with 52·3 ± 3·01 (49–58) ovarioles, a heart-shaped spermatheca, and a pair of clustered female accessory glands. The components of the internal reproductive organs of the infected female adults were similar to those of the uninfected female adults. The average number of ovarioles in each ovary of the infected female adults was 52·2 ± 2·74 (42–57), which is similar to that of the uninfected adult females ($t = 0·0624$, $P = 0·9519$) (Fig. 4A). Although the components of the internal reproductive organs were not influenced by worms, no mature eggs (Fig. 3B) were discovered in the infected female adults, whereas all 5 uninfected adult females contained mature eggs, which can be judged by having the yellow yolk inside (Fig. 3A) (4 females contained 1–221 mature eggs each, 1 laid eggs with an ootheca before dissection).

The uninfected male adults possessed a pair of testes with a long vas deferens, paired fusiform seminal vesicles (Figs 3C–D) and a cluster of male accessory glands (Fig. 3C). The area of a testis is 10·96 ± 4·53 (5·34–20·83) mm^2. The seminal vesicle (area of the seminal vesicle is 2·56 ± 0·80 (1·053–3·849) mm^2) is located at the end of the vas deferens. The male accessory glands are located directly above the seminal vesicles. In the infected male adults, testes disappeared (Fig. 3E) (in 9 infected males) or were extremely atrophic (in 4 infected males); only 1 infected male adult contained testes of normal size. The average area of each testis in the 5 infected male adults was 5·491 ± 7·94 (1·05–19·653) mm^2, which was slightly smaller than that in the uninfected male adults ($U = 45$, $P = 0·052$) (Fig. 4B). Seminal vesicles were identified in all the infected male adults (Fig. 3F), but the average size (0·67 ± 0·55 (0·086–2·6) mm^2) was smaller than that of the uninfected male adults ($U = 149$, $P < 0·001$) (Fig. 4C) and the shape was nearly round. The male accessory glands were fewer and scattered in most of the infected male adults.

Antennal characteristics (Figs 1, 5, 6; Table 1) Morphology of the 3 types of sensilla. According to the description provided by Hurd *et al*. (2004), 3 types of antennal sensilla (large trichoid sensilla, small trichoid sensilla and grooved basiconic sensilla) exist (Fig. 1A) based on SEM observations. The sensilla can be easily distinguished by the length and characteristics of the surface. The large trichoid sensilla are the longest and thickest tapered sensilla (76·35 ± 33·656 (35·02–152·09) μm) with longitudinal ridges. They form a single ring

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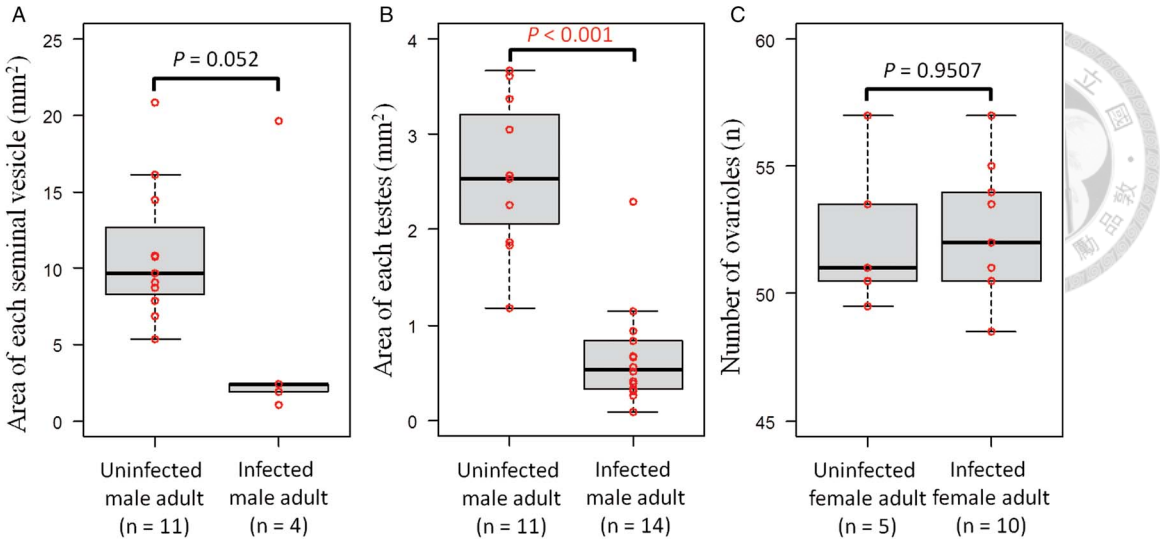

Fig. 4. Parasitic effect of the horsehair worm (*Chordodes formosanus*) on the size and number of internal sex organs (seminal vesicles (A), testes (B), and ovarioles (C)) of infected and uninfected mantid adults (*Hierodula formosana*). The gray boxes indicate the median and 25th and 75th percentiles of trait values (red dots). Whiskers indicate the maximal and minimal data values, except for outliers. *P* values indicate the results of testing the differences in median between the uninfected and infected individuals by using the Mann–Whitney *U* test.

around the distal third of each segment. Small trichoid sensilla and grooved basiconic sensilla are scattered over a segment. Small trichoid sensilla are long (38·77 ± 7·717 (21·81–56·78) μm) tapered sensilla with a pitted surface, and grooved basiconic sensilla are short (11·39 ± 1·808 (6·69–14·75) μm) and nearly columelliform with slightly longitudinal grooves. These 3 types of antennal sensilla were observed on the antennae of all the examined samples. In the following comparison of density and distribution of the grooved basiconic sensilla, number of the sensilla shorter than 18 μm were counted.

Density of grooved basiconic sensilla on each segment. The results of statistical analysis are displayed in Table 1 and Fig. 5. The average numbers of grooved basiconic sensilla in each $25 \times 25\ \mu m^2$ were sexually dimorphic in uninfected adults, and the patterns differed among the male samples (uninfected adults, infected adults and nymphal individuals). The density of the grooved basiconic sensilla was significantly higher in the uninfected male adults than in the uninfected female adults in most segments (20th to 100th), except for the 10th segment. The densities of all the female samples (uninfected adults, infected adults and nymphal individuals) displayed no significant differences in most segments, except for the 80th segment of nymphal females, which possessed fewer grooved basiconic sensilla than did the 80th segment of uninfected and infected adult females. The antennae of infected male adults exhibited higher similarity to those of the female samples than to those of the uninfected male adults, because of the significantly low density of the grooved basiconic sensilla. The grooved basiconic sensilla appeared only on the distal antennal segments of the male nymphs, and the densities after the 90th segment did not differ significantly from those of the female samples and the infected male adults. During these analyses, we discarded the antennal data from 2 of the infected male adults because they differed significantly from the other 9 infected male adult samples. The 2 discarded samples exhibited characteristics similar to those of the uninfected adult males, rather than the other infected male adults. We suspect that probably some male hosts were not or slightly affected after being infected. Therefore, we removed them from this comparison, but maintained the data isolated in Table 1.

First segment bearing grooved basiconic sensilla. The results of the statistical analysis are shown in Fig. 6A. After discarding the antennae without sufficient segments, the antennae of 26 uninfected male adults, 34 infected male adults, 17 uninfected female adults, 61 infected female adults, 5 male nymphs and 4 female nymphs were analysed. The grooved basiconic sensilla typically begin distribution from a specific segment to the end of an antenna. This starting segment was more anterior on the antenna of the uninfected male adults (segment 16·42 ± 0·81 (16–18)) than on those of the uninfected female adults (segment 47·10 ± 3·85 (38–55)). Similar to the results for the sensillum densities, the starting segment of the uninfected female adults was similar to that of the infected female adults (segment 46·51 ± 2·8 (40–52)), and the female nymphs (segment 47·25 ± 1·26 (46–49)). The starting segment of most

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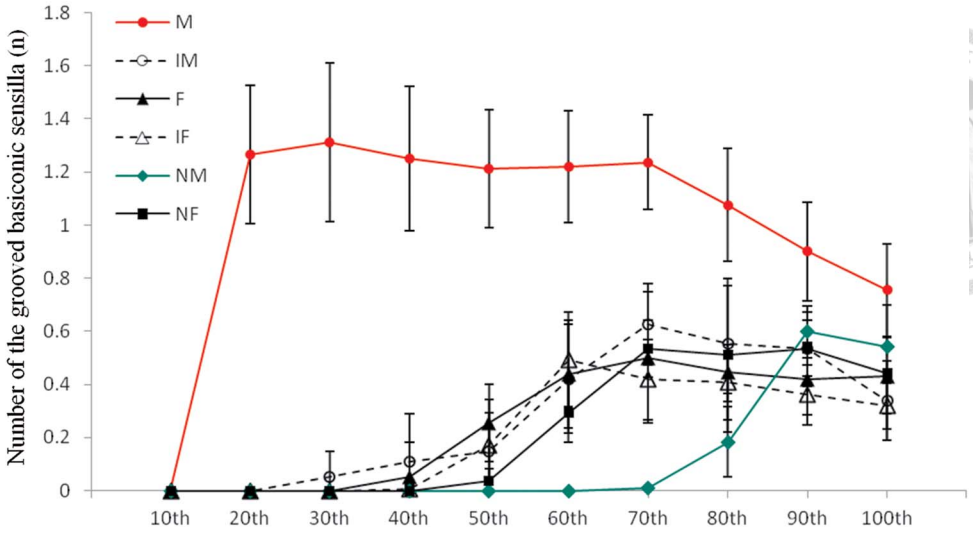

Fig. 5. Parasitic effect of the horsehair worm (*Chordodes formosanus*) on the mean (± standard deviation) number of grooved basiconic sensilla per 25 μm^2 on 10 selected segments of antennal flagella of infected and uninfected mantid adults (*Hierodula formosana*). M: uninfected male adult, IM: infected male adult, F: uninfected female adult, IF: infected female adult, NM: last-instar male nymph, NF: last-instar female nymph.

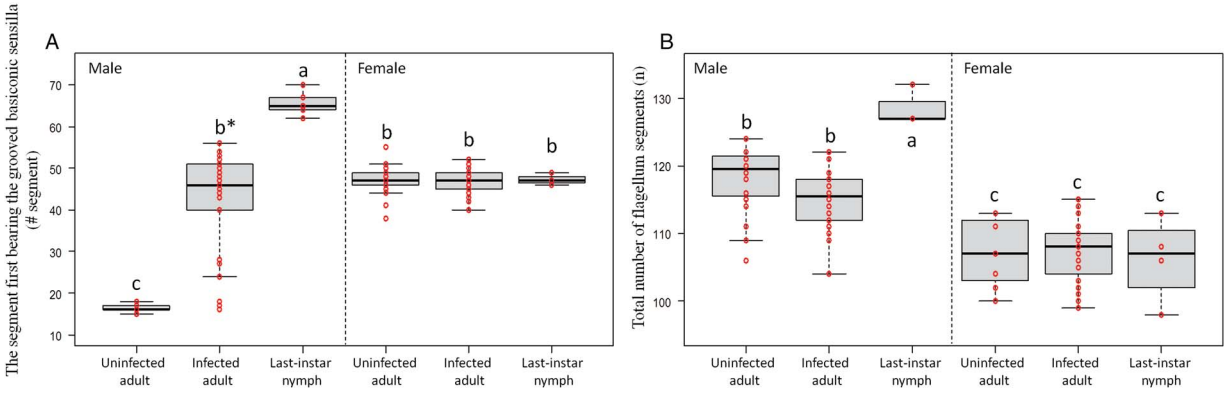

Fig. 6. Parasitic effect of the horsehair worm (*Chordodes formosanus*) on the antennal characteristics of the first flagellum segment bearing the grooved basiconic sensilla (A) and the total numbers of flagellum segments (B) of infected and uninfected mantids, *Hierodula formosana*. The gray boxes indicate the median and 25th and 75th percentiles of trait values (red dots). Whiskers indicate the maximal and minimal data values, except for outliers. Letters indicate significant pairwise differences (multiple comparisons conducted using a Student's *t*-test with Bonferroni correction, $P < 0{\cdot}05$). Statistical analysis of the infected adult males (asterisks) in (A) was performed using the Mann–Whitney *U* test because of the violation of normality.

of the infected male adults (26 individuals, segment 40–56) was between the values of the female samples, but 8 infected male adults exhibited extremely low starting segment values (segment 16–28). The average starting segment of the total infected male adults was 47·62 ± 4·53 (16–56). The starting segment of the grooved basiconic sensilla on the antennae of the male nymphs was located on the posterior segment (segment 65·60 ± 3·05 (62–70)) in our samples.

Total number of flagellum segments. The results of statistical analysis are displayed in Fig. 6B. After discarding the antennae without full segments, antennae of 20 uninfected male adults, 22 infected male adults, 7 uninfected female adults, 35 infected female adults, 4 male nymphs and 4 female nymphs were analysed. The numbers of flagellum segments were sexually dimorphic and not affected by infection in either sex. The antennae of the male samples possessed higher numbers of flagellum segments than those of the female samples. Among the male samples, the numbers of antenna of the uninfected male adults (118·00 ± 4·99 (106–124)) and the infected male adults (114·50 ± 4·30 (104–122)) exhibited no significant difference, but were lower than those of the male nymphs (128·25 ± 2·50 (127–132)). Among the female samples, no difference was observed among the uninfected female adults (107·14 ± 5·34 (100–113)), the infected female adults (107·17 ± 3·99 (99–115)) and the female nymphs (106·25 ± 6·24 (98–113)).

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Table 1. Densities of grooved basiconic sensilla (number per $25 \times 25\ \mu m^2$) on antennal flagella of *H. formosana*

Antennal segment	Male					Female		
	Adult	Infected adult	IM[1]	IM[2]	Nymph	Adult	Infected adult	Nymph
10th	0	0	0	0	0	0	0	0
20th	$1{\cdot}27 \pm 0{\cdot}26^{a}$	0^{b}	1·72	1·25	0^{b}	0^{b}	0^{b}	0^{b}
30th	$1{\cdot}31 \pm 0{\cdot}30^{a}$	$0{\cdot}05 \pm 0{\cdot}09^{b}$	1·63	1·28	0^{b}	0^{b}	0^{b}	0^{b}
40th	$1{\cdot}25 \pm 0{\cdot}27^{a}$	$0{\cdot}11 \pm 0{\cdot}18^{b}$	1·50	1·65	0^{b}	$0{\cdot}05 \pm 0{\cdot}13^{b}$	$0{\cdot}01 \pm 0{\cdot}02^{b}$	0^{b}
50th	$1{\cdot}21 \pm 0{\cdot}22^{a}$	$0{\cdot}15 \pm 0{\cdot}14^{b}$	1·63	1·64	0^{b}	$0{\cdot}26 \pm 0{\cdot}15^{b}$	$0{\cdot}17 \pm 0{\cdot}17^{b}$	$0{\cdot}04 \pm 0{\cdot}04^{b}$
60th	$1{\cdot}23 \pm 0{\cdot}21^{a}$	$0{\cdot}42 \pm 0{\cdot}21^{b}$	1·53	1·64	0^{c}	$0{\cdot}44 \pm 0{\cdot}20^{b}$	$0{\cdot}49 \pm 0{\cdot}18^{b}$	$0{\cdot}30 \pm 0{\cdot}11^{bc}$
70th	$1{\cdot}24 \pm 0{\cdot}18^{a}$	$0{\cdot}63 \pm 0{\cdot}15^{b}$	1·36	1·27	$0{\cdot}01 \pm 0{\cdot}03^{c}$	$0{\cdot}50 \pm 0{\cdot}25^{b}$	$0{\cdot}42 \pm 0{\cdot}15^{b}$	$0{\cdot}54 \pm 0{\cdot}11^{b}$
80th	$1{\cdot}08 \pm 0{\cdot}21^{a}$	$0{\cdot}55 \pm 0{\cdot}22^{b}$	1·28	1·38	$0{\cdot}18 \pm 0{\cdot}12^{bc}$	$0{\cdot}45 \pm 0{\cdot}08^{bc}$	$0{\cdot}41 \pm 0{\cdot}14^{bc}$	$0{\cdot}51 \pm 0{\cdot}29^{c}$
90th	$0{\cdot}90 \pm 0{\cdot}18^{a}$	$0{\cdot}53 \pm 0{\cdot}14^{b}$	1·07	1·11	$0{\cdot}60 \pm 0{\cdot}07^{b}$	$0{\cdot}42 \pm 0{\cdot}14^{b}$	$0{\cdot}36 \pm 0{\cdot}11^{b}$	$0{\cdot}54 \pm 0{\cdot}04^{b}$
100th	$0{\cdot}75 \pm 0{\cdot}17^{a}$	$0{\cdot}34 \pm 0{\cdot}15^{b}$	0·92	0·58	$0{\cdot}54 \pm 0{\cdot}10^{ab}$	$0{\cdot}43 \pm 0{\cdot}14^{b}$	$0{\cdot}32 \pm 0{\cdot}09^{b}$	$0{\cdot}44 \pm 0{\cdot}14^{b}$

Densities were calculated for 10 selected segments of antennal flagella (one was selected for every 10 segments from the 10th to the 100th segment, and 10 antennal segments were selected from each sample).
[a–c] Letters indicate significant pairwise differences (multiple comparisons conducted using a Student's *t*-test with Bonferroni correction, $P < 0{\cdot}05$) across the various categories for each segment. The Mann–Whitney *U*-test was performed to verify the bias caused by the violation of normality.
[1–2] Datasets from 2 infected male adults, which were considered outliers because they exhibited higher similarity with the uninfected male adults than with the infected male adults.

Table 2. Sexual dimorphism of *H. formosana*

	Parameter of linear regression			Comparison			
				Slope		Vertical shift	
	β_0	β_1	R^2	*F*-value	*P*-value	*F*-value	*P*-value
Forewing							
Male	14·5474	1·9938	0·5724	0·069	0·7942	230·437	**<0·001**[a]
Female	−0·3260	1·8553	0·5877				
Hindwing							
Male	14·6951	1·6874	0·549	0·0001	0·9907	273·5850	**<0·001**
Female	−2·6865	1·6927	0·6455				
Raptorial-leg							
Male	13·639	1·422	0·41	6·1609	**0·01704**	NA	0·001278[b]
Female	−16·5649	2·7174	0·7284				
Mid-leg							
Male	13·4368	1·4274	0·4974	2·2883	0·1377	1·9930	0·1652
Female	−4·9364	2·0929	0·6905				
Hind-leg							
Male	13·1812	1·9503	0·6476	2·477	0·122850	12·338	**0·001057**
Female	−6·9486	2·6084	0·8216				

Results of ANCOVA using pronotum length as a covariate and the length of wings and legs as independent variables. Bold fonts indicate those results of significant difference with *P* value less than 0·05.
[a] The difference in the ratio of the trait to the pronotum length was analysed using a Student's *t*-test because the homogeneity of variances was violated.
[b] The difference in the ratio of the trait to the pronotum length was analysed using the Mann–Whitney *U*-test because the homogeneity of β_1 was violated.

Pronotum, wing and leg characteristics (Tables 2–3, Figs 7 and 8)

Pronotum length. The pronotum length was sexually dimorphic and influenced by infection in both sexes. The pronotum lengths of the uninfected male adults (23·20 ± 1·281 (20·40–25·58) mm) were shorter than those of the uninfected female adults (27·07 ± 1·320 (24·74–29·67) mm) ($t = 9{\cdot}98$, $P < 0{\cdot}001$) and both infected male adults (21·38 ± 1·661 (18·66–24·68) mm) and infected female adults (24·43 ± 2·186 (20·67–30·39) mm) exhibited decreases in pronotum length after being infected (male: $t = 4{\cdot}97$, $P < 0{\cdot}001$; female: $t = 6{\cdot}51$, $P < 0{\cdot}001$).

Leg length. After being rescaled to body size, the uninfected male adults possessed longer hind legs than did the females, and the uninfected female adults possessed longer raptorial legs than did the

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Table 3. Parasitic effects on male and female mantids (*H. formosana*)

	Parameter of linear regression			Comparison			
				Slope		Vertical shift	
	β_0	β_1	R^2	*F*-value	*P*-value	*F*-value	*P*-value
Forewing							
M	14·5474	1·9938	0·5724	0·4473	0·5062	134·8036	**<0·001***[a]
IM	−4·8089	2·3742	0·5431				
F	−0·3260	1·8553	0·5877	0·032	0·858453	15·875	**<0·001**
IF	−4·0926	1·9154	0·87				
Hindwing							
M	14·6951	1·6874	0·549	1·2805	0·2623	128·7185	**<0·001**
IM	−9·534	2·309	0·5353				
F	−2·6865	1·6927	0·6455	0·0319	0·8587	17·8215	**<0·001**
IF	−3·28767	1·63952	0·855				
Raptorial-leg							
M	13·639	1·422	0·41	0·713	0·40181	3·292	0·07462*[b]
IM	6·4317	1·6975	0·8091				
F	−16·5649	2·7174	0·7284	8·7240	**0·004122**	NA	0·6568*[c]
IF	4·88023	1·89349	0·9236				
Mid-leg							
M	13·4368	1·4274	0·4974	1·3151	0·256	29·7760	**<0·001**
IM	2·4682	1·7885	0·7754				
F	−4·9364	2·0929	0·6905	2·2940	0·133817	8·8713	**0·003833**
IF	5·19939	1·66527	0·8763				
Hind-leg							
M	13·1812	1·9503	0·6476	0·0119	0·9135	61·560	**<0·001**
IM	9·5333	1·9117	0·7206				
F	−6·9486	2·6084	0·8216	3·5140	0·06450	6·7642	**0·01108**
IF	9·105	1·956	0·8365				

Results of ANCOVA using pronotum length as a covariate and the length of wings and legs as independent variables. Bold fonts indicate those results of significant difference with *P* value less than 0·05. (M: male, IM: infected male, F: female, IF: infected female).

[a] The difference in the ratio of the trait to the pronotum length was analysed using a Student's *t*-test because the homogeneity of variances was violated.

[b] The difference between uninfected males and infected males was marginally significant based on ANCOVA analysis; therefore, the ratio of raptorial leg length to pronotum length was verified using the Mann–Whitney *U*-test, which indicated no significant difference ($U = 612$, $P = 0{\cdot}147$).

[c] The difference in the ratio of the trait to the pronotum length was analysed using a Student's *t*-test because the homogeneity of β_1 was violated.

males (Table 2, Fig. 7). The horsehair worm infection caused shortening of the walking legs (mid and hind legs) in the hosts of either sex (Table 3, Fig. 7). In contrast to the walking legs, no parasitic effects on the raptorial legs were observed. The differences between the infected and uninfected males were not significant ($P = 0{\cdot}07462$); therefore, we re-examined the ratio of raptorial leg length to pronotum length using the Mann–Whitney *U*-test and obtained the same result ($U = 612$, $P = 0{\cdot}147$).

Wing length. One broken forewing each from 2 uninfected female adults, and 1 hindwing each (for a total of 2 hindwings) from 1 uninfected and 1 infected female adult were discarded. After being rescaled to body size, the wing lengths were sexually dimorphic and influenced by infection in both sexes. The length of the forewings and hindwings were longer in the uninfected male adults than in the uninfected female adults (Table 2 and Fig. 7). Similar to the results for the walking legs, the horsehair worm infection caused shortening of the wings (forewings and hindwings) in hosts of either sex (Table 3). The decrease in wing length in the infected female adults was less obvious than that in the infected male adults (Fig. 7).

Forewing shape. One broken forewing each from 2 uninfected female adults, and 1 hindwing each (for a total of 2 hindwings) from 1 uninfected and 1 infected female adult were discarded. Forewing shape was sexually dimorphic and influenced by infection in the infected males. The shape indices were higher in the uninfected male adults than that in the uninfected female adults (male, $0{\cdot}621 \pm 0{\cdot}012$; female, $0{\cdot}43 \pm 0{\cdot}014$, $t = 68{\cdot}68$, $P < 0{\cdot}001$), which indicates the smaller AR area (area above the radial vein) in the male forewings than in the female forewings. Parasitic effects on forewing shape were only observed in the infected male adults. The shape indices of the infected female adults ($0{\cdot}43 \pm 0{\cdot}013$) exhibited no significant

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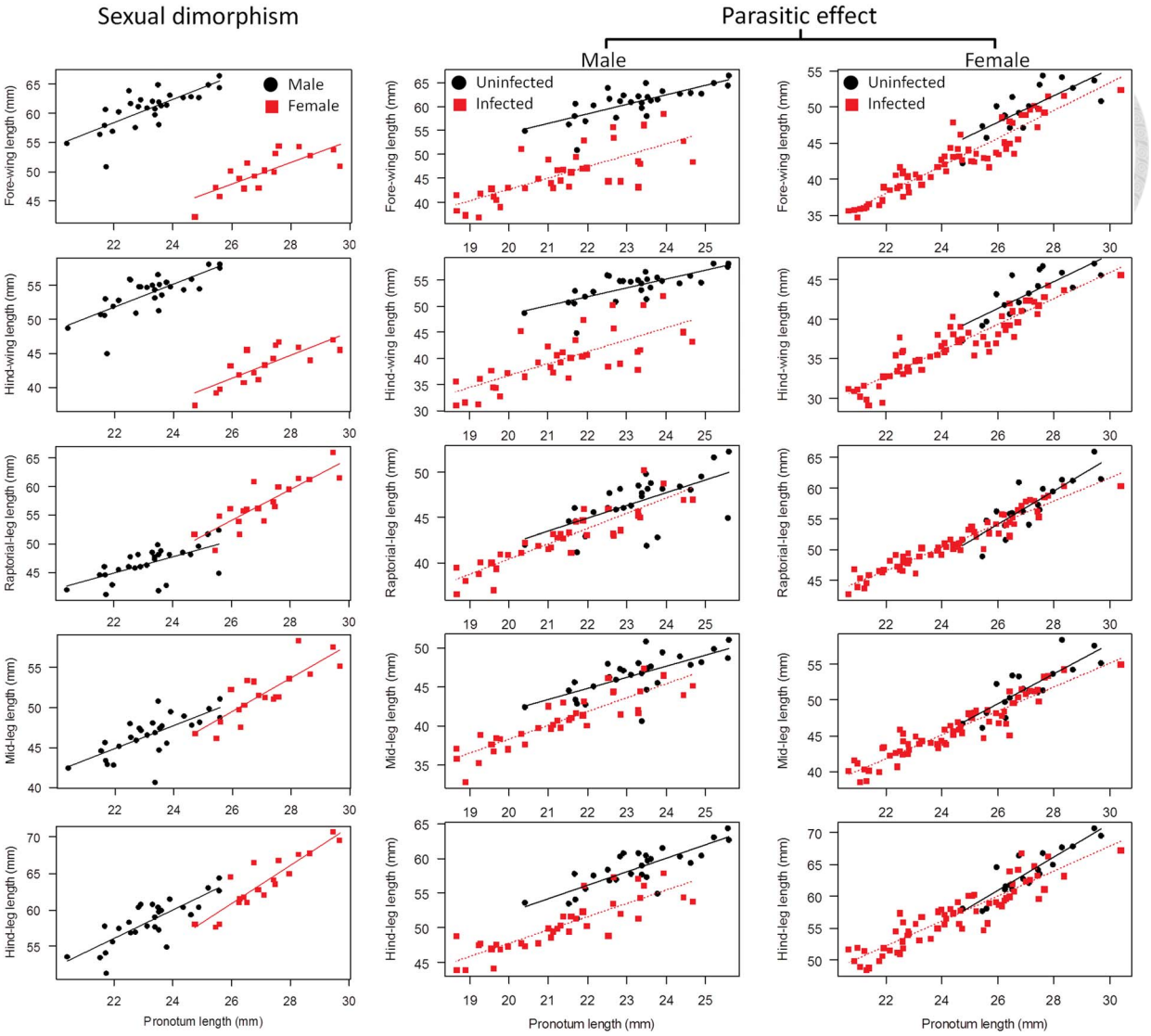

Fig. 7. Sexual dimorphism and parasitic effects of the horsehair worm (*Chordodes formosanus*) on the legs and wing lengths of infected mantids (*Hierodula formosana*). The lines inside the plots are linear regression lines generated using the data set of each plot.

difference from that of the uninfected female adults ($t = 137{\cdot}06$, $P = 0{\cdot}589$). The average shape indices of the infected male adults ($0{\cdot}55 \pm 0{\cdot}036$) were significantly less than those of the uninfected male adults ($U = 951$, $P < 0{\cdot}0001$). This indicated relatively smaller BR areas in the infected specimens than in the uninfected specimens (Fig. 8).

DISCUSSION

In the present study, we determined that horsehair worms (*C. formosanus*) cause castration, allometry and intersexuality (feminized male) in mantid hosts (*H. formosana*).

Influence on the internal sex organs of hosts

Castration induced by horsehair worms was confirmed by the absence of or extremely small testes in most of the infected males. Lacking of matured eggs in the infected females might support the parasitic castration but it could also be caused by the delaying egg development, especially for those of ovariole number which did not change. These 2 phenomena suggest that the parasitic effect on gonads was likely to be a developmental alteration instead of direct destruction: first, the number of ovarioles in the infected females was almost the same as that in the uninfected females (Fig. 4C). Second, 1 infected male mantid harbouring 3 mature worms maintained normal-sized internal organs. The presence of ovarioles may also explain the rapid recovery of egg production in previously infected crickets after releasing harboured worms (Biron *et al*. 2005).

Morphological change: allometry and intersexuality

Regarding changes in external morphologies, 2 parasitic effects were observed in the infected

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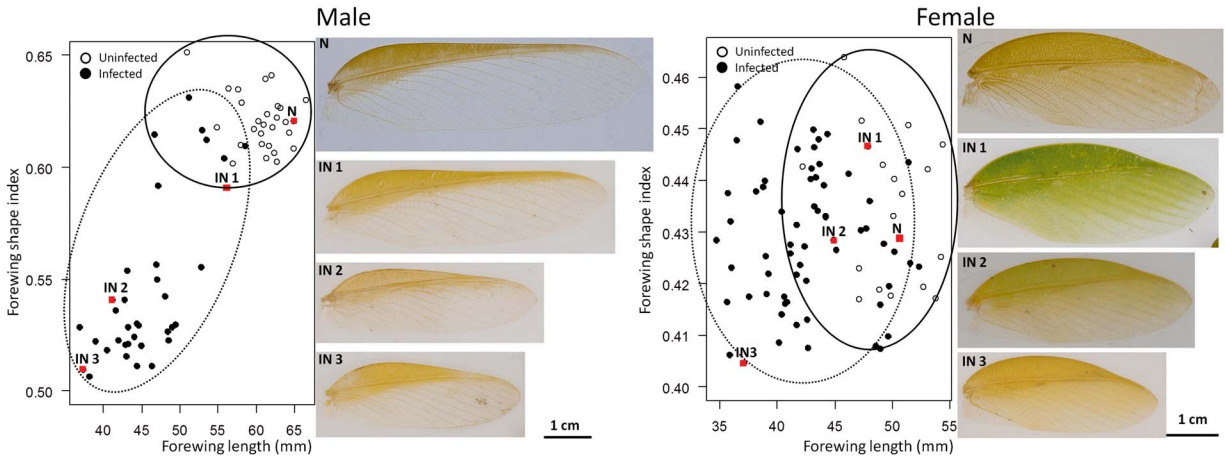

Fig. 8. Parasitic effects of the horsehair worm (*Chordodes formosanus*) on the forewing shapes of infected mantids (*Hierodula formosana*). The forewing shape index was calculated to determine the difference between the area above (*AR*) and below (*BR*) the radial vein. The formula for calculating the forewing shape index is $(BR - AR)/(BR + AR)$. The black solid circles indicate the infected hosts, and the open circles indicate the uninfected hosts. The large circles surrounding each group (sold line for uninfected hosts, and dashed line for infected hosts) were determined subjectively to approximately include all data points of each group. The four red squares (N: uninfected hosts, IN1–3: infected hosts) in each graph are the trait values corresponding to the pictures of wings on the right side.

samples. Allometric growth occurred in infected hosts of either sex and intersexuality (feminization) occurred in the infected males. Allometric growth originates from the nonproportional decrease in the size of wings and walking legs against the body size of the infected hosts (Fig. 7). Roy (2003) previously recorded an extremely short wing length of an infected male mantid, and considered the phenomenon to be a result of intersexuality. Wings and walking legs are sexually dimorphic characteristics (Fig. 7), and the changes in them theoretically correlate with the definition of intersexuality (Wülker, 1964). However, these changes could be a general effect of the infection. 'Real' intersexuality, in which the changes are sex dependent, occurred in the distribution of antennal sensilla (Fig. 6A) and wing shape (Fig. 8). These parasitic effects, which did not occur in the infected females, caused a decrease in the density of antennal sensilla and abnormal wing shape in infected males, causing the males to become partially feminized.

Hypothesis of the juvenilization that cause intersexuality

The feminine characteristics of the infected males suggested that the sexual differentiation of an insect is regulated by external factors. However, the sexual differentiation of an insect is typically considered autonomous and determined by the insect's own genes (Negri and Pellecchia, 2012), despite recent evidence still being considered controversial (de Loof and Huybrechts, 1998; DeFalco *et al*. 2008). To resolve this conflict, several researchers have suggested juvenilization to explain parasitic intersexuality or reproductive inhibition (Baudoin, 1975; Hurd, 2009). The hypothesis of juvenilization suggests that parasites can block the structural development of a host at the early stage of ontogeny (Baudoin, 1975). Similar to most bisexual organisms, male mantids typically evolve structures related to mating, and females conserve energy to produce eggs (Hurd, 2009). Therefore, the structures of adult males could be changed before sexual maturity through parasitic juvenilization, rather than these changes being the result of femalization.

In the present study of *H. formosana*, the horsehair-worm-infected adults maintained several nymphal characteristics, including the total number of antennal segments in both sexes (Fig. 6B), and the first segment bearing grooved basiconic sensilla in the females (Fig. 6A). These characteristics were not influenced by the parasite. The number of flagellum segments in the last-instar male nymphs was significantly higher than that of the adult males. Schafer (2005) also observed this phenomenon, which may be caused by mechanical damage. The antennal characteristic affected by the infection was the first segment bearing grooved basiconic sensilla in the infected males, whose trait values were between those of the last-instar nymphs and the uninfected male adults (Fig. 6A), and may have been caused by incomplete juvenilization.

Our evidence suggests that the hypothesized juvenilization may act through a pathway similar to that of the insect hormone that causes development. Juvenile hormones (or analogues) can block insect metamorphosis (Klowden, 2007). Treatment using a juvenile hormone analogue has been demonstrated to create nymphal antennae in adult cockroaches (Ramaswamy and Gupta, 1981; Kotaki *et al*. 2011)

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and abnormal wings in adult mantids (Harron and Yager, 1996). These hormonal influences are similar to the parasitic effects on the antennae (Fig. 6A) and wings (Fig. 8) in the infected males observed in the present study. Parasites of microsporidia (Fisher and Sanborn, 1962; Down *et al*. 2008) and cestodes (Hurd, 2009) also increase the hormone titre in the infected hosts, and cause juvenile characteristics to occur in pupal and adult mealworms infected by microsporidia (Fisher and Sanborn, 1962).

Considerable evidence supports the hypothesis of juvenilization, but further study is required because no solid and direct evidence has been obtained. In addition, the antennal characteristics of infected males may not completely match the scenario of juvenilization. Regardless of how the parasites affect hosts, castration and intersexuality may reflect the success of parasites in achieving virulence tradeoff by exploiting the resources of the host reproductive system.

Allometry as an index for the strategy of unbalanced host energy exploitation

In addition to intersexuality induced by horsehair worms, the nonproportional reduction in the lengths of wings and walking legs (morphological allometry) may indicate unbalanced energy exploitation. Based on the concept of reduced host fecundity by parasites, reduction in structures contributing to locomotion is relatively harmless to host survival. Such morphological allometry, which retains resources and supports host survival, can be triggered by parasites, or automatically activated by the host itself under specific circumstances. A decrease in the length of the walking legs (mid and hind legs) but not in the raptorial legs has been observed in giant water bugs (*Lethocerus deyrolli*), not as a result of parasitic infection but as a result of reduced food supply (Ohba *et al*. 2006).

Concluding remarks

Unbalanced host energy exploitation, regardless of whether it is initiated by parasites or the hosts themselves, could be used to explain the castration and the 2 patterns of morphological alteration, intersexuality and allometry, observed in the present study. In this paper, we discussed the morphological changes that occurred in mantids because of parasitic influences. However, the horsehair worm typically drives the host into water (Thomas *et al*. 2002); therefore, we did not eliminate the possibility that the horsehair worm alters host behaviour through the morphological changes in the host. Although no reasonable connection has been established between them, the functional changes caused by the altered morphology should be considered further. Maximizing resource exploitation without killing the host is most likely a common approach used in most host–parasite systems. In future studies, determining the role of sophisticated chemical and physical pathways, particularly the mechanism of intersexuality could be useful in elucidating the coevolution of these parasites with their hosts.

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Appendix 3.

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ANNUAL SURVEY OF HORSEHAIR WORM CYSTS IN NORTHERN TAIWAN, WITH NOTES ON A SINGLE SEASONAL INFECTION PEAK IN CHIRONOMID LARVAE (DIPTERA: CHIRONOMIDAE)

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ABSTRACT: The life cycle of the freshwater horsehair worm typically includes a free-living phase (adult, egg, larva) and a multiple-host parasitic phase (aquatic paratenic host, terrestrial definitive host). Such a life cycle involving water and land can improve energy flow in riparian ecosystems; however, its temporal dynamics in nature have rarely been investigated. This study examined seasonal infection with cysts in larval Chironominae (Diptera: Chironomidae) in northern Taiwan. In the larval chironomids, cysts of 3 horsehair worm species were identified. The cysts of the dominant species were morphologically similar to those of *Chordodes formosanus*. Infection with these cysts increased suddenly and peaked 2 mo after the reproductive season of the adult horsehair worms. Although adult *C. formosanus* emerged several times in a year, only 1 distinct infection peak was detected in September in the chironomid larvae. Compared with the subfamily Chironominae, samples from the subfamilies Tanypodinae and Orthocladiinae were less parasitized. This indicates that the feeding behavior of the chironomid host likely affects horsehair worm cyst infections; however, bioconcentration in predatory chironomids was not detected.

The life cycle of freshwater horsehair worms (Nematomorpha: Gordiida) involving water and land habitats has a unique role in riparian ecosystems (Sato et al., 2011). The life cycle begins with eggs laid in the aquatic environment. The hatched larvae stay at the bottom of the river until being ingested and encysted in the body cavities of aquatic animals called paratenic hosts. Some of the cysts in the paratenic hosts are carried to the terrestrial environment and enter terrestrial definitive hosts when the infected paratenic host is consumed as prey. Inside the definitive host, the larvae excyst within the gut of the definitive host and penetrate into the hemocoel to develop into wormlike juveniles, where they continue to develop. At the end of their parasitic phase, adult horsehair worms might manipulate the behavior of their hosts to enter aquatic environments and then emerge to reproduce (Hanelt et al., 2005).

Each stage of the life cycle of the horsehair worm has been studied in the natural environment by detecting cysts (White, 1966; Winterbourn, 2005; Hanelt, 2009a) and adult worms in various hosts (Schmidt-Rhaesa et al., 2005) and environments (Cochran et al., 2004; Salas et al., 2011) and confirmed in the laboratory environment by examining artificial infections (Inoue, 1962; Hanelt and Janovy, 2003, 2004a; Bolek et al., 2013b). A laboratory-reared population has also been successfully established for more than 10 yr (Hanelt and Janovy, 2004b). However, their annual temporal patterns have rarely been studied. In some horsehair worm species, the seasonal occurrence of adult worms has been described on the basis of direct observation of free-living adults (Cochran et al., 2004; Salas et al., 2011), manipulated hosts (Schmidt-Rhaesa et al., 2005), and dead host remains from the stomach contents of fishes (Sato et al., 2008). The occurrence of adult worms, which reflects the final stage of the parasitic phase of horsehair worms, was recently determined to be the crucial event mediating energy flow in riparian ecosystems (Sato et al., 2011). Nevertheless, using the adult stage as an indicator is problematic because adult horsehair worms and infected definitive hosts are difficult to detect because of the short life span, hiding behavior during mating, variable host species (Hanelt et al., 2001), and low infection rate (Looney et al., 2012) of the worms. In contrast to the adults, the immature stages in paratenic hosts are more typically encountered in the natural environment (Bolek et al., 2013b). The definitive host range of horsehair worm cysts is narrow; however, the cysts are found in a wide range of animals, including vertebrates and invertebrates (Schmidt-Rhaesa and Ehrmann, 2001; Schmidt-Rhaesa, 2013). Although the morphology of the cysts is not as well described as that of the adult stage (Szmygiel et al., 2014), it has been recently applied as an indicator for estimating their geographic distribution (Hanelt et al., 2001) and species composition (Bolek et al., 2013b). In the terrapin–trematode system, the metacercarial cysts of the trematode have been used to estimate the abundance of terrapins (Byers et al., 2010).

The short life span of a paratenic host is a potential indicator of the seasonal reproduction of horsehair worms. On the basis of the known life cycle, we presume that the seasonal infection dynamics of larval chironomids are associated with the reproductive patterns of horsehair worms in a stream. In Taiwan, *Chordodes formosanus* is a typical horsehair worm species that mainly parasitizes the mantids *Hierodula formosana* and *Hierodula patellifera* (Chiu et al., 2011). These 2 mantids are tree living but can typically be found on the ground with adult horsehair worms inside. In our field survey during 2009–2010, these infected mantids were found mainly in summer (adult *H. formosana* from late June to early August) and occasionally in autumn (adult *H. patellifera* during September) and spring (nymphal *H. formosana* during late January; Chiu and Wu, 2008; Chiu et al., 2011). The offspring of the horsehair worm produced in these periods theoretically create intensive infection risks for their aquatic paratenic hosts. Larval chironomids, the aquatic immature stages of nonbiting midges, were frequently observed to be infected by horsehair worm cysts (de Silva et al., 2008). Unlike perennial snails, which have been commonly used as indicators of the horsehair worm population (Hanelt et al., 2001; Bolek et al., 2013b), most larval chironomids spend only approximately 1 mo developing and foraging in water. The short-term developmental period strongly associates their infection dynamics with the amount of infective horsehair worm larvae in water. Technically, their small size, large number, and easy sampling make the survey and estimation of the larval chironomid population feasible.

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In this study, we recorded the seasonal infection of horsehair worm cysts in larval chironomids and the appearance of infected mantids in the main reproductive season of *C. formosanus* in northern Taiwan. To reduce the bias caused by different foraging behaviors of larval chironomids, the parasite metrics of different functional groups, Chironominae (collector–gatherer or collector–filterer) and Tanypodinae (predator) (Merritt and Cummins, 1996), were measured separately.

MATERIALS AND METHODS

Seasonal parasite metrics of the cyst stage (field survey in 2013 and 2014)

Collection of larval chironomids: Larval chironomids were collected from Lujiaokeng Ecological Protected Area, Yangmingshan National Park, Taipei (25.19015°N, 121.5579°E; approximately 40 km from the type locality of *C. formosanus* in Jiaushi; collection permission: 20130740 [Yangmingshan National Park]). In a 20×10 m^2 aquatic area, where the water was 30–60 cm deep, 6 sites (30×30 cm^2) were randomly selected, and the aquatic insects were collected using a D-net (DR7412D, BioQuip, Rancho Dominguez, California). Sampling was first performed on 14 August 2013 to confirm the existence of horsehair worms. Because of stream remediation (desilting work in the sediment basin of the water purification station for 1 mo) in November, regular sampling was performed beginning in January 2014 every 2 to 3 wk. The aquatic insects collected were temporarily preserved in 75% ethanol. After being brought to the laboratory, the larval chironomids were isolated and preserved in 95% ethanol at –20 C.

The larval chironomids were identified on the basis of morphological characteristics according to the keys for North America (Merritt and Cummins, 1996; Epler, 2001) and Australia (Madden, 2010). The specimens were first examined and grouped using a stereomicroscope (Leica S8 APO, Leica, Wetzlar, Germany) according to the characteristics of head capsules, antennae, mouth parts, anal setae, and posterior parapods. Within each group, 1–6 individuals were treated with a 20% KOH water solution at 60 C for 5–20 min and then examined using a light microscope (Olympus BH-2, PM-10AD, Olympus, Tokyo, Japan). All individuals were then grouped into 3 subfamilies (representing 3 functional groups): Chironominae (collector–gatherers or collector–filterers), Orthocladiinae (collector–gatherers or scrapers), and Tanypodinae (predators) (Merritt and Cummins, 1996). Individuals within each subfamily were further identified to the genus level. However, because the midge fauna has not yet been completely studied in Taiwan, the characteristics described in the keys might not completely fit those of our samples. Thus, the generic status that we identified was temporarily expressed as "*genus*-like," which can be corrected.

Examining cysts in larval chironomids: Larval chironomids belonging to the subfamily Chironominae were used for examining seasonal infection with horsehair worm cysts because they were the most abundant group that could be stably collected. From each collection, more than 20 chironomid larvae were examined for infection, except for samples collected during winter and early spring (December to March). The sample sizes in the other 2 subfamilies (Orthocladiinae and Tanypodinae) were too small to examine their seasonal infection dynamics. Only 2 dominant genera (*Corynoneura*-like and *Cricotopus*-like) were chosen from the subfamily Orthocladiinae, whereas all samples were chosen from the subfamily Tanypodinae to compare the infection among different functional groups at the highest infection period of Chironominae (August, September, October; see the Results section).

Horsehair worm cysts inside each larval chironomid were counted under the light microscope after the tissues were cleared by heating the samples in a lactophenol solution (75 ml of lactic acid, 14 ml of acetic acid, 7 ml of phenol, and 4 ml of distilled water; Winterbourn, 2005) or Nesbitt's fluid (40 g of chloral hydrate, 25 ml of distilled water and 2.5 ml of concentrated hydrochloric acid; Walter and Krantz, 2009) for 15–20 min at 60 C or 40 C, respectively. Cysts with clear images were photographed to measure the lengths of the preseptum, postseptum, and stylet.

Molecular identification of 5 collected horsehair worms: During our aquatic insect survey, 5 adult horsehair worms were collected. They were fixed and preserved in 95% ethanol for DNA amplification. Genomic DNA was extracted from fragments of the mid-body part using an ALS Tissue Genomic DNA Extraction Kit (Pharmigene, Kaohsiung, Taiwan). A partial mitochondrial DNA cytochrome oxidase subunit I (COI) gene sequence was amplified using a universal primer pair (LCO1490 and HC02198; Folmer et al., 1994) according to the method used by Chiu et al. (2011). The 528-nucleotide base pairs (not including the base pairs with low sequencing quality in the head and tail) of the COI sequence were compared with 39 sequences of *C. formosanus* (Chiu et al., 2011). By these 528 base pairs, pairwise genetic distances between samples collected in Lujiaokeng Ecological Protected Area and 39 sequences from *C. formosanus* published by Chiu et al. (2011) were calculated using MEGA 4.0.2 (Tamura et al., 2007) by employing the neighbor-joining method with the Kimura 2-parameter model.

Emergence of adult horsehair worms in summer (field survey in 2013)

The numbers of emerging adult horsehair worms were estimated by collecting and counting mantids fallen on the ground. Because of the abnormal behavior of the infected mantids, we considered all fallen individuals to be infected with horsehair worms, including the road-killed mantids that had no horsehair worms inside or around them. These mantids (alive and dead individuals) were searched for and recorded along the road of a type locality of *C. formosanus* in Jiaushi, Yilan, Taiwan (24.83212°N, 121.7473°E), during summer (June 8 to August 12) in 2013.

Data analyses

Horsehair worm cyst infection in the larval chironomid population was represented by 3 parasite metrics: prevalence (infected host number/total examined host number), mean intensity (total cyst number/infected host number), and mean abundance (total cyst number/total examined host number). All continuous data were expressed as the mean ± standard deviation. The parasite metrics of different functional groups were compared by performing a Fisher's exact test for prevalence and bootstrap Student's t test for mean intensity and mean abundance (Rózsa et al., 2000).

All statistical analyses were performed using R version 3.1.0.R Development Core Team, 2014).

RESULTS

Cysts in larval chironomids

Population dynamics of larval chironomids: During pilot sampling in August 2013, 20 blood worms were collected. During 25 regular samplings from January 2014 to January 2015, 1,595 larval chironomids were collected in total. Twelve morphogenera were identified as belonging to the 3 subfamilies (Chironominae: *Polypedilum*-like, *Cladotanytarsus*-like, *Micropsectra*-like, *Stenochironomus*-like; Orthocladiinae: *Corynoneura*-like, *Cricotopus*-like, *Parametriocnemus*-like, *Thienemanniella*-like, *Stictocladius*-like; and Tanypodinae: *Australopelopia*-like, *Nilotanypus*-like, *Paramerina*-like; see Supplementary materials). The 3 most abundant morphogenera belonged to the subfamily Chironominae. Dead remains of other aquatic insects were found in the guts of Tanypodinae larvae in most of the samples.

Cyst morphology: In total, 832 cysts were found inside 958 larval chironomids and morphologically classified as 3 types.

More than 95% (806/832) of the cysts (type 1; Figs. 1–6) were morphologically similar to the larvae of *C. formosanus* (Chiu et al., 2011). Their body lengths ($n = 31$) (preseptum: 29.69 ± 2.98 [24.39–37.69] μm, postseptum: 29.84 ± 2.71 [23.63–35.62] μm) were slightly higher than those described by Chiu et al. (2014) (preseptum: 20.55 [16.32–24.78] μm, postseptum: 24.91 [22.52–27.44] μm); however, the stylet lengths (11.66 ± 1.35 [7.41–14.10] μm) were similar to those described by Chiu et al. (2011) (11.04 [9.59–13.25] μm). Some of these cysts were enclosed by cyst walls

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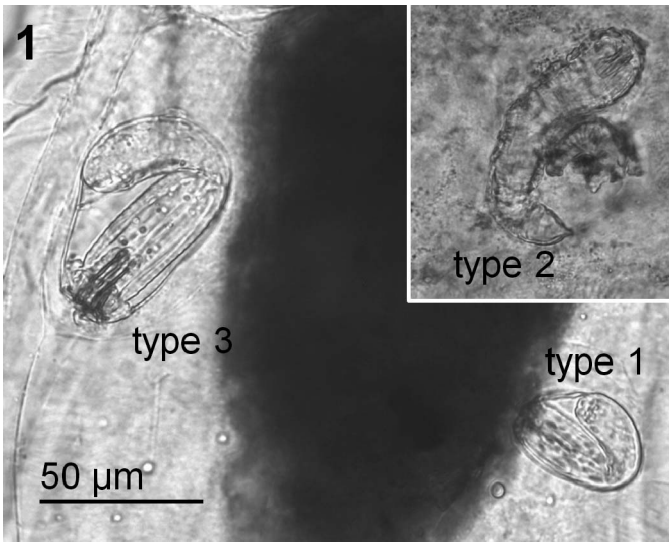

FIGURE 1. Three cyst types found in larval chironomids. Type 1: *Chordodes formosanus*; type 2: *Acutogordius* sp.; type 3: a cyst of an unknown nematomorph.

(Fig. 3), and some of them were dark in color, likely because of the host immune response (Fig. 6). On the basis of the morphology, adult worms collected near the river, and the cyst images of *C. formosanus* we took in the laboratory (Supplementary materials), we considered the type-1 cysts as belonging to the species *C. formosanus*.

The type-2 cysts had long postsepta that were 2 times longer than the presepta (preseptum: 26.02 ± 3.58 [18.88–30.85] μm, postseptum: 56.81 ± 4.38 [50.45–65.32] μm, stylet: 11.17 ± 2.66 [5.95–14.43] μm) (n = 10). On the end of the postseptum, there was a single spine (Fig. 1). The morphology was similar to that of the larval *Gordius* described by Hanelt and Janovy (2002) and Szmygiel et al. (2014) but with a considerably shorter body. None of these cysts had surrounding cyst walls; approximately half of these individuals were dark in color and appeared to be attacked by host immune responses. According to our field observation, these cysts should belong to the *Acutogordius* sp., which is the sister group of *Gordius* and commonly distributed in the low-altitude areas of Taiwan (unpubl. data). All 19 type-2 cysts were found in only 4 of the 20 larval chironomids from the pilot sampling in 2013. Three of the infected larval chironomids were found to harbor both type-1 and type-2 cysts.

The type-3 cysts (Fig. 1) were few in number. Only 7 cysts were found in 6 chironomid larvae. Their morphology was similar to that of the type-1 cysts; however, they were larger (preseptum: 45.69 ± 7.87 [38.50–54.12] μm, postseptum: 44.02 ± 5.91 [37.64–49.30] μm, stylet: 19.82 ± 2.04 [17.54–21.50] μm) (n = 3). This type of cyst might be a novel horsehair worm species that has never been identified in Taiwan. Among the 7 cysts of this type, 4 coexisted with type-1 cysts in 3 larval chironomids.

Other than complete cysts, dead remains of larval horsehair worms were also found inside the examined larval chironomids (Figs. 4, 5). In total, 49 dead remains of larval horsehair worms were found, and most of them (30/49) were collected during early September 2014.

Seasonal parasite metrics of Chironominae larvae

Because cysts belonging to the species *Acutogordius* (type-2 cysts) were not regularly collected in our survey, and only a few type-3 cysts were found, only parasite metrics of *C. formosanus* (type-1 cyst) were used to compare the infections among different seasons. The prevalence, mean intensity, and mean abundance of the samples are shown in Figures 7 and 8, and the detailed prevalence data are shown in the Supplementary materials.

The annual infection can be roughly divided into 3 phases: moderate infection (January to March), low infection (April to July), and high infection (August to December). The infection was lowest from late May to early July (no cyst was found during this period), dramatically increased from late July, and reached the highest value in mid-September according to the 3 parasite metrics. The infection then decreased and remained at a moderate level until December and decreased again in the following January. The values of mean intensity and mean abundance showed an infection peak in September; however, no obvious peak in prevalence was observed because the prevalence value did not decrease drastically until the following January. The infection in mid-September was as high as 94.59% in prevalence, 8.03 per infected host in mean intensity, and 7.59 per host in mean abundance. The highest number of cysts inside a single host individual was 31.

Parasite metrics in different functional groups

The parasite metrics of *C. formosanus* belonging to the subfamilies Chironominae and Tanypodinae and 2 dominant genera (*Corynoneura*-like and *Cricotopus*-like) of the subfamily Orthocladiinae collected in August, September, and October 2015 were compared. Typically, infections with the samples belonging to the subfamilies Orthocladiinae and Tanypodinae were significantly lower than those with the samples belonging to the subfamily Chironominae. Moreover, no cyst was found in *Corynoneura*-like species (Table I).

Emergence of adult horsehair worms in summer

The total number of mantids collected in Jiaushi was 95 (male: 32, female: 63). The highest numbers of mantids were collected on 4 July, and more than 80% of mantids were collected during 10 days between 27 June and 9 July (Fig. 8, vertical bar).

During the survey of aquatic insects in the Lujiaokeng Ecological Protected Area, 5 adult horsehair worms (male: 3, female: 2) were collected after visual observation near the sampling site. Except 1 male worm found in the small pool on 21 August 2014, the others were collected on 6 July 2014 from 2 adult female *H. formosana* individuals on the ground. The genetic distances between these 5 horsehair worms (GenBank numbers: KU509484–KU509488) and the 39 sequences from *C. formosanus* ranged from 0 to 0.017, while the genetic distance within these 39 *C. formosanus* sequences averaged 0.00979 (0.000–0.01922; Chiu et al., 2011).

DISCUSSION

The analysis of horsehair worm cyst infection in larval chironomids from northern Taiwan revealed the following: (1) 3 species of horsehair worms can parasitize chironomid larvae; (2) a

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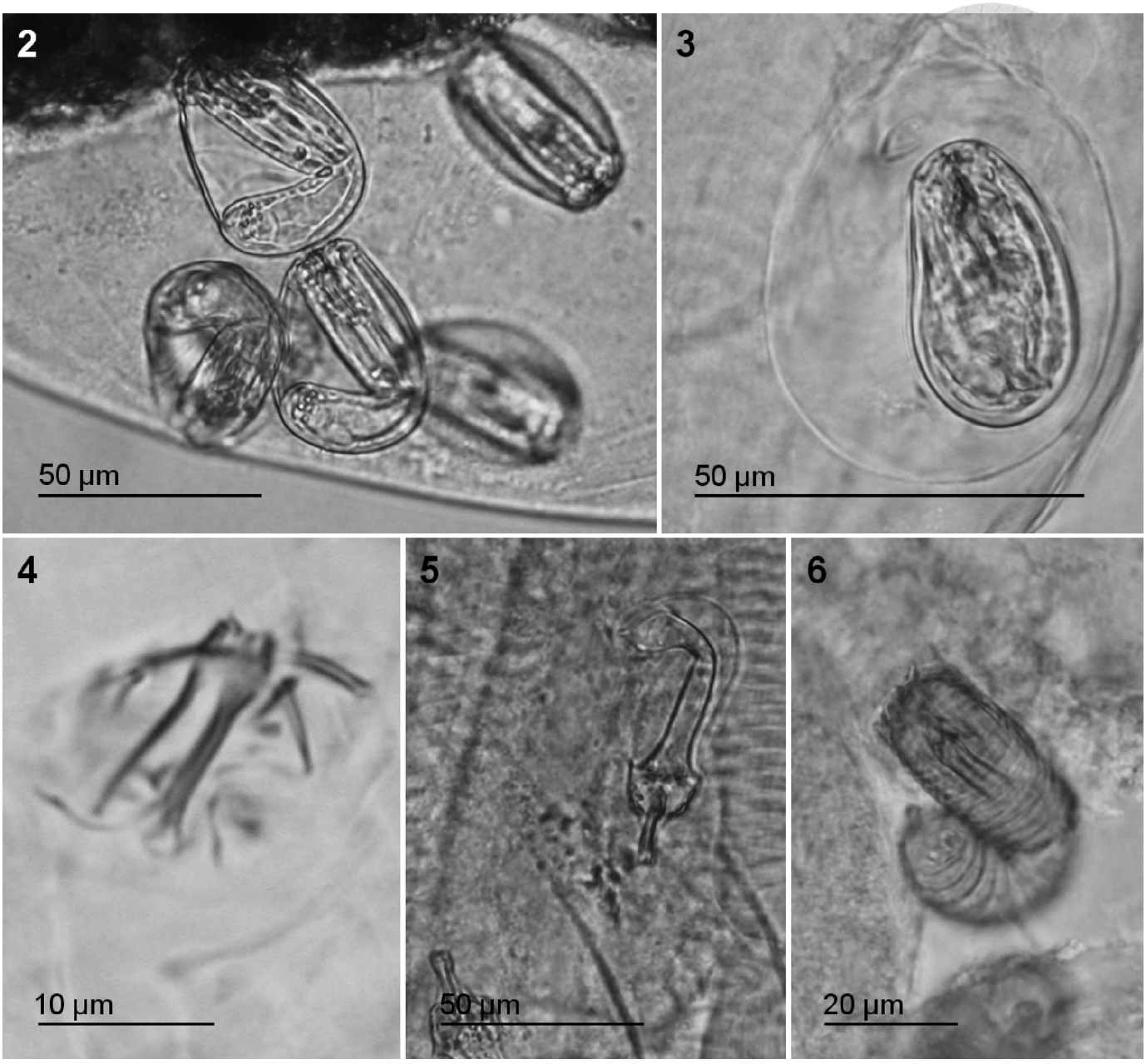

FIGURES 2–6. Different appearances of *Chordodes formosanus* (type-1 cyst) in larval chironomids. (**2**) Normal appearance; (**3**) cyst with a clear cyst wall; (**4, 5**) dead remains of larval horsehair worms; (**6**) a cyst attacked by the host immune response.

single infection peak was detected in September, which is 2 mo after the known dominant reproductive season of adult horsehair worms; and (3) different infection patterns were detected among different functional groups of larval chironomids. These results not only support the use of paratenic hosts for monitoring the biodiversity of horsehair worms, but they also improve the understanding of the life history of horsehair worms in nature.

Species and relative abundance of horsehair worms

In the survey of the Lujiaokeng Ecological Protected Area, where the nematomorph fauna has never been formally studied, only 1 adult species, *C. formosanus*, was found, whereas 3 species were confirmed on the basis of cyst morphology.

The relative abundance of cysts is likely to reflect the population size of these 3 species. Although it can be influenced by host preference, we speculate that the relative abundance of cysts can still provide a reliable estimate because horsehair worm larvae can become encysted in almost any aquatic animal that ingests them, because of their wide host range (Schmidt-Rhaesa and Ehrmann, 2001; Hanelt and Janovy, 2003). Hence, type-3 cysts represent a rare species for which cysts constitute only less than 1% of the total cyst number.

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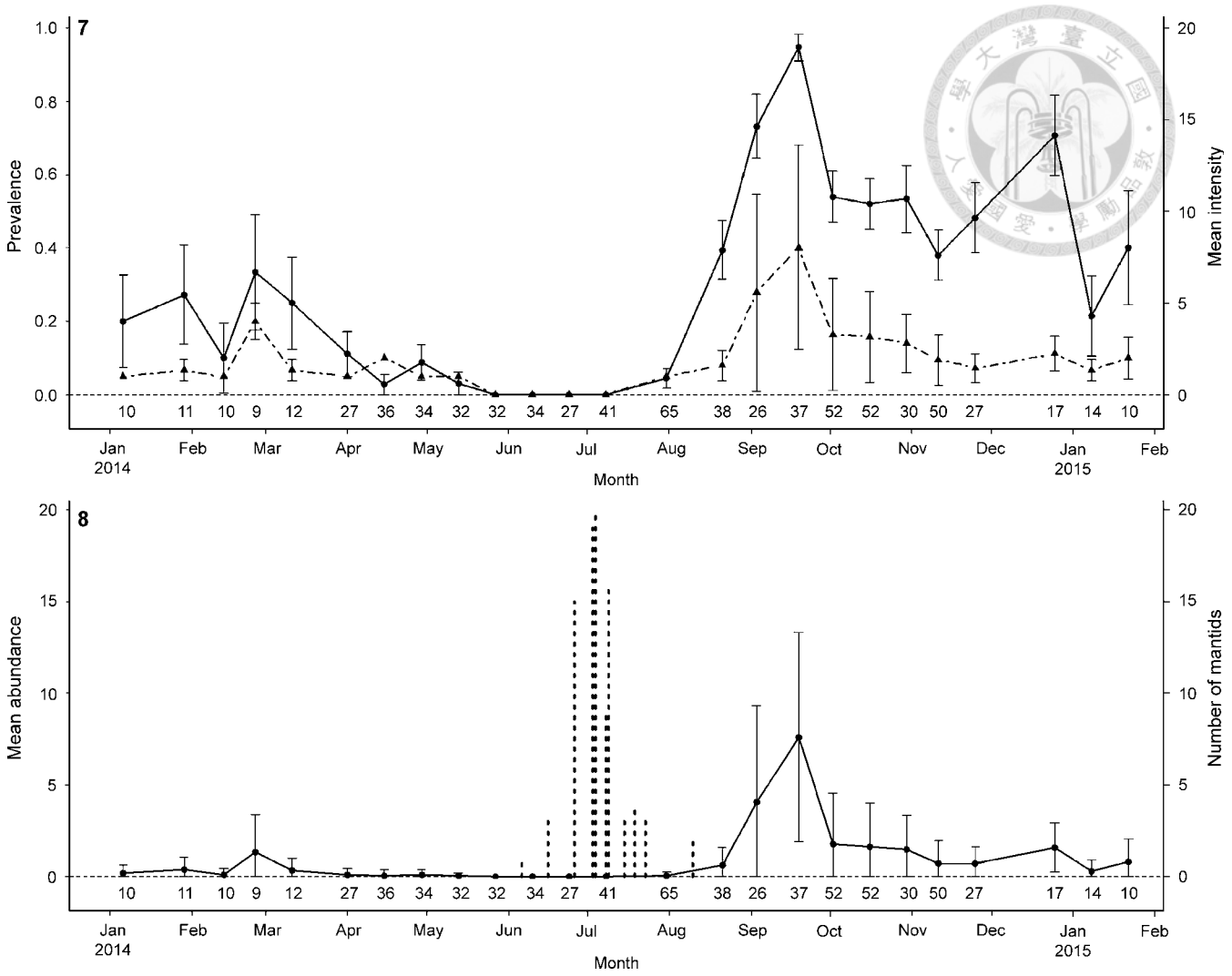

FIGURES 7, 8. Seasonal parasite metrics of *Chordodes formosanus* (type-1 cyst) in larval Chironominae and the collected numbers of horsehair worm–manipulated mantids, *Hierodula formosana*. (**7**) Seasonal dynamics of prevalence (circles) and mean intensity (triangles); (**8**) seasonal dynamics of mean abundance (circles) and number of manipulated mantids fallen on the ground (vertical bar). Numbers on the bottom indicate the samples examined.

The disappearance of the *Acutogordius* cysts in 2014 might indicate a distinct decrease in their local population. Sato et al. (2014) noted that the horsehair worm population underwent local extinction after clear-cut logging in artificial forests. Although no intensive human activity on the terrestrial environments around our sampling area was noted, the effect of stream remediation on horsehair worms should be further investigated for understanding their water–land life cycles. In contrast to the *Acutogordius* cysts, the population of *C. formosanus* seems to be much more tolerant to interference. Because the habitats and aquatic hosts of the 3 horsehair worms overlap, a fluctuation in 1 population is likely to influence the others.

Infection dynamics of *C. formosanus* in paratenic hosts

Infection peak in September: This is the first survey of the seasonal dynamics of horsehair worm infection in paratenic hosts in Taiwan. The *C. formosanus* infection broke out in September and rapidly decreased in October. This infection peak indicates that a large amount of larval horsehair worms suddenly appeared in the water. The infection peak was significant in mean intensity and mean abundance because these 2 parameters represent the amount of parasites rather than only the number of infected hosts. Other evidence suggested that this suddenly increased number of larvae was because of the dead remains of larval horsehair worms in host guts. Larval horsehair worms invade host body cavities through the intestines (de Villalobos et al., 2003). This process exposes the larvae to damage from host digestive systems and internal defense reactions (Hanelt and Janovy, 2004a). The dead remains of larval horsehair worms inside larval chironomids are likely to be those of larval horsehair worms destroyed but not yet egested or digested. Because larval horsehair worms encyst 3–15 days after being swallowed (Inoue, 1962; Hanelt and Janovy, 2004b), the appearance of dead remains of larval horsehair worms might provide a more precise estimation of the time when the larval horsehair worms appeared.

The 2 mo free-living period in the life cycle of horsehair worms: The sudden increase in larval number in September indicates a mass reproduction of the horsehair worms. Among their 3 known

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TABLE I. Parasite metrics in different subfamilies of Chironomidae.

Subfamily	Chironominae	Orthocladiinae (*Corynoneura*)	Orthocladiinae (*Cricotopus*)	Tanypodinae
21 August				
Prevalence	15/38 (39.47%)	0/7 (0%)	0/5 (0%)	**0/17 (0%)***
Mean intensity	1.6 ± 0.83	0†	0†	0†
Mean abundance	0.63 ± 0.94	**0***	**0***	**0***
3 September				
Prevalence	19/26 (73.08%)	—	2/6 (33.33%)	**1/13 (7.69)***
Mean intensity	5.58 ± 5.37	—	**1 ± 0***	4†
Mean abundance	4.08 ± 5.21	—	**0.33 ± 0.52***	**0.31 ± 1.11***
19 September				
Prevalence	35/37 (94.59%)	—	**3/12 (25.00%)***	**2/13 (15.38%)***
Mean intensity	8.03 ± 5.55	—	**1.67 ± 0.58***	3 ± 2.83
Mean abundance	7.59 ± 5.70	—	**0.42 ± 0.79***	**0.46 ± 1.39***
2 October				
Prevalence	28/52 (53.85%)	**0/10 (0%)***	**3/25 (12%)***	**0/7 (0%)***
Mean intensity	3.29 ± 3.07	0†	**1 ± 0***	0†
Mean abundance	1.77 ± 2.78	**0***	**0.12 ± 0.33***	**0***
16 October				
Prevalence	27/52 (51.92%)	**0/20 (0%)***	**3/35 (8.57%)***	1/5 (20.00%)
Mean intensity	3.15 ± 2.46	0†	**1 ± 0***	1†
Mean abundance	1.63 ± 2.37	**0***	**0.09 ± 0.28***	**0.2 ± 0.45***
30 October				
Prevalence	16/30 (53.33%)	**0/11 (0%)***	**1/17 (5.88%)***	0/2 (0%)
Mean intensity	2.81 ± 1.60	0†	1†	0†
Mean abundance	1.5 ± 1.83	**0***	**0.06 ± 0.24***	**0***

* The boldface numbers indicate significant differences ($P < 0.05$) compared with infection values in Chironominae (Fisher's exact test for prevalence and bootstrap Student's t test for mean intensity and mean abundance; Rózsa et al., 2000).
† The sample size is less than 2 and not sufficient for statistical comparison.

reproductive seasons, summer, autumn, and spring, the larvae reproducing in summer are more likely to create this infection peak than those reproducing in autumn or spring. The time interval between the reproduction and infection in paratenic hosts is the free-living period of the life cycle of horsehair worms. During this period, horsehair worms undergo mating, oviposition, embryogenesis, and encystment. Laboratory examinations have revealed that embryogenesis takes approximately 0.5–1 mo (30 days in *Neochordodes occidentalis* [Poinar and Doelman, 1974], 20–25 days in *Chordodes nobilii* [Zanca et al., 2007], 14–21 days in *Chordodes janovyi*, *Chordodes kenyaensis*, and *Paragordius varius* [Bolek et al., 2015]) at room temperature. The hatched larvae maintain their infectivity for 2 wk in water (Hanelt and Janovy, 2004a). The period of cyst formation lasts approximately 3–15 days (Inoue, 1962; Hanelt and Janovy, 2004a). In summary, horsehair worms, after being laid as the egg strings, spend approximately 30 free-living days in the aquatic environment. The time interval between the reproduction in July and infection peak in September was 2–2.5 mo (Fig. 8). Thus, female horsehair worms might generally remain in water for approximately 1–1.5 mo until mating and laying eggs.

Dominant season of horsehair worm reproduction: The single infection peak in chironomid population in September suggests a dominant reproductive season of *C. formosanus*, which conforms to our previous observations (Chiu and Wu, 2008). Among the 3 known reproductive periods, reproduction during autumn and spring seems not to lead to obvious infections in larval chironomids. Although we do not have solid evidence yet to explain this seasonally unequal reproduction, notably, adult horsehair worms emerge from different species and different stages of mantids during these 3 periods. During the reproductive seasons of summer and autumn, adult horsehair worms emerge from adult *H. formosana* and *H. patellifera* hosts, respectively. Thus, *H. formosana* seems to be a more important host of *C. formosanus* in northern Taiwan. During spring, adult worms emerge from nymphal *H. formosana*; thus, the length of the worms is only half that in summer and autumn (Chiu et al., 2011), and they are likely to produce fewer offspring (Hanelt, 2009b).

Does the infection peak indicate the beginning of horsehair worm development in the mantid host?: The imagoes of infected larval chironomids are expected to bring the cysts to the terrestrial environment and create a high infection risk for nymphal *H. formosana*. The mantid *H. formosana* is a monovoline insect that mainly reproduces in July, hatches after 1–2 mo, and overwinters as a nymph (Chiu and Wu, 2008). It is plausible that nymphal mantids are infected by preying on infected aquatic insects, and they release the adult worms in the following summer. However, the real situation seems to be more complicated because the adult worms mature and emerge from nymphal *H. formosana* in spring (Chiu and Wu, 2008). During our laboratory rearing, we found that some horsehair worms parasitizing early-instar mantids matured before the host emerged (unpubl. data). This might suggest the existence of 2 generations of a horsehair worm population per year. However, the scale of reproduction in spring

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is too small to cause the large number of larval worms produced in July. Thus, we suggest the following possible scenario, which is not yet confirmed. Only some of the cysts enter the mantids directly. The remaining cysts enter the food web of aquatic animals through paratenesis (Hanelt and Janovy, 2004a) and maintain their infectivity for several months (Bolek et al., 2013a). These cysts might enter nymphal mantids at different times during the following spring and mature together when the mantids develop to adulthood. In this scenario, the mass reproduction in July is because of the synchronized development of the hosts and parasites rather than the simultaneous infection of the mantids.

Infection among different functional feeding groups of larval chironomids

The hatching of horsehair worm larvae after the reproductive season created a high infection risk for larvae belonging to the subfamily Chironominae, whereas only a few larvae belonging to the subfamily Tanypodinae and one to the dominant genus of the subfamily Orthocladiinae (*Cricotopus*) were parasitized from July to October, and no cyst was found in *Corynoneura*. This can be attributed to the feeding behaviors of those chironomid larvae. The foraging type of the host is the critical factor affecting horsehair worm infection (Hanelt and Janovy, 2003). As collectors (Merritt and Cummins, 1996), Chironominae larvae are likely to ingest horsehair worm larvae at the bottom of the river, whereas horsehair worms are less encountered by the *Cricotopus* species, which feed as scrapers or collectors, and *Corynoneura* species, which feed on algae (Pillot, 2014). The members of the subfamily Tanypodinae are predators; they do not directly ingest horsehair worm larvae from the environment but are infected by preying on infected aquatic animals (Hanelt and Janovy, 2004a). Such predators concentrate parasites; hence, they have a high infection risk in their own population (Moravec and Skoríková, 1998; Brown et al., 2001). However, this was not the case in this study. The significantly lower infection in larval Tanypodinae might suggest that the paratenesis of horsehair worms was less efficient, or their prey was less parasitized. In addition to the feeding behavior, the life history of larval chironomids, such as the diapause for overwintering (Pillot, 2014), might also influence infection with horsehair worms.

The different infection risks might indicate different parasitic effects of horsehair worms among chironomid populations. The cysts are now considered harmless to their hosts because the paratenic host is considered a carrier. However, their parasitic effects, including inhibiting (or delaying) host pupation (White, 1969) and inducing internal defense reactions, are still detected (Poinar and Doelman, 1974; Hanelt and Janovy, 2004a). Although we did not find a direct relationship between fluctuations in the chironomid population and infection dynamics, further investigation on the ways in which horsehair worms affect the populations of aquatic animals during the season of their high infection will be valuable.

CONCLUSION

The seasonal infection dynamics of *C. formosanus* in the present study suggested that infection of paratenic hosts can be used to estimate the reproduction of adult horsehair worms. The infection dynamics of *C. formosanus* revealed an infection peak of larval horsehair worms during September, the 2-mo free-living period in the life history of *C. formosanus*, and the dominant reproductive season. Although the findings for the cysts of the other 2 horsehair worm species were not sufficient for building a seasonal infection dynamic model, the horsehair worm biodiversity was more efficiently estimated through the survey of paratenic hosts. The differences in infection among chironomids with different feeding behaviors support the prediction that the host foraging type influences horsehair worm infection; however, predator concentration was not found in the Tanypodinae functional group.

To date, the life-history traits of horsehair worms in the aquatic environment remain obscure. Here, we present the first field survey data on the aquatic life stage of *C. formosanus*. It would be valuable to gather more detailed information on this infection dynamic model and the related life-history biology of other horsehair worm species, especially those with a wider definitive host range, and their infection dynamics in other paratenic hosts.

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Running title: A new species of *Acutogordius* and novel hosts of *Chordodes*

A new horsehair worm *Acutogordius formosanus* sp. n. (Nematomorpha, Gordiida) parasitizing orthopteran hosts, with the redescription of *Chordodes formosanus* and novel host recordings from Taiwan

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Abstract

A new species of horsehair worm, *Acutogordius formosanus* sp. n. and redescription of the *Chordodes formosanus* with novel host records are provided here. The *Acutogordius formosanus* sp. n. is morphologically similar to *A. protectus* with moderately flat areoles on tail tips but distinguishable by the small ornamentations on its mid body. Despite the distinct differences on the postclocal crescents of the 14 male samples, their conspecific status with other 9 female samples were still suggested by the comparison of partial cytochrome oxidase subunit I (COI) sequence. Another commonly-found horsehair worm species of *C. formosanus* in Taiwan was previously believed to specifically parasitize *Hierodula* mantids, but five worms emerged from one *Acromantis* mantid and two long-horned grasshopper hosts (*Leptoteratura* sp. and *Holochlora japonica*) were found belonging to *C. formosanus* according to their COI sequences. One of these worm samples bears the abnormal crowned areoles which have never been found on *C. formosanus* and might be attributed to its incomplete development.

Keywords: *Acutogordius formosanus*, *Chordodes formosanus*, new species, immature stages, novel hosts.

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Introduction

Acutogordius formosanus sp. n. and *Chordodes formosanus* Chiu, 2011 are two most common horsehair worm species and usually found to be sympatric at the low-altitude of Taiwan. With the parasitic life cycle passing through the aquatic and terrestrial environments, their cysts have been found super-parasitic in the aquatic chironomids (Chiu et al. 2016), whereas the adults generally parasitize different terrestrial definitive hosts.

The definitive host of *C. formosanus* is *Hierodula* mantids (Chiu et al. 2011), while adults of *Acutogordius formosanus* sp. n. are generally found to emerge from orthopteran insects in Taiwan. *Acutogordius* is a small genus which consists of ten described species (de Miralles and de Villalobos 1998, Schmidt-Rhaesa and Geraci 2006, Schmidt-Rhaesa and Schwarz 2016) in the family Gordiidae (Poinar 2008). Only two genera in Gordiidae, *Acutogordius* and *Gordius*, are characterized by the postcloacal crescent closely locating on the base of two tail lobes, and they are distinguishable by the distinctly pointed tips on the male's tail lobes of *Acutogordius* (Schmidt-Rhaesa 2002). Classification of *Acutogordius* species is mainly based on the characters on male tails. However, the inter-specific variation has less been mentioned, and still confuses the separation of species (Schmidt-Rhaesa and Geraci 2006).

In the present study, we suggested the conspecific status of 23 *Acutogordius* samples collected from 11 species of orthopteran hosts according to their minor differences in the sequences of the partial mitochondrial DNA cytochrome oxidase subunit I (mtDNA-COI) gene, and considered the morphological differences as intraspecific variations among these samples. In addition, the morphologies of their immature stages were also described. In this study, we confirmed five horsehair

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63 worm samples which were emerged from *Acromantis* mantids and long-horned
64 grasshoppers, *Leptoteratura* sp. and *Holochlora japonica*, to be *C. formosanus*
65 according to the morphological and molecular evidences, and thus added these
66 insect species as their novel definitive hosts.
67

Materials and methods

In total, the morphologies and DNA sequences of 29 adult horsehair worms (24 *Acutogordius* and five *Chordodes*) were examined. Two pairs of *Acutogordius* were reared in the laboratory for breeding and laying eggs for two weeks. The morphologies of their larvae were examined using a light microscope (Olympus BH-2, PM-10AD, Tokyo, Japan). Specimens (partial bodies with their hosts) were preserved in the Department of Entomology, National Taiwan University, Taipei; National Museum of Natural Science, Taichung, Taiwan; and Lake Biwa Museum, Shiga, Japan. Specimen examination follows our previous methods as in Chiu et al. 2011.

Collection and preservation of horsehair worms

The insect hosts infected with horsehair worms were hand-collected in the riparian environment. The horsehair worms inside were first checked by examining the posteriors of their hosts and then collected by immersing the hosts in water or by dissection. Except two pairs of *Acutogordius* were kept for breeding, all the other horsehair worms were first killed by hot water (>80°C) and fixed in the 75% alcohol solution with their hosts for days and then kept in a 95% alcohol solution. The collection data and host data are given in Table 1 and Table 2.

The *Acutogordius* pairs for laying eggs were reared with 800 ml aerated tap water and maintained at room temperature (~28°C). The mated male was then removed and fixed and preserved in 75% and 95% alcohol solution, respectively. After the mated females laying eggs for one month, they were then also fixed and preserved respectively in 75% and 95% alcohol solution. Egg strings were found around three days and the hatched larvae were detected about 2-3 weeks after egg laying. The live larvae were observed under a light microscope.

Snails (*Physa* sp.) infected by the horsehair worms were collected with the

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other nine non-infected ones in a small pond in Wufengqi Waterfalls, Jiaushi Township, Yilan County, Taiwan, where the free-living adult horsehair worms have ever been seen inside. The live snails were reared together with 2000 ml aerated tap water and dissected in five days.

Morphological examination

Adult specimens. Fragments (~0.5 cm in length) of the anterior end, mid body, and posterior end of the preserved adult horsehair worm samples were first examined under the stereomicroscope (Leica S8 APO, Leica, Wetzlar, Germany). These fragments were dehydrated with a series of ethanol and acetone solutions (95%, and 100% ethanol (twice), and ethanol/acetone mixtures of 2:1, 1:1, 1:2, and 0:1), then critical-point-dried and gold-sputter-coated before being examined under a scanning electronic microscope (SEM) (JEOL JSM-5600, Tokyo, Japan) at a magnification of 100–15,000×.

Immature stages. Eggs and newly hatched larvae were examined and photographed alive on the microslides using a light microscope (Olympus BH-2, PM-10AD, Olympus, Tokyo, Japan) (at a magnification of 200× and 400×). For examining the cysts inside the snail hosts, the snail shells were removed first and the soft tissue was flatted by two glass slides, then the slides were examined under a light microscope at 200× magnification.

The terminology of larvae using in this study mainly followed that of Hanelt and Janovy (2002) and Szmygiel et al. (2014).

Phylogenetic analysis

Genomic DNA of the adult horsehair worm was extracted using an ALS Tissue Genomic DNA Extraction Kit (Pharmigene, Kaohsiung, Taiwan). A set of universal primers (LCO1490 and HC02198) (Folmer et al. 1994) were applied to amplify and sequence the partial COI sequence. The 14 *Acutogordius* samples whose COI

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sequences cannot be well amplified by the universal primers were prepared for a newly designed primer set (AcCOiF: TGAGCTGCCTTTTTAG, AcCOiR: TGTATTAATGTTTCGGTC). The PCR with both primer sets were initiated at 95℃ for 5 min, and the amplification was conducted for 40 cycles, at 95℃ for 1 min, 50 ℃ for 1 min, and 72℃ for 1 min, with a final extension at 72℃ for 10 min.

For phylogenetic analysis, the pairwise genetic distances and a phylogenic tree reconstructed by the Neighbor-joining (NJ) method based on the Kimura 2-parameter model were used to justify the conspecific status of the horsehair worm samples. The COI sequence (450 nucleotide base pairs of high quality) were first aligned using CLUSTALX 2.0.10 (Thompson et al. 1997) and the analysis was conducted by using MEGA 6.0 (Tamura et al. 2013). The COI sequence of *Gordius balticus*, *G. attoni*, and *G.* cf. *robustus*. (Hanelt et al. 2015, GenBank ref. no. KM382320, KM382318, KM382277), *C. formosanus* and *C. japanesis* (Chiu et al. 2011, GenBank ref. no. HM044105, HM044124, JF808206), and *Paragordius* sp. (GenBank ref. no. AY428843) were also used in the comparison and the 5000 replicates of the bootstrapping method were used to access the branch supports in this NJ tree.

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Results

Acutogordius formosanus Chiu *et al.* sp. n.

Type locality. Wufengqi Waterfalls (24°49'55.62"N, 121°44'50.10"E), Jiaushi Township, Yilan County, Taiwan (Holotype and Allotype). Paratypes were collected from Sindian, New Taipei City, and Fushan botanical garden, Yilan County, see Table 1 for detailed information.

Type material. Partial bodies of holotype, and allotype deposited at the National Museum of Natural Science with their hosts. Paratypes were deposited at the Department of Entomology, National Taiwan University, Taipei, National Museum of Natural Science, Taichung, Taiwan and Lake Biwa Museum, Shiga, Japan, see Table 1 for detailed information.

Type-host. *Eugryllacris* sp., *Neanias magnus* Matsumura and Shiraki, 1908 (Orthoptera: Gryllacrididae). *Deflorita apicalis* (Shiraki, 1930), *Elimaea* sp., *Hexacentrus japonicus* Karny, 1907, *H. unicolor* Serville, 1831, *Isopsera* sp., *Mecopoda elongata* (Linnaeus, 1758), *Phaulula* sp., *Pyrgocorypha formosana* Matsumura and Shiraki, 1908, *Sinochlora longifissa* (Matsumura and Shiraki, 1908) (Orthoptera: Tettigoniidae), see Table 2 for detailed information.

Etymology. The specific name refers to the type locality, Taiwan.

Description. (Figs 1–7)

Male adults (n = 14) (Figs 2, 3). Body length 288.3 ± 90.1 (133–428) mm, width (widest after dehydration) 623.48 ± 173.18 (404.70–1079.88) µm. Body light-brown, smooth, and slightly mucous before fixed by alcohol, alcohol-preserved specimens significantly flat and hard.

Anterior end columned and slightly narrowed at tip; anterior tip white (white cap) and followed by dark brown collar (Fig. 1A); white spots scattered on brown collar (Fig. 1B, C) in some samples (3/14); under SEM, surface of anterior end

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wrinkled (Fig. 1F) (4/14), smooth (Fig. 1D) (7/14), or smooth but wrinkled on the tip (Fig. 1E) (3/14); short bristles or holes scattered on some samples; no obvious boundary between white cap and dark brown collar.

Cuticle in mid body smooth, slightly wrinkled, or cracked; short or cone-like bristles (Fig. 3D, E) scattered on some samples (6/14).

Posterior end divided into two tail lobes, each 360.25 ± 53.30 (303.70–489.58) µm in length; lobe tips generally tapered, wrinkled or covered by moderately flat areoles with short spines among areoles; inner side of tail lobes smooth; tiny spines scattered around tip; cone-shaped spines or flat areoles scattered on base behind postclocal crescent.

Ventral side of posterior end structured with postclocal crescent, clocal opening, and tiny bristles. One postclocal crescent not evident since covered by larval cuticle, postclocal crescent length 275.48 ± 68.84 (195.78–417.03) µm, width (widest) 44.81 ± 16.21 (18.73–83.01) µm, located near base of tail lobes; postclocal crescent slightly curved (Fig. 2B, E) (5/13), near to right angle (Fig. 2A, D) (5/13, including 2 samples reared for laying eggs), or semicircular which slender than curved and angled ones (Fig. 2C, F) (3/13). Two ends of postclocal crescent extending over (Fig. 2 A, C, D, F) (11/13) or anterior to (Fig. 2B, E) (2/13) starting point of bifurcation of tail lobes. Clocal opening circular or slightly oval-shaped, 26.61 ± 7.86 (14.63–43.23) µm in diameter, 55.50 ± 19.71 (32.55–89.90) µm away from anterior margin of postclocal crescent, surrounding area depressed in four samples, no circumcloacal spine. Four clocal openings totally invisible since covered by larval cuticle or mold. Tiny bristles scattered over ventral side of posterior end except two samples which covered by larval skin or mold; tiny bristles scattered over ventral posterior end and concentrative on tail lobes (Fig. 3B) (3/13), anterior of postclocal crescent (Fig. 3A) (1/13), or randomly scattered on cuticle (Fig. 3D, E) (9/13).

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Female adults (n = 10) (Fig. 4). Body length 271.80 ± 99.14 (73–432) mm, width (widest, after dehydration) 896.65 ± 171.51 (578.54–1120.80) µm, light-brown, smooth, and mucous. Alcohol-preserved specimens flat in egg-laying samples. Anterior end (Fig. 4A) columned and slightly narrowed at tip, white cap and dark brown collar present. Under SEM, surface of anterior end smooth or wrinkled; one sample has hole-like structures (Fig. 5J); small spines scattered on surface of three samples; no obvious boundary between white cap and dark brown collar. Cuticle in mid body smooth, wrinkled, or crack-like; most with small spines scattered on cuticle (7/10). Posterior end (Fig. 4B) columned and round, smooth without spine or bristles. Clocal opening on terminal end circular, 24.70 ± 5.88 (16.80–30.62) µm in diameter, no circumcloacal spine.

Eggs (Fig. 6E). Egg strings (Fig. 6E) length 12.04 ± 3.91 (4.94–19.13) mm, width 0.61 ± 0.11 (0.343–0.708) mm (n = 11), white or light yellow in color, deposited as short pieces without sticking onto substrate. Eggs (12 days after being laid, nearly hatch) oval-shaped, length 31.93 ± 3.08 (28.79–34.67) µm, width 25.69 ± 1.25 (24.04–27.71) µm (n = 6).

Larvae (Fig. 6A-C, F, G). Newly hatched larvae near eggs presented as "worm-form" (Fig. 6B, C) or "cyst-form" (Fig. 6A). Under light microscopy, worm form (n = 13) larvae's preseptum length 31.25 ± 2.83 (24.66–34.14) µm, width 13.18 ± 0.44 (12.30–14.13) µm; postseptum length 80.75 ± 3.87 (77.16–89.13) µm, width 11.17 ± 0.70 (9.76–2.60) µm. Proboscis (same as stylet in our previous description in Chiu et al. (2011)) length 11.77 ± 0.87 (10.14–12.46) µm, width 3.29 ± 0.39 (2.79–4.02) µm; pseudointestines unequally subdivided oval with length 48.22 ± 2.86 (44.69–54.32) µm, width 7.99 ± 0.87 (6.57–9.17) µm. Cyst form (n = 15) larvae folded their postseptum forming oval shaped, length 25.64 ± 1.66 (22.34–27.88) µm and width 17.41 ± 1.40 (14.91–19.38) µm; only proboscis obvious with length 11.19

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± 1.25 (8.22–13.23) μm and width 2.60 ± 0.63 (1.38–3.21) μm.

Under SEM (worm-form larvae), larvae superficially annulated with 13 segments on preseptum and 35 on postseptum, ectodermal septum not distinguishable (Fig. 6C). Hooks arranged in three rings on anterior preseptum: outer ring containing seven hooks, including two ventral double hooks closed to each other; middle and inner rings containing six hooks and six inner spines, respectively, located between each outer hook. A proboscis inside preseptum covered by sheath, ornamented with two sets of spines: seven larger spines on each of lateral sides arranged in two lines expect the largest terminal spine; seven smaller spines on dorsal side, no spine on ventral proboscis (Fig. 6G). One single posterior spine located on end of postseptum; exterior openings of pseudointestine might present but not clear (Fig. 6F).

Field-collected cysts (Fig. 6D) Three cysts inside a snail length 23.59–24.35 μm, width 15.33–16.45 μm; proboscis length 11.42–11.91 μm, width 1.67–2.047 μm. Shape of cysts similar to cyst-form larvae, no cyst wall found, probably ruined during sample preparation.

Phylogeny. Except one female whose DNA condition was not suitable to be sequenced, the 23 *Acutogordius* COI sequences (GenBank ref. no. KX591922, KX591926–35, KX591937–48) contained 8 haplotypes with 442 invariable sites, six singletons, and two parsimoniously informative sites. The genetic distance among them was 0.0025 with a range of 0.0000–0.0112. The phylogenetic tree revealed a polytomic topology in which some clades were not highly supported due to low bootstrap values and short genetic distances (Fig. 7). The genetic distance of the COI sequences between these 23 *Acutogordius* with that of *G. balticus* was 0.27948 comparing with that of *G. attoni* and *G.* cf. *robustus*, 0.25455 and 0.27439 respectively.

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242 **Diagnosis.** The 23 *Acutogordius* samples from orthopteran hosts were justified
243 to a single species according to their low genetic distances, which is similar to the
244 intraspecific pairwise distances of *G.* cf. *robustus* (0.64–2.63%) (Hanelt et al. 2015)
245 and *C. formosanus* (0–1.92%) (Chiu et al. 2011), and lower than the interspecific
246 pairwise distances among species of genus *Gordius* (8.0–24.3%) (Hanelt et al. 2015)
247 and *Chordodes* (16.84%) (Chiu et al. 2011).

248 *Acutogordius formosanus* sp. n. is morphologically similar to *A. protectus*
249 Schmidt-Rhaesa and Geraci, 2006 by (1) the distribution pattern of tiny bristles on
250 ventral posterior end, (2) moderately flat areoles (rounded elevation) covering tail
251 tips, and (3) cone-shaped spines scattered on the base of tail lobes of the male
252 samples, but distinct from small ornamentations on the mid body of *Acutogordius*
253 *formosanus* sp. n.

254 All the three morphological types of postclocal crescents on individuals of *A.*
255 *protectus* are appeared on the samples of *Acutogordius formosanus* sp. n.
256 Nevertheless, postclocal crescent significantly extending onto some tail lobes were
257 only described in *Acutogordius formosanus* sp. n., and previously on *A. acuminatus*
258 de Miralles and de Villalobos 1998, *A. feae* (Camerano, 1897), *A. obesus* (Camerano,
259 1895), and *A. sulawensis* Schmidt-Rhaesa and Geraci, 2006. The higher intraspecific
260 variation makes the postclocal crescent not a suitable diagnostic characteristic in the
261 species level despite it is the most obvious structure which can be examined by
262 stereomicroscope.

263 The short bristle on mid body is the newly described character which was first
264 found in *A. finni* (Schmidt-Rhaesa and Schwarz 2016). This character is not likely to
265 be examined by stereomicroscope, but in *Acutogordius formosanus* sp. n., the short
266 bristle is still not stably present in all individuals examined under SEM. One of the
267 possible reason is the bristles were covered by the mucus on the cuticle surface. The

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surface of *Acutogordius* has been generally described as totally smooth (Schmidt-Rhaesa et al. 2006; de Miralles and de Villalobos 1998). However, various structures were found in *Acutogordius formosanus* sp. n. including the wrinkled, cracked, or hole-like structures. The similar structure (wrinkled structure which was described as fine grooves in Schmidt-Rhaesa and Schwarz 2016) has been found in *A. finni* and in the Fig. 4D, E in Schmidt-Rhaesa and Schwarz (2016), some of the bristles can be seen like "sticks" on the cuticle surface. In addition, the areole-like structure on the anterior end on one female sample also suggest the possibility that the moderately flat areoles covering male tail tips are caused by the mucus. Thus, although the moderately flat areoles and the short bristle are applied as the main diagnostic characters for *Acutogordius formosanus* sp. n. and *A. protectus*, more information might be necessary for confidently distinguishing these two species.

***Chordodes formosanus* Chiu, 2011**

Material examined. Taipei Zoo (24°59'44.70"N, 121°34'49.49"E), Taipei City, Taiwan (three males from an *Acromantis japonica*); Wufengqi Waterfalls (24°49'55.62"N, 121°44'50.10"E), Jiaushi Township, Yilan County, Taiwan (two males from two Tettigoniidae species). Specimen information see Table 1 for details.

Host. *Acromantis japonica* Westwood, 1889 (Mantodea: Mantidae). *Leptoteratura* sp., *Holochlora japonica* Brunner von Wattenwyl, 1878 (Orthoptera: Tettigoniidae). Host information see Table 2 for details.

Redescription. (Fig. 8)

Male adult (n = 5). Body length 109 ± 64 (43–204) mm, width (widest after dehydration) 0.56 ± 0.29 (0.32–0.88) mm, dark-brown, rough and flat with dorsal and ventral grooves in alcohol-preserved specimens.

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293 Expect one samples whose posterior end broken, posterior end (Fig. 8A)
294 in remaining four samples not lobed, ornamented areoles on margin with short
295 spines among them. Oval cloacal opening subterminal, 61.14 ± 27.61
296 (44.41–93.00) μm long and 31.23 ± 11.42 (22.00–44.00) μm wide, circumcloacal
297 spines present. A pair of oval regions free with areoles posterior to cloacal
298 opening with scattered bristles over it. Paired oval bristlefields 171.94 ± 48.32
299 (127.84–223.59) μm long and 55.94 ± 10.08 (46.34–66.43) μm wide, not found
300 on one sample, located on lateral side of cloacal opening between areas
301 distributed by flat areoles and normal areoles. Anterior end (Fig. 8B) tapered,
302 with white tip (white cap) under stereomicroscopy. Under SEM, anterior tip
303 smooth or wrinkled, covered with abundant small spines and scattered, thick
304 bristles; mouth open on terminal end of anterior extremity.

305 Mid body covered by areoles with more or less ornamentation on surface.
306 Areoles characterized into five types (simple, tubercle, thorn, circumcluster,
307 and crowned areoles). Simple areoles, most abundant, covering entire cuticle
308 of mid body, 9.70 ± 1.84 (8.18–12.51) μm in diameter, circular, surface smooth
309 or uneven. Tubercle areoles and thorn areoles scattered among simple areoles,
310 similar in shape but with a tubercle (ca. 3.96–8.09 μm long) or a solid thorn
311 (ca. 7.35–16.92 μm long), respectively, on latter or top of areoles; thorn
312 areoles less abundant than tubercle areoles and not found in one sample.
313 Crowned areoles (Fig. 8C, E) (each 14.90 ± 3.40 (9.72–21.81) μm in diameter)
314 surrounded by 7–12 circumcluster areoles with a central tubercle in between;
315 each areole with flat top and medium filaments (13.11 ± 4.96 (7.41–27.62) μm)
316 originating from apical center to edges; few long filaments (55.43–179.70 μm)
317 found in one sample. In one smaller sample from *Acromantis* mantid,

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crowned areoles (Fig. 8D, F) smaller than usual (10.84 ± 0.84 (9.60–11.83) μm) as well as the apical filaments (6.56 ± 1.11 (5.03–8.36) μm), with almost the same size with circumcluster areoles.

Phylogeny. The genetic distances among all COI sequences of horsehair worms from *Acromantis japonica* (GenBank numbers: KX591949-KX591951), *Leptoteratura* sp. (GenBank numbers: KX591952), *Holochlora japonica* (GenBank numbers: KX591953), and *Hierodula* mantids (sequences from Chiu et al. (2011)) were ranged from 0.000 to 0.010. The phylogenetic tree (Fig. 7) revealed a polytomic topology while the five horsehair worms' sequences from *Acromantis* matid and Tettigoniidae hosts were randomly inserted in this clade.

Comments.

The five male horsehair worms are all judged as *C. formosanus* due to the low genetic distances with *C. formosanus* described in Chiu et al. (2011). Their morphologies were also similar to that described in Chiu et al. (2011) which have five areole types on male adults, while slight differences were found on the bristlefield and crowned areole.

The size of bristlefields is smaller in the present study than that described in Chiu et al. (2011) (70–77 μm wide and 145–243 μm long) and not found in an extremely smaller sample. Although the difference in bristlefield has ever been considered as a diagnostic character of *Gordionus kii* to distinguish from its related species, *G. chinensis* (Schmidt-Rhaesa and Sato 2009), this character might be more diverse in genus *Chordodes*.

Another abnormal morphology is the similar size of the paired crowned areoles with their surrounding circumcluster areoles. This "abnormal crowned areole" was

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only found on one extremely smaller sample but not on the other horsehair worms, including the two larger ones emerging from the same host individual. As this sample is suggested by the molecular data to be conspecific to *C. formosanus*, we believe this abnormal crowned areole might be caused by the incomplete development in the synchronized maturation (see Discussion for details).

Discussion

In this article, a new species, *Acutogordius formosanus* sp. n. from 11 species of orthopteran insect hosts with its immature stages were described and three novel hosts of *C. formosanus*, *A. japonica* and *Leptoteratura* sp., *Holochlora japonica*, were proposed.

***Acutogordius formosanus* sp. n.**

The intra-specific variation

Finding stable diagnostic characters is a crucial step in distinguishing horsehair worm species (Schmidt-Rhaesa and Geraci 2006; Hanelt et al. 2015). It includes two main challenges: 1) finding a stable diagnostic characters and 2) setting the boundary of intra- and inter-specific variation. Postclocal crescent is the main diagnostic character in distinguishing the species of *Acutogordius* and *Gordius* (Schmidt-Rhaesa 2001). This structure, despite unclear for its function, is easy to be examined under both SEM and stereomicroscope. However, its morphology might be more unstable than it was considered. Such potential polymorphism was also pointed out in *A. protectus* which was mainly characterized by tail areoles instead of the postclocal crescent (Schmidt-Rhaesa and Geraci 2006). In *Acutogordius formosanus* sp. n., the distinct intra-specific variation on postclocal crescent is further confirmed. Likewise, the small ornamentations on the body cuticle currently described as diagnostic character in *Acutogordius formosanus* sp. n. and *A. finni*

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(Schmidt-Rhaesa and Schwarz 2016) might also appear in other *Acutogordius* species, especially those described without examining with SEM before Schmidt-Rhaesa et al. (2006) and de Miralles and de Villalobos (1998).

Using SEM in describing the nematormorph species currently becomes a standard method (Schmidt-Rhaesa 2001). However, the mucus which might be present on *Acutogordius* (might be as well as *Gordius*) is likely to cause the observation problem by its various morphology under SEM. This is the first report proposed the horsehair worm might secrete mucus on its body surface. The function of the mucus on the horsehair worm is unknown. As an aquatic animal which parasitizes terrestrial host, the horsehair worm is known to avoid to emerge and be dehydrated on the ground by manipulating the hosts (Thomas et al. 2002). The mucus secretion might be an additional strategy for moisture retention under the high risk before going back to the water.

Since the high intra-specific variation, the conspecific status of the 28 examined samples of *Acutogordius formosanus* sp. n. is primarily suggested by the comparison of barcoding sequence and secondarily supported by the similarity of their host and habitat. The DNA sequencing, as the application of SEM since 1980 (reviewed in Chandler and Wells 1989), provides a new and standard tool in horsehair worm taxonomy. Although the database of horsehair worm's sequences now is not complete enough to support determining a new species, it is useful in judging the conspecific status of a set of samples. Combination of the information from molecular and morphological data has now also improved the understandings of intra-specific variation and cryptic species (Chiu et al. 2011; Hanelt et al. 2015). Thus, although here we suggested *Acutogordius formosanus* sp. n. as a newly introduced species to science, its phylogenetic relationship with another nine *Acutogordius* species (or seven species, see Schmidt-Rhaesa 2002) is still unclear

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and worth further investigation.

The immature stages of *Acutogordius*

Studies in immature stages of the horsehair worms have caught more attention in the recent years. Although the morphological identification of the immature stage is now only roughly approaching to genus level (Szmygiel et al. 2014), cysts in wide range of aquatic hosts have recently applied to estimate their geographic distribution (Hanelt et al. 2001; Harkins et al. 2016), species composition (Bolek et al. 2013a) and annual reproductive season (Chiu et al. 2016). The immature morphology of *Acutogordius* in the present study also supports the cysts we found in our previous survey (Chiu et al. 2016, Fig. 1, type 2 cyst) are belonged to *Acutogordius* despite no adult worm was found in that sampling field.

This is the first time to find the newly hatched larvae fold their body in the environment. However, we have not yet known what factor triggers the newly hatched larvae to become worm-shaped or cyst-shaped, since both of them were found under similar living conditions. The larval worms are now believed to stay on the river bottom and be passively ingested by the paratenic hosts (Hanelt et al. 2005). Folding their bodies might be benefit in reducing the water flow or making them easily to be ingested, since the postseptum of *Acutogordius*, as well as *Gordius*, are significantly longer than the other genera (Hanelt and Janovy 2002; Szmygiel et al. 2014).

Hosts of *Acutogordius formosanus* sp. n.

The only reported host of *Acutogordius* is *Acanthodis* sp. (reviewed in Schmidt-Rhaesa 2013). In the case of *Acutogordius formosanus* sp. n., they were found to emerge from several families of Orthoptera with different foraging behaviors. Most of the hosts are obligate or facultative predators and frequently found to prey other smaller insects in the field (e.g. *Eugryllacris* sp., *Neanias*

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magnus, *Hexacentrus japonicus*, and *H. unicolor*), but some of them were mainly herbivorous in Taiwan (e.g. *Deflorita apicalis*, *Elimaea* sp., *Isopsera* sp., and *Phaulula* sp.). The adult horsehair worms emerged from non-carnivorous host, herbivores (*Barbitistes serricauda*, *Leptophyes punctatissima*) or detritivores (*Cambala annulata*), have been reported (Schmidt-Rhaesa et al. 2005, 2009). These horsehair worms challenge our current knowledge since the horsehair worm typically invade its definitive host through the paratenic hosts, which needs a carnivorous host to ingest the horsehair worm's cyst with infected paratenic host (Hanelt et al. 2005). The possible pathway to parasitize these non-carnivorous hosts is via the larvae/cysts in the water or on the vegetation (Schmidt-Rhaesa et al. 2005, 2009). The horsehair worm's cyst is now known to maintain partial infectiousness after the paratenic hosts die and dry for 30 days (Bolek et al. 2013b), this makes them possible to be accidentally ingested by the herbivores or detritivores arthropods with the dead paratenic hosts in the water or on the vegetation. In addition, it is also possible the herbivorous hosts facultatively prey the weak or newly emerged paratenic hosts. No matter which pathway the horsehair worm cysts go through, the infection rates of those non-carnivorous hosts is theoretically lower than that of the predators, and it meets our collection experiences.

Chordodes formosanus

The novel hosts

The *Acromantis* mantid and *Leptoteratura* sp. are general predators which can easily ingest the cysts of *C. formosanus* in the paratenic hosts, but *C. formosanus* is previously believed to specifically develop inside *Hierodula* mantids (Chiu et al. 2011). The novel hosts suggest the flexibility of its host use. Such phenomenon might also happen to the *C. japanensis* which mainly parasitizes *Tenodera* mantids (Inoue 1952, 1955; Chiu et al. 2011) but found in the longhorn grasshoppers,

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Hexacentrus japonicus japonicus (Inoue 1955).

We believe the longhorn grasshoppers and *Acromantis* mantids are novel hosts of *C. formosanus* due to that *Chordodes* is generally parasitize mantid hosts (Schmidt-Rhaesa and Ehrmann 2001) and their lower contribution to the parasite population. The hosts of horsehair worms have been recorded covering almost all the horsehair worm genera (Schmidt-Rhaesa 2013), but the understandings of host preference, the extent to which a particular host taxon is used by a parasite (Lymbery 1989) is generally lacking. We believe *Acromantis* mantids and tettigoniids, compared to *Hierodula* mantids, are rarely parasitized by *C. formosanus* due to the seasonal infection of aquatic paratenic hosts in Taiwan. Our previous survey of the horsehair worm's infection in aquatic paratenic host suggests a single infection peak after the adult *C. formosanus* emerges from *Hierodula formosana*. Such infection peak did not, or not significantly, appear after the season when the adult worm emerged from *Acromantis* mantids and tettigoniids in spring (Chiu et al. 2016, Fig. 1). Thus, the contribution of *Acromantis* mantids and tettigoniids to *C. formosanus*'s population might be less than that of the *Hierodula* mantids. It is not clear that developing in the novel hosts is ecologically significant or accidental, but such flexibility of the host use might improve our understandings of physiological mechanism in triggering the cyst metamorphosis.

Abnormal morphologies in the smallest individual

Length of the horsehair worm is strongly correlated to its host size and number of individuals in one host (Hanelt 2009). However, it has never been mentioned about the relationship in horsehair worm's size and its own morphology. The only one individual bearing the "abnormal crowned areoles" and no bristlefields is only half in length (58 mm in length) to the other two (125 and 115 mm in length) from the same host individual. One possibility to cause the abnormal morphology,

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even though we have no direct evidence, might be the incomplete development caused by the resource competition or synchronized maturation. The horsehair worms inside one host individual might compete their own fecundity through resource competition, or ensure their survival by synchronized maturation before the host suicide behavior. These consequently cause the horsehair worm, which might enter the host latter than its neighbors, to become mature without completing for its development. Thus, we suggest that the abnormal crowned areoles might be a result of incomplete development instead of the smaller size. This hypothesis could also be supported by another extremely small *C. formosanus* (43 mm in length) singly parasitized a long-horn grasshopper (9.63 mm in length) in this study whose crowned areoles are more likely to be normal.

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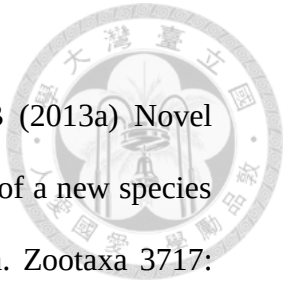

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582

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583 Table 1. Specimen information of *Acutogordius formosanus* sp. n. and *Chordodes formosanus* examined

Horsehair worm									
Species	**Collecting date**	**GenBank no.**	**Locality**	**Longitude and latitude**	**Collector**	**Depository**	**Sex**	**Length (mm)**	**Host code**
A. formosanus	16-XI-2014	KX591947	Xindian, New Taipei, Taiwan	24°50'47.70"N 121°32'41.20"E	Shipher Wu	NTU	F	283	HAc23302
A. formosanus	2-VIII-2009	KX591922	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	M	334	HAc26201
A. formosanus	29-VII-2009	KX591948	Fushan botanical garden, Yilian, Taiwan	-	Ming-Chung Chiu	NTU	M	278	HAC26401
A. formosanus	10-VII-2011	KX591926	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	M	312	HAc26206
A. formosanus	5-VII-2011	**KX591927**[1]	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	M	410	HAc26207
A. formosanus	5-VII-2011	KX591928	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	M	428	HAc26208
A. formosanus	18-VIII-2011	KX591929	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	F	360	HAc26209
A. formosanus	20-VII-2010	KX591930	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	M	387	HAc26210
A. formosanus	24-IX-2011	KX591931	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	M	262	HAc26211-12
A. formosanus	24-IX-2011	KX591932	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	F	272	HAc26211-12
A. formosanus	5-VIII-2012	**KX591933**[2]	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	F	288	HAc26214
A. formosanus	21-VII-2012	KX591934	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	M	133	HAc26215
A. formosanus	21-XI-2012	KX591935	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	M	241	HAc26217
A. formosanus	31-VIII-2012	KX591937	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	M	222	HAc26219-20
A. formosanus	31-VIII-2012	KX591938	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	M	216	HAc26219-20
A. formosanus	31-VIII-2012	KX591939	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	F	322	HAc26221-21A
A. formosanus	31-VIII-2012	NA[3]	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	F	73	HAc26221-21A
A. formosanus	31-VIII-2012	KX591940	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NMNS	F	285	HAc26222

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A. formosanus	26-VII-2014	KX591941	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	LBM	M	369	HAc26223
A. formosanus	26-VI-2015	KX591942	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	LBM	M	164	HAc26225-26
A. formosanus	26-VI-2015	KX591943	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	LBM	F	166	HAc26225-26
A. formosanus	17-VII-2015	KX591944	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	LBM	M	280	HAc26228
A. formosanus	17-VII-2015	KX591945	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	LBM	F	432	HAc26231
A. formosanus	17-VII-2015	KX591946	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	LBM	F	237	HAc26232
C. formosanus	11-II-2015	KX591949	Taipei Zoo, Taipei City, Taiwan	24°59'44.70"N, 121°34'49.49"E	Long-Chun Huang	NMNS	M	58	HCH11606-8
C. formosanus	11-II-2015	KX591950	Taipei Zoo, Taipei City, Taiwan	24°59'44.70"N, 121°34'49.49"E	Long-Chun Huang	NMNS	M	125	HCH11606-8
C. formosanus	11-II-2015	KX591951	Taipei Zoo, Taipei City, Taiwan	24°59'44.70"N, 121°34'49.49"E	Long-Chun Huang	NMNS	M	115	HCH11606-8
C. formosanus	4-III-2015	KX591952	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	M	43	HCH26207
C. formosanus	10-XI-2015	KX591953	Jiaushi, Yilian, Taiwan	24°49'55.62"N, 121°44'50.12"E	Ming-Chung Chiu	NTU	M	204	HAc26216

LBM: Lake Biwa Museum; NMNS: National Museum of Natural Science; NTU: National Taiwan University.

[1] Holotype.

[2] Allotype.

[3] The female sample whose DNA condition was not enough to be sequenced.

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589 Table 2. Host information of the horsehair worms examined

Host code (in Table 1)	Host species	Host sex	Host length (mm)
HAc23302	*Mecopoda elongata*	M	31.5
HAc26201	*Eugryllacris* sp.	M	27.5
HAC26401	*Neanias magnus*	F	20.5
HAc26206	*Neanias magnus*	M	21.9
HAc26207	*Eugryllacris* sp.	M	27.2
HAc26208	*Eugryllacris* sp.	M	25.9
HAc26209	*Hexacentrus japonicus*	F	29.4
HAc26210	*Sinochlora longifissa*	F	33.5
HAc26211-12	*Hexacentrus unicolor*	F	28.8
HAc26214	*Elimaea* sp.	F	27.1
HAc26215	*Deflorita apicalis*	M	22.3
HAc26217	*Pyrgocorypha formosana*	F	41.3
HAc26219-20	*Phaulula* sp.	F	23.1
HAc26221-21A	*Hexacentrus unicolor*	F	23.9
HAc26222	*Hexacentrus unicolor*	F	29.1
HAc26223	*Elimaea* sp.	F	27.2
HAc26225-26	*Neanias magnus*	M	17.9
HAc26228	*Hexacentrus unicolor*	M	28.4
HAc26231	*Eugryllacris* sp.	F	30.6
HAc26232	*Isopsera* sp.	M	24.1
HCH11606-8	*Acromantis japonica*	F	29.4
HCH26207	*Leptoteratura* sp.	F	9.6
HAc26216	*Holochlora japonica*	F	39.2

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Figure legends:

Figure 1. Anterior end of *Acutogordius formosanus* sp. n. **A-C** Anterior end with the white cap and the dark brown collar (A) and the Anterior end with white spots scattered on the brown collar (B-C) **D-F** SEM image of the anterior end whose surface is smooth (D), smooth but wrinkled on the tip with holes scattered on dark brown collar (E), and wrinkled (F). A-D, B-E, C-F are images from the same individual.

Figure 2. Posterior end of male *Acutogordius formosanus* sp. n. **A-C** Posterior end with the postclocal crescent extending over (A, C) or anterior to (B) starting point of bifurcation of tail lobes **D-F** SEM image of the posterior end with angled (D), slightly curved (E), and semicircular postclocal crescent (F). A-D, B-E, C-F are images from the same individual.

Figure 3. Detailed diagnostic characteristics of male *Acutogordius formosanus* sp. n. **A** Tiny bristles scattered anterior of postclocal crescent **B** tiny bristles scattered concentratedly on tail lobes **C** lobe tips covered by moderately flat areoles with short spines among areoles **D-E** short bristles scattered on the cuticle of mid body.

Figure 4. Female *Acutogordius formosanus* sp. n. **A** Anterior end **B** posterior end. Clo, cloacal opening.

Figure 5. Morphological variation of the cuticle which might be resulted from the mucous. **A** Smooth cuticle **B-C** wrinkled cuticle **D-E** cracked surface of cuticle **F** areole-like structures on the anterior end **G-J** hole-like structures on the cuticle surface of mid body (G-I) and anterior end (J).

Figure 6. Immature stages of *Acutogordius formosanus* sp. n. **A-B** Live larva in water of the cyst-form (A) and larval-form (B) **C** larval-form larva under SEM **D** cysts in an infected snail **E** egg strings **F** posterior view of a larva **G** anterior view of

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a larva showing the proboscis and hook arrangement. Cys, cysts in a snail; Ho, hooklet; Peo, pseudointestine exterior opening; PostS, postseptum; PreS, preseptum; Pro, proboscis; PsI, pseudointestine gland.

Figure 7. Neighbor-joining tree of *Acutogordius formosanus* n. sp. and *Chordodes formosanus* with the comparison of *C. japonensis*, *Gordius attoni*, *G.* cf *robustus*, *G. balticus*, and *Paragordius* sp. Numbers at the nodes represent the percentage of 5000 bootstrap values.

Figure 8. Male adult of *Chordodes formosanus* from novel hosts. **A** Posterior end of the male **B** anterior end of the male **C-D** different morphologies of crowned areoles from different individuals **E** closed view of (C) with the usual crowned areoles of *C. formosanus* **F** closed view of (D) with the smaller crowned areoles. Ca, crowned areole.

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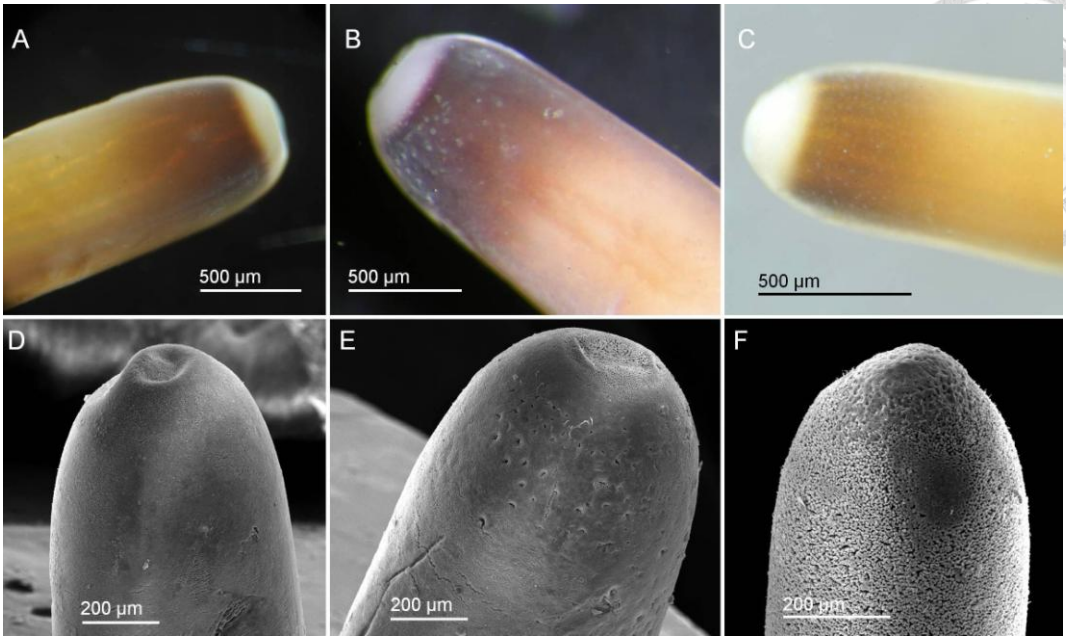

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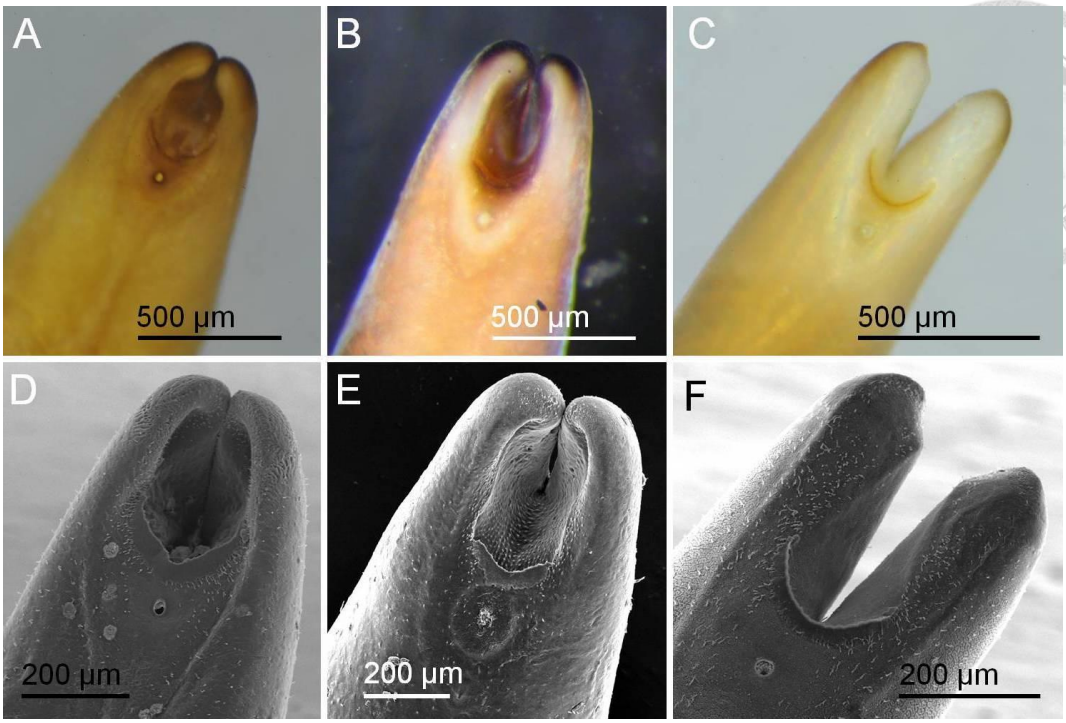

639

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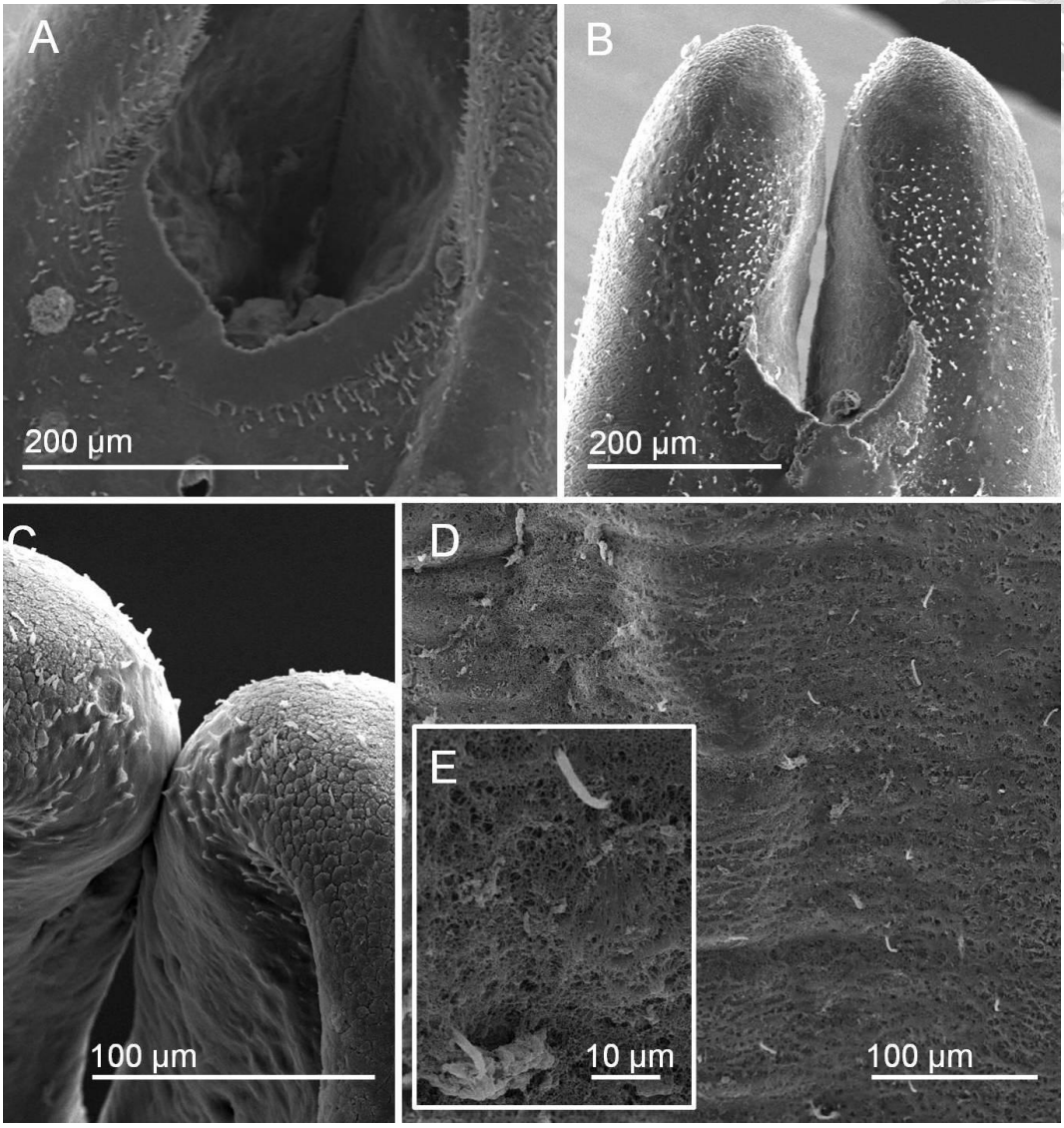

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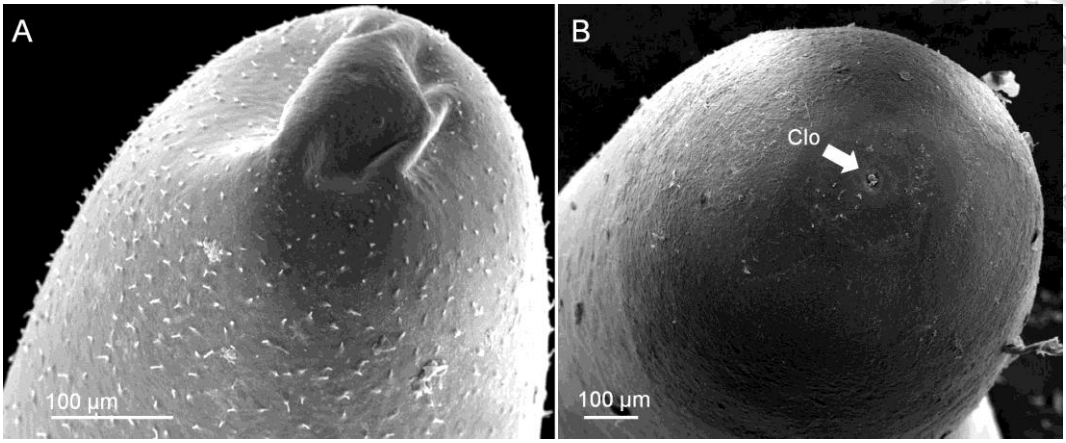

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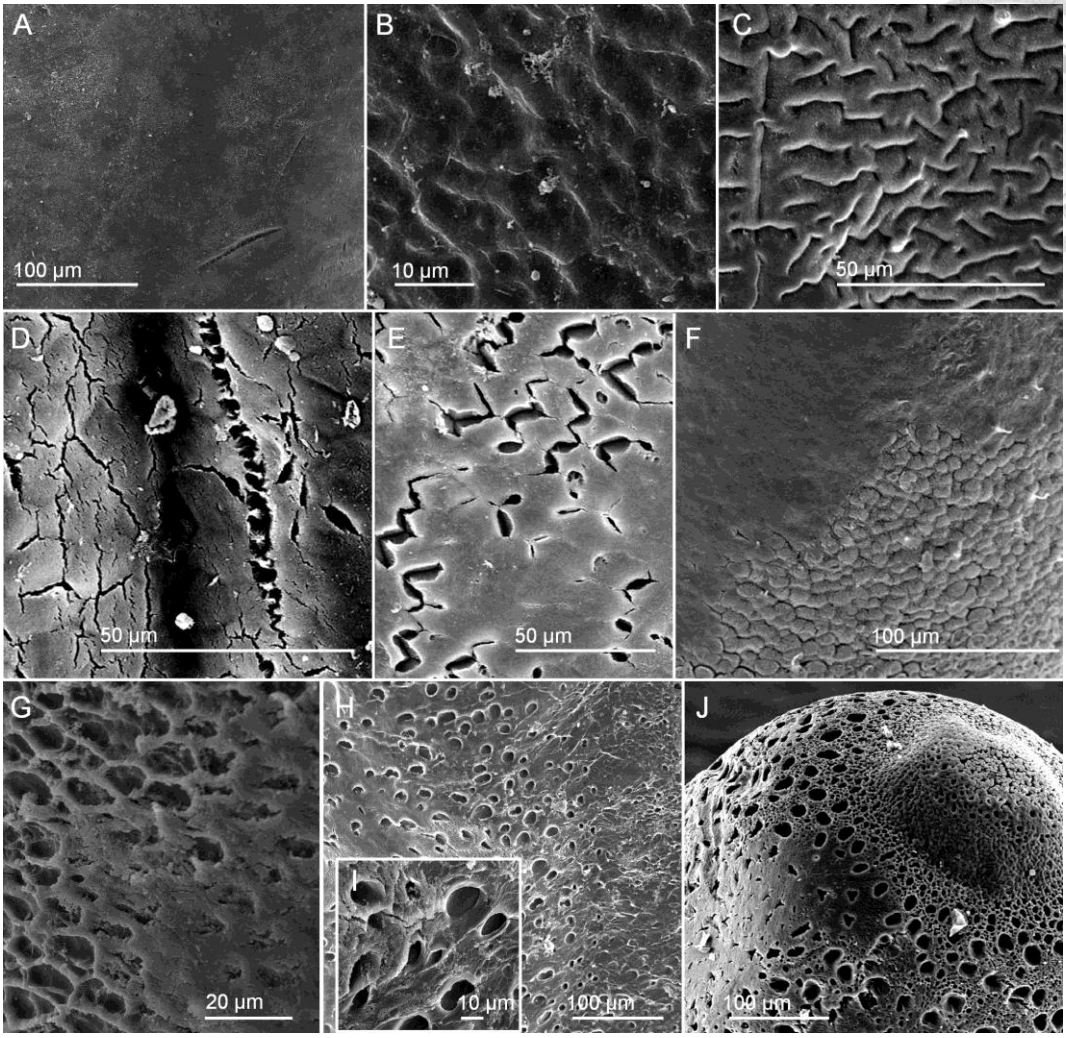

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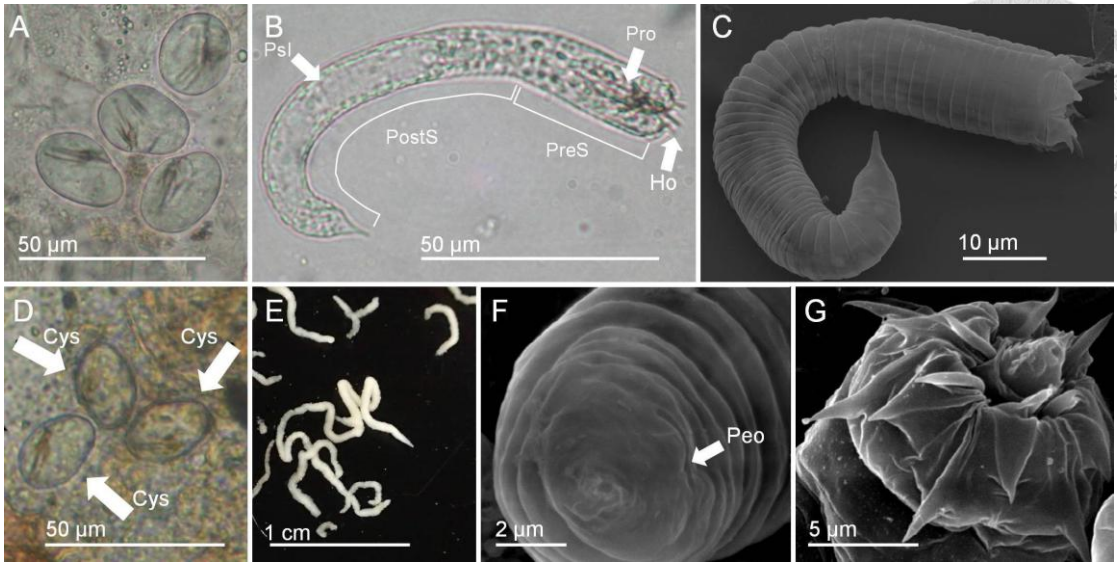

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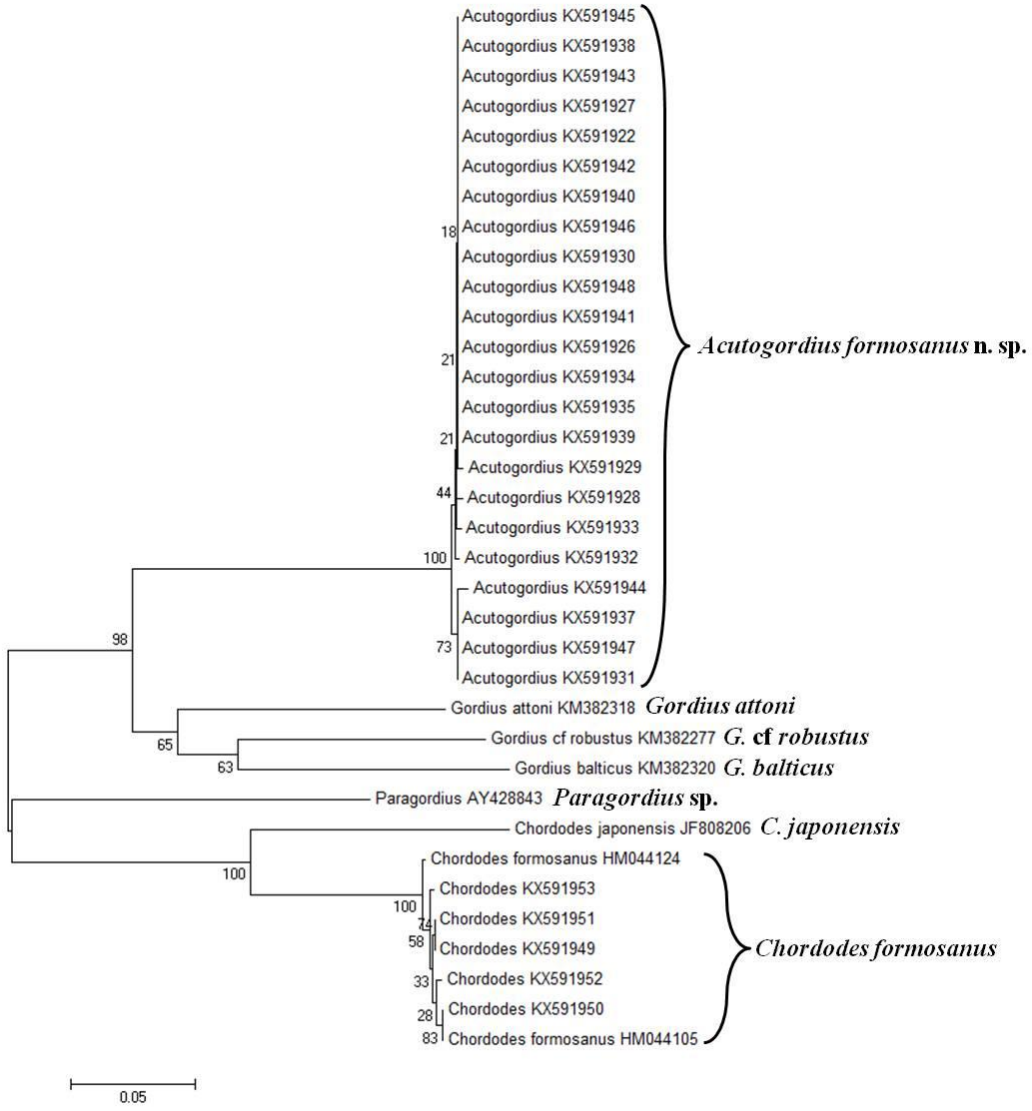

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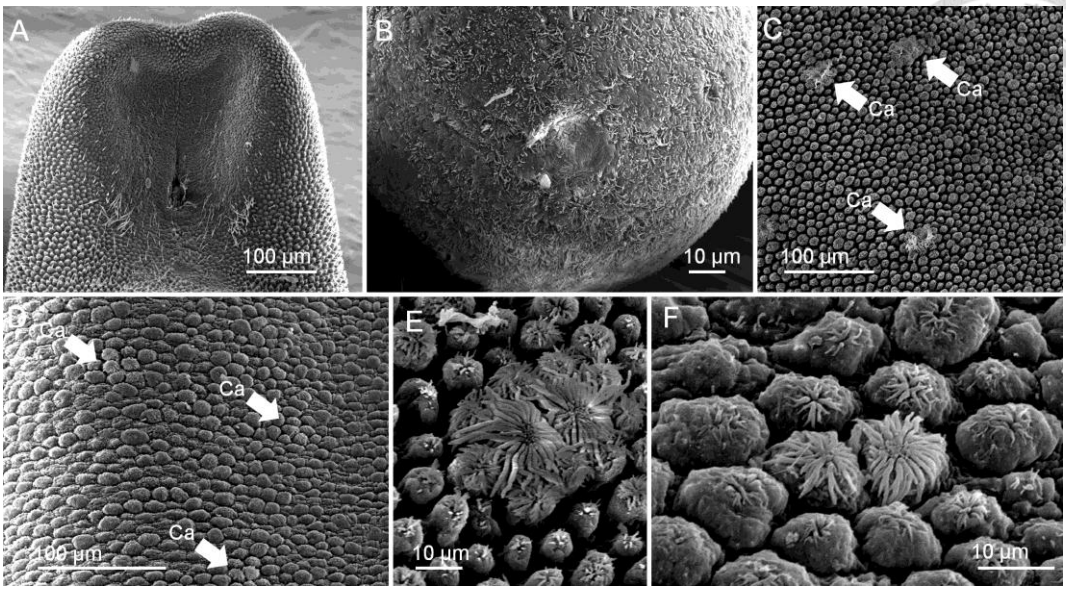

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688

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Appendix 5.

Ministry of Science and Technology Overseas Project

for Post Graduate Research

Mechanism in host morphological manipulation: Mimetic Wnt protein alters host sexual differentiation

Final Report

Project level: ■Ph.D. student □Post doctoral scholar

Grant ID number: 104－2917－I－002－001－

Project full duration (dd-mm-yyyy): 25-08-2015~21-08-2016

Domestic institute/department/chair or advisor affiliated (for Ph.D. student only): 台灣大學/昆蟲學系/吳文哲，蕭旭峰

Report written by (same as the grantee): 邱名鍾

Project-associated (overseas) advisor: 佐藤拓哉

Research abroad destination (country/state): 日本，神戶市

Research abroad institute: 神戶大學，大學院理學研究科

Institute address: 1-1 Rokkodai-cyo, Nada-ku, Kobe city, Japan 657-8501

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一、摘要

寄主的形態變化反應了寄生蟲感染對發育造成的影響，也因此成為研究寄生蟲生存策略的重要指標。在我們的研究中，臺灣斧螳 (*Hierodula formosana*) 雄成蟲受到台灣索鐵線蟲 (*Chordodes formosanus*) 感染後出現間性的現象，造成雌性化的翅膀形狀及觸角感覺毛分布。這些變化理論上造成雄性寄主交配上的弱勢，但卻使寄主得以將投資在生殖行為的資源轉而支持鐵線蟲發育所必須消耗的能量。這類寄生蟲藉由掠奪寄主的生殖資源來避開毒性權衡中藉由降低資源利用效率來減緩寄主死亡率所造成的浪費，並因此干擾寄主性徵的發育。然而昆蟲的性徵分化長久以來被認為是細胞自主發育的結果，即不受細胞外因子的調控。因此在寄生蟲造成的間性案例中，幼年化 (juvenilization) 傳統上被用來解釋寄生蟲干擾昆蟲性徵分化的現象。許多生物的性徵在幼期雌雄之間並沒有明顯差異，而是在發育過程中漸漸分化。因此鐵線蟲可能藉由幼年化來阻斷性徵的發育而造成雄臺灣斧螳出現雌性化特徵。臺灣斧螳雌成蟲則因維持了末齡若蟲的形態，因此鐵線蟲的感染無法對其形態造成影響。在本試驗中，我們利用寬腹螳螂 (*H. patellifera*) 的性徵變化來檢驗幼年化假說是否是鐵線蟲造成寄主間性的機制。儘管觸角感覺毛的分布在寬腹螳螂成蟲也呈現和臺灣斧螳一樣的雌雄二型性，但寬腹螳螂的雌成蟲並沒有完全維持幼期的形態，因此寄生蟲造成的幼年化理應在雌成蟲身上也造成影響。在飼養試驗中，我們首先以索鐵線蟲 (*Chordodes* sp.) 感染不同發育時期的雄寬腹螳螂。在所有的雄成蟲中，鐵線蟲入侵到寄主羽化之間間隔的時間與形態改變 (觸角感覺毛的分布) 的幅度呈現明顯的正相關。而所有感染 24 天內羽化的雄成蟲其形態均與未感染的個體無明顯差異。在邏輯斯迴歸模型的預測下，9 成以上受鐵線蟲感染的寄主在 36 天內便被啟動形態變化的機制。而在雌寬腹螳螂螳螂的感染中，儘管本次成功感染的樣本數稀少，但所有的 4 個受感染樣本均未出現形態變化，其中也包含一個鐵線蟲發育超過 36 天的個體。而受感染的雄螳螂末齡若蟲形態一樣不因感染而有所改變。這些現象顯示鐵線蟲僅對雄成蟲的形態產生影響，且非單純的造成特徵的幼年化。寬腹螳螂的觸角感覺毛分布在羽化時產生雌雄分化，因此鐵線蟲的影響可能與寄主性徵分化的時間相關。這個推測在寬腹螳螂雌蟲腹部特有的紅斑發育得到進一步的支持。該特徵從螳螂進入三齡若蟲後便出現在雌蟲腹部背板並維持至成蟲，但雄蟲不具此特徵。然而 4 隻受感染的雄若蟲身上卻在感染後 32-35 天後被觀察到紅斑的出現。這顯示鐵線蟲對寄主的形態影響並非限定在寄主羽化時，而是在寄主性徵分化之後。這些結果使得間性的發生無法單純的以幼年化來做解釋，進而支持鐵線蟲調控昆蟲性徵分化的可能性。現今昆蟲性徵分化的調控機制尚未明瞭，鐵線蟲的研究很可能能協助釐清昆蟲性徵分化的調控因子。目前 Wnt 蛋白被認為可能是調控果蠅的性徵分化因子之一，而該蛋白質的含量也曾被發現在受鐵線蟲感染的寄主身上明顯提高。在鐵線蟲造成間性的機制漸漸被解開的情況下，Wnt 蛋白與間性之間的關聯將是下一步我們要釐清的目標。

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二、Abstract

Host morphological changes caused by parasite-altered developmental process reflect the parasite's adaptation in infecting the host. In our previous study, male adult of the mantid, *Hierodula formosana*, showed intersexual characteristics including feminized wing shape and distribution of antennal sensilla after being infected by the horsehair worm, *Chordodes formosanus*. These manipulations on the host morphology logically reduce the mating ability in the male adult, but divert the energy resource in host reproductive investment to supply parasite development. The reduction of host reproductive investment is believed to be the strategy to avoid the increasing host mortality during parasite's exploitation, which is known as the virulence trade-off. However, although the intersexuality has been found in wide parasitism cases, its functional relationship with host fecundity reduction is unclear. The insect sexual differentiation has long been believed to be the cell-autonomous process which lacks regulation outside a cell, including the parasitic effect. Thus, juvenilization, as the alternative hypothesis, is frequently applied to explain the occurrence of the parasitic intersexuality. The juvenilization suggests that parasites inhibit the ontogeny of host sex characteristics and maintain it in the stage earlier than diverging in the opposite sexes. This hypothesis well explains the gender-dependent effect of the infected *H. formosana* whose female adult was not manipulated since it maintains the same antennal morphology with its last-instar nymph. In the present study, we tested the hypothesis of juvenilization by examining the parasitic effect on the antennal character of *H. patellifera*. As that happened on *H. formosana*, the distribution of antennal sensilla is sexually dimorphic in *H. patellifera*, but the adult female of *H. patellifera* does not completely maintain the nymphal chatacter. Thus, the parasite-induced juvenilization is expected to affect adult of both sexes instead of only males. To investigate the beginning time of parasitic effect launched after the horsehair worm enter the host, we first infected the male mantids by the horsehair worm, *Chordodes* sp., and compared the sensilla distribution (the first flagellum segment bearing the grooved basiconic sensilla) on the adults of the infected mantid and the control one. The antennal distribution was significantly different in the infected adult and the difference increased with the horsehair worm developmental time before the last molting of the mantid host. All the infected mantids with horsehair worm developed less than 24 days showed the similar antennal characteristic with that on the control ones. Under the estimation of the logistic regression model, most of the infected hosts (90%) were manipulated 36 days after the infection. In contrast to the male adults, all the infected adult females and infected last-instar males showed non-manipulated antennal character even the horsehair

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worms had developed for more than 36 days inside the hosts. Since the sensilla distribution of mantid antennae sexually diverges during the last molting, the parasitic effect specifically happened on the male adult suggests the intersexuality occurring only after the time of sexual differentiation, which is hard to be simply explained by the juvenilization. This hypothesis is supported by the change in red pigments on female abdomen. This characteristic appears on only the female manitids since the 3rd instar, while 4 infected nymphal males in this study also showed this characteristic 32-35 days after infection. All these evidences suggest the horsehair worms intervene in the regulation of insect sexual differentiation. Since the factor in regulating insect sexual differentiation has not been well investigated, studies in the horsehair worm's parasitism might provide the different point of view in this issue. The Wnt protein, which has been known to be concentrated in the horsehair worm-infected crickets, is recently found to regulate the development of sex characteristic in male *Drosophila*. In the next step, we are going to examine the role of Wnt protein in the horsehair worm-induced host intersexuality.

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三、報告內容

1. Introduction

Parasite's extended phenotype related to host fecundity reduction

Under natural selection, the genotype is selected via its expression known as phenotype. This concept is broadly accepted but divided in opinion that "how far" can the reach of genes expressed (Hunter, 2009). The phenotype has never been confinedly defined to be appeared on the individual carrying its gene, but the adaptive effect of a gene expressing outside its carrier was not well noted until "the extended phenotype" suggested by Dawkins (1982). Such extended phenotypes on the biotic or abiotic materials also determine the gene favored in the natural selection, like the phenotype traditionally considered, and thus evolved through evolutionary processes. Parasite's extended phenotype on its host is known in wide parasitism cases which alters host's original phenotypes (Poulin and Thomas, 1999). These phenotypical changes on the infected host visualizes the adaptation of the parasites by creating the new function on the manipulated structures or as the indicative symptom showing the host physiological change. Unfortunately, under the great interest in the new function created by the parasite's extended phenotype, significance of the symptoms are sometimes overlooked or mislinked with an imaginary adaptation (Poulin, 2000).

The symptom related to host fecundity reduction, including the alterations in host morphology, mating behavior, and life history trait, has been found on the phylogenetically diverse host-parasite systems (Hurd, 2001). Such symptoms might not directly benefit to the parasites but the host fecundity reduction is one parasite strategy to avoid virulence tradeoff during their development (Hurd, 2001). The pareasite virulence tradeoff comes from the unavoidable host mortality during infection, which is increased with parasite’s exploitation and consequently cause parasite’s low transmission ability or high risk in premature death (Jensen *et al.*, 2006). With the side effect of host survival damage, unlimited increase in exploiting is unallowable, but it is unrestrained in those who intercept the resource in host reproduction. This hypothesis has been earlier noted according to the symptoms such as alteration of host gonad tissue (Baudoin, 1975) and supported by mathematical models (O'Keefe and Antonovics, 2002; Hall *et al.*, 2007). Since these parasitic symptoms reveal the altered host's investment in reproduction and survival, it might be a valuable not only in host-parasite interaction, but also, as Beckage and Gelman (2004) noted, in host development, reproductive strategy and sexual differentiation.

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Intersexuality in horsehair worm-infected host

Intersexuality is one common morphological symptoms associated with host fecundity reduction. These hosts show the altered sexually dimorphic characters which sometimes makes them resemble the opposite sexes in appearance (Baudoin, 1975). In few cases, these intersexual structure are suggested to be functional and benefit to parasite's adaptation (Rodgers-Gray *et al.*, 2004), while most of them are generally believed to be the side-effect of altered host development or outcome of castration (Wülker, 1964).

In the parasitism cases of horsehair worms and their ecological closed mermithids, intersexuality is commonly found on the infected hosts (*Metrioptera brachyptera*, *Pholidoptera* sp., *Pterostichus niger*, *Blaps mucronata*, *Vespa germanica* (Wülker, 1964), midges (Chironomidae) (Rempel, 1940), biting midges (Ceratopogonidae) (McKeever *et al.* 1997), grasshoppers (Acrididae) (Rowell, 2000), mayflies (Baetidae) (Vance, 1996), and mantids (Roy, 2003)). The large body size is believed to promote the parasite to consume host fecundity (Lafferty and Kuris, 2009). In the case of the horsehair worms, their body size increase from a 50 μm cyst to tens of center meters juvenile worm occupying almost whole body cavity of the host (Hanelt *et al.*, 2005). With the high consumption of host energy and spatial resource, these parasites are believed to avoid host mortality risk by consuming the resource from host reproduction (Lafferty and Kuris, 2009).

Our study resolved that the adult mantids, *Hierodula formosana*, infected by the horsehair worms, *Chordodes formosanus*, showed intersexuality (abnormal fore-wing shape and lower density of antennal sensilla) in the adult male, whereas the infected female adult maintained its normal morphology (Chiu *et al.*, 2015). The mantids are sexually dimorphic in their adult stage. The males are structured by longer wings and walking legs (mid-legs and hind-legs), and higher number of antennal sensilla (Robinson and Robinson, 1979; Roy, 2003; Béthoux, 2010; Lombardo and Umbriaco, 2011). These characters makes scientists believe the male mantids are pheromone receptors since Slifer (1968), which is confirmed when the female calling behavior were found by Robinson and Robinson (1979).

The mechanism of intersexuality: Sexual differentiation or juvenilization

The horsehair worm's infection inhibits the development of the reproductive characteristics in its male mantid host. In contrast to the male, the female mantid maintains more nymphal characteristics (Chiu *et al.*, 2015). Maintaining the nymphal characteristics help the female to invest more energy resource in producing eggs, while the male tend to develop structures related to

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mating behaviors (Hurd, 2009). It is one of the possible reasons that the horsehair worm makes the obvious morphological symptoms in the infected male and raise the hypothesis of juvenilization as the mechanism of parasitic intersexuality (review of Baudoin,1975). Juvenilization caused by the parasites maintain the sexually dimorphic structures in the early step of ontogeny (review of Baudoin,1975). This hypothesis has widely applied to explain the origin of intersexual characteristics especially in the insect hosts, which can be approached by the increased juvenile hormone titer in the host (Fisher and Sanborn, 1962; Down *et al.*, 2008; Hurd, 2009). Other than the juvenilization, parasites also cause intersexuality by interference in host sexual differentiation. In the parasitism of murine and amphipod hosts, parasites manipulate sex hormone titer and lead to the intersexuality on their hosts (Larralde *et al.*, 1995; Rodgers-Gray *et al.*, 2004).

These two hypotheses indicate different developmental processes that the horsehair worm might intervene during infection. Despite the insect sexual differentiation has long time been believed to be the cell-autonomous process which lacks regulation outside a cell (Negri and Pellecchia, 2012), the current studies is now challenging this viewpoint (de Loof and Huybrechts, 1998; DeFalco *et al.*, 2008). The critical evidence in distinguishing these two hypotheses is if the host characteristic stop developing when the parasitic effect begins. In this study, we used the distribution of mantid antennal sensillum as morphological indicator to examine "the beginning of parasitic effect on host morphology" by examining the morphology of the adult male mantids infected in different developmental stages, and to test "the hypothesis of juvenilization" by examining the morphology of the last-instar male and adult female mantids.

2. Materials and methods

(1) Mantid rearing

A mantid ootheca, *Hierodula patellifera*, was collected from the Kobe University, Kobe, Japan. Each newly hatched nymph was reared alone in a box under 30°C, 50-70% RH. Food (*Drosophila* sp. and *Tenebrio molitor*) were provided 3 times a week.

(2) Infection of the horsehair worms

Horsehair worm egg strings with the female laying eggs were collected from Yamaguchi prefecture, Japan. The females were fixed by 99% alcohol and sequenced to examine their species. The egg strings on a stick were reared in aerated tap water under room temperature. The eggs nearly hatching turn dark in color and were cut into a 2-3 mm piece for the infection. Physid snails (*Physa* sp.) collected from a aquatic plant greenhouse in the Kobe University were applied as intermediate hosts.

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Each snail was exposed to an egg piece for 4 nights in a well of the 26-well culture plate. Then all the snails were reared together in aerated tap water and fed with the cabbage for 15-20 days. The snails survived for 15-20 days (71 of the 236 infected snails) were removed and preserved under -80°C until examined or fed to the mantid host (Bolek *et al.*, 2013).

The 23 preserved snails were randomly sampled for examining the infection. Their shells were removed and the soft tissue was flatted by two glass slides before counting the cyst number under light microscope. All these 23 snails were all infected with 153±136 (19-616) cysts. The left preserved snails were used to infect the mantids. Their shells were removed and the soft tissue was separated into 2-3 pieces. Each piece was expected to contains 65±60 (6-308) cysts. During the infection, a piece of the infected snail was mixed with the fat body of *T. molitor* and touched the mouth part of the mantid. Thirty-nine nymphal males and 8 nymphal females (4^{th} and 8^{th} instar) were randomly selected to do the infection for twice with the interval of 8 days. The remained 14 males and 6 females at the similar stage were fed with the snail piece without horsehair worm cysts and reared under the same condition as control. These mantids were reared to be adults and recorded the adult instar, the date to eclosion, and number of the horsehair worms inside their body.

(3) Examination of the antennal characteristic

The horsehair worm's manipulation was indicted by the distribution of antennal sensilla. The grooved basiconic sensilla on mantid antennae were found to be sexually dimorphic in total amount and its distribution (Slifer, 1968). It spreads not all over antenna but from a given flagellum segment to the tip. The first flagellum segment bearing the grooved basiconic sensilla on the female adult is posterior than that on the male adult in *Hierodula*. Such sexual dimorphism is disappeared on the infected adults while the male shows the feminized antennal character whereas the female maintains the similar characteristic with the non-infected mantid (Chiu *et al.*, 2015).

In the present study, we quantify the horsehair worm's manipulation by the first flagellum segment bearing the grooved basiconic sensilla. The antennae of the adult mantids and the molting skin of the last instar nymph were collected and preserved in 75% alcohol. Number of sensilla on each segment were counted under the light microscope. The first flagellum segment bearing more than 3 grooved basiconic sensilla (BS) was recorded and considered as the beginning of their distribution.

(4) Statistical analysis

The basic infection parameters were measured by the infection rate (infected mantids/total mantids fed with the infected snail), mean intensity (total horsehair worm number/infected mantids). The mortality rate of the infected and control mantids before the adulthood were compared by Fisher's exact test.

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a. The beginning of parasitic effect on host morphology

The beginning of parasitic effect on host morphology was examined by the time when male hosts show the manipulated antennal characteristic. The BS values of the last instar, and adult males were first compared between the control and infected mantids by Mann-Whitney *U* test. The infected adults were characterized by their BS into "manipulated group" whose BS is outside of two standard deviations of the control mantids and "non-manipulated group" whose BS is inside of one standard deviation of the control ones. The BS in each group were analyzed by linear regression model against the "number of days from the date of infection to last molting (MT)". These two categories were coded as 0 (Non-manipulated group) and 1 (Manipulated group) and analyzed by logistic regression model against MT to get the time when 50% and 90% of the infected mantids showed the manipulated antennal characteristic.

b. The hypothesis of juvenilization

Since the female *H. fromosana* maintains its nymphal antennal characteristic in the adult stage, the juvenilization is one possible explanation of the unchanged antenna on infected female (Chiu *et al.*, 2015). In the antenna of *H. patellifera*, the distribution of the grooved basiconic sensilla is distributed toward the antennal base (the BS value decreases during the last molting), while the sexual dimorphism is still showed since the distribution in the male approach more than that in the female (the BS value is lower in the male adult than that in the female adult) (Chiu *et al*., 2013). The difference between the adult and the last instar nymph in the female *H. patellifera* is here used to test the hypothesis of juvenilization and gender-depended interference of the sexual differentiation. BS values of the female adult and the last instar nymph were compared by paired Student's *t*-test. The parasitic effect was also tested by comparing the BS values of infected female adults and control female adults by Mann-Whitney *U* test.

All statistical analyses were performed using R (version 3.1·0, R Development Core Team, 2014) with the base packages.

3. Results

(1) The beginning of parasitic manipulation

a. Infection rate

Amount the 47 mantids consuming the infected snails, 35 mantids survived to the last molting. All these 35 mantids were infected with at least one horsehair worm inside. Except 10 dead mantids whose horsehair worms were too rotten to be counted, the mean intensity of the left 25 infected mantids is 11.76±7.25 (2-29) worms. Sixteen of the 20 control mantids survived to the last molting, no worm was found inside them. The mortality rate of the mantids before the adulthood was not significantly different

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between the normal and infection groups (Fisher's exact test, P = 0.76), most of the infected mantids (33/35) died before the worm became mature.

b. Parasitic effect on the host antennal sensilla

The distribution of grooved basiconic sensilla is significantly posterior to the antennal tip (Mann-Whitney U test: U = 64, P = 0.009) in the adult of infected mantid (BS: 34.26±7.04 (23-48)) than the control one (BS: 27.73±2.94 (23-32)). Among 27 infected mantid's with clear antennal character, 13 of them were considered as the "manipulated group" and 11 were the "non-manipulated group", while 3 antenna whose BS values fall between one to two standard deviations of the control mantids were removed in the following analysis (Fig. 1). The BS values belong to the "non-manipulated group" are not significantly correlated to the MT (Linear regression model: β = 0.10, t = 0.89, P = 0.397), whereas the BS values of the "manipulated group" show the significant correlation to the MT (Linear regression model: β = 0.37, t = 2.63, P = 0.02). The BS value in the last-instar nymphs showed no significant difference (Mann-Whitney U test: U = 147, P = 0.9469) in the infected (BS: 56.48±1.97 (52-60)) and control mantids (BS: 56.69±2.36 (54-61)) (Fig. 2). Almost all the BS value in the infected mantids fall into one standard deviation of the control mantids. Only three of infected mantids with the BS values outside one standard deviation of the control one but inside two standard deviations. The BS values of the infected mantids are not significantly correlated to the MT (Linear regression model: β = -0.06, t = -1.65, P = 0.113).

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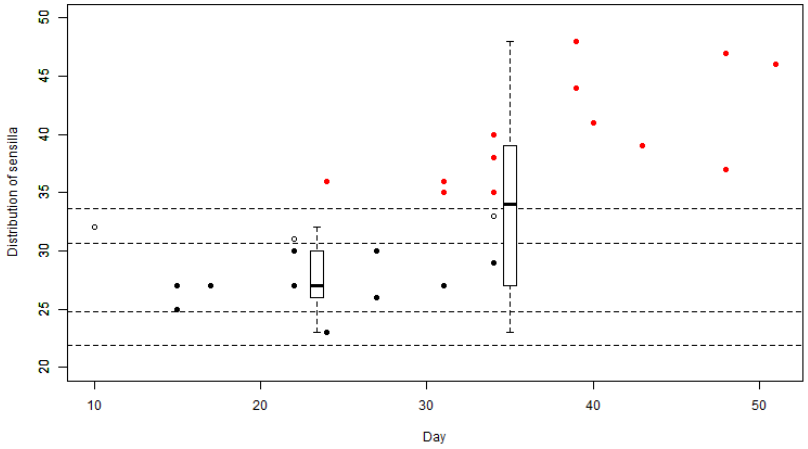

Fig. 1. Distribution of the grooved basiconic sensilla in the adult of infected and control mantids. Y-axis is the first flagellum segment bearing the grooved basiconic sensilla. X-axis is developmental time between the date of infection and the last molting. The box-plots are the values of the control mantids (left, n = 11) and the infected mantids (right, n = 26). The dots are values the infected mantids against the developmental time (black: non-manipulated group; red: manipulated group; hollow: infected mantids with the value fallen between 1-2 standard deviations of the control mantids).

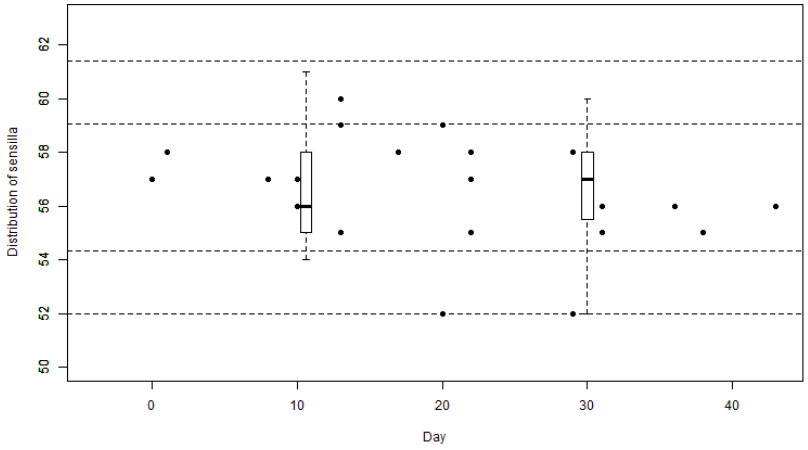

Fig. 2. Distribution of the grooved basiconic sensilla in the last instar of infected and control mantids. Y-axis is the first flagellum segment bearing the grooved basiconic sensilla. X-axis is developmental time between the date of infection and the last molting. The box-plots are the values of the control mantids (left, n = 13) and the infected mantids (right, n = 23). The dots are values the infected mantids against the developmental time.

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c. The time when the host start to show the manipulated antennal characteristic

The probability of mantids showing manipulated antennal characteristic increases with the MT (Logistic regression model: $\beta = 0.32$, $z = 2.37$, $P = 0.02$). The parasitic effect was showed from the mantids which were infected 24 days before becoming adult. The estimated time when 50% of the host start to be manipulated is around 30 (95CI: 26.21-32.95) days while 90% of the host start to be manipulated is around 36 (95CI: 32.24-40.53) days (Fig. 3). Since all the infected mantids were fed with the infected snails for twice, the estimated time might be revised to 22 days for 50% manipulation and 28 days for 90% manipulation.

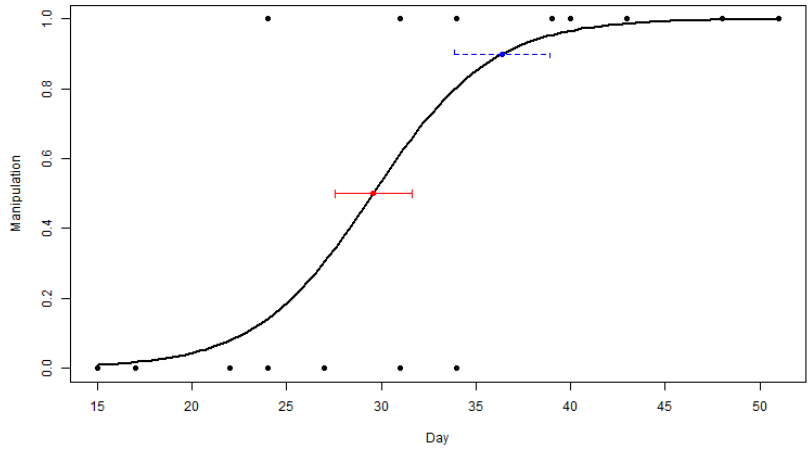

Fig. 3. The beginning of the manipulation after the horsehair worms enter the mantid host. Y-axis is the manipulated mantids (1) or non-manipulated mantids (0) against the developmental time between the date of infection and the last molting (X-axis). The black line is the regression line estimated by logistic regression model. Red dot and black dot are 50% and 90% individuals been manipulated, respectively, estimated by the logistic regression model with ± SD.

(2) The hypothesis of juvenilization

The distribution of grooved basiconic sensilla is not manipulated by the horsehair worm (Mann-Whitney *U* test: $U = 9$, $P = 0.373$). The BS value is 36.75± 2.36 (35-40) in the infected female adult and 35.00±2.65 (33-38) in the control female adult. The distribution of grooved basiconic sensilla is changed during the last molting while the BS values significantly decrease in both infected (paired Student's *t*-test, $t = 7.81$, $P = 0.004$) and control females (paired Student's *t*-test, $t = 10.03$, $P = 0.010$).

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4. Discussion

The beginning of parasitic manipulation

Our experiment reproduce the antennal manipulation, which was found on the field-collected *H. formosana* (Chiu *et al.*, 2015), in the artificially infected *H. patellifera*. Such parasitic effect was first found from 24 days after infecting the host and more than 90% mantids were manipulated 36 days after being infected.

The horsehair worm is widely known to manipulate the host behavior when the worms nearly become mature (Sanchez *et al.*, 2008). However, this is the first time to investigate the horsehair worm's effect during their early development. In our experience, *Chordodes* spends 97±22 (54-143) days developing from cysts to adults (unpublished data, n = 18). Our data show that most of the horsehair worms start to manipulate their hosts in the first one-third (30-36 days) of their developmental period. Unlike the behavior manipulation which is advantageous to horsehair worm's reproduction (Thomas *et al.*, 2002), the host morphological manipulation is more likely to promote the host energy exploitation for the parasite's development (Chiu *et al.*, 2015). Launching the manipulation in the early stage is benefit for the parasite to gain more resource from the host. The length of developmental period might also cause the intensity of the morphological change on the host. In our pervious field survey of *H. formosana*, the infected male showed different levels of change on their wing shape (Chiu *et al.*, 2015). The similar pattern was also found in the antenna of the "manipulated group" in the present study. These two phenomena might indicate the increasing parasitic effect with infection time or earlier stage of host ontogeny. In the parasitism case of midge infected by mermithid, parasites invading in different stages of host larvae create different level of intersexual characteristics on the male hosts (Rempel, 1940). Since the ontogeny of some characteristics on the hosts are irreversible, parasites are unable to manipulate such characteristics whose fate is already determined (review of Baudoin,1975). This hypothesis is supported by the mantids belonging to the "non-manipulated group". These infected mantids were infected when they nearly became adulthood whose antennae might be already developed before the parasitic effect was carried out.

The increasing parasitic effect on the male antenna implies the gradually diverged process in the host sexual differentiation. However, this speculation does not completely match the known sexual differentiation of *Hierodula*'s antennae. In nymphal stages of *H. parellifera*, the sensillum distribution changes during each molting but is identical in the both sexes. This characteristic is diverged only during the last molting (Chiu *et al.*, 2013). The gradual antennal change might be caused by the accumulation of parasitic effect on the time point of the host

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sexual differentiation. However, this explanation needs more evidence to be confirmed.

Juvenilization and sexual differentiation

Under the hypothesis of juvenilization, the adult antenna of female *H. formosana* was not influenced since it maintains the nymphal characteristic (Chiu *et al.*, 2015). Thus, the female adult of *H. patellifera* might suffer the morphological manipulation since its antennal characteristic changes during the last molting (Chiu *et al.*, 2013). However, all the four infected females showed the significantly different antennae in their nymphal and adult stages. As that happened on the female *H. formosana*, female *H. patellifera* were also free from the parasitic manipulation on its antennae. The infected female with the horsehair worms developing for more than 36 days before its last molting (90% of the infected hosts are expected to be mnaipulated), although there is only one sample, also showed the obviously change from the last instar nymph to adult (BS from 50 to 40). It is undoubted that more samples are necessary, but the current evidences seem to challenge the hypothesis of juvenilization in horsehair worm's effect. Except the female adult which is non-manipulated under the horsehair worm's effect, the last instar male is also free from the manipulation. The BS values in the last-instar infected male are not correlated with the developmental time of the horsehair worm and the 3 infected males with horsehair worms developing for more than 36 days still maintain the normal antennal characteristic.

This phenomenon also suggested the parasitic manipulation on the mantid antennae was carried out at the critical window of host development. The developmental window is the periods to receive the information in regulating organism development (DeWitt and Scheiner, 2004). In the infected host, although the parasitic effect is already launched, the host morphology is manipulated when it start to receive the cue in developmental regulation. As both of the female adult and last-instar male of *H. patellifera* are free from the manipulation on their sensillum distribution, we believe such developmental window only opens during the last molting of the male, when the sexual differentiation of the sensillum distribution happened (Chiu *et al.*, 2013). Another phenomenon supporting the opening of the developmental window of the sexual differentiation is the red pigment on the abdomen of female. During the experiment, we found the red pigment exits from 3rd-instar to adult of the control female mantids but not in the control male. This characteristic existing in only females appeared in 5 infected male nymph which were 32-35 days after being infected showed the similar characteristic (Fig. 4). The parasitic manipulation in the infected male nymphs on the red pigment instead of the sensillum distribution also support the horsehair worm create intersexual characteristics by regulating the host sexual differentiation instead of simply

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juvenilizing their hosts.

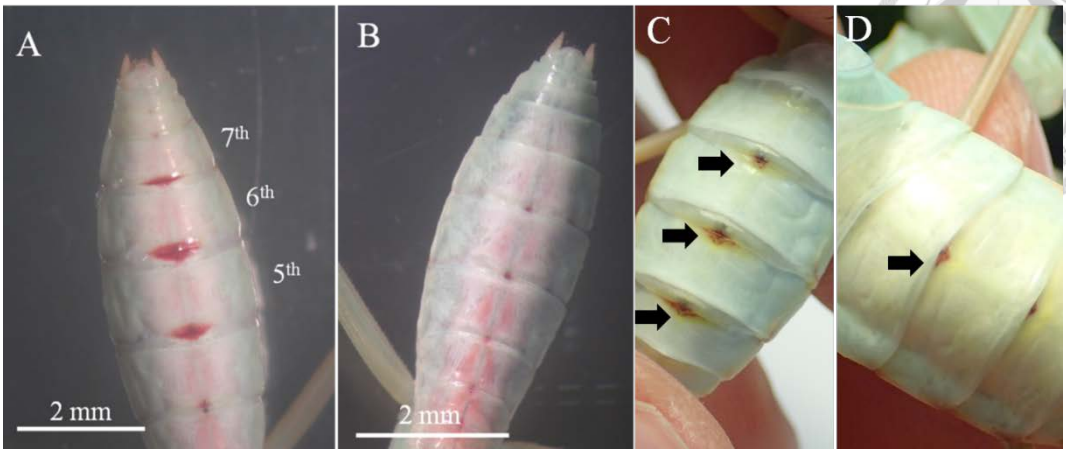

Fig. 4. Red pigments on 5th-7th tergum of female *Hierodula patellifera* (3rd instar) (A). The pigment does not exist in the normal male (3rd instar) (B) but appears in the horsehair worm-infected males in both nymph (C) and adult (D). The black arrows indicate the red pigments in the infected male mantids.

The present study suggests the horsehair worm, *Chordodes formosanus*, starts to affect development of its mantid host, *Hierodula patellifera*, around 36 days after infection. Such parasitic effect manipulates the morphology of mantid sex characteristics and expresses when the host sexual differentiation is launched. This phenomenon implies the parasite intervenes in the regulation of insect sexual differentiation. Traditionally, the insect sexual differentiation is believed to be the cell-autonomous process (Negri and Pellecchia, 2012). In other word, no or only weak evidences were found in regulating the sexual differentiation during insect embryonic and post-embryonic development. DeFalco *et al.* (2008) found the male-specific characteristic in *Drosophila* embryo was expressed in the female cell surrounded by the male cell through the regulation of *Wnt2* gene. The amount of Wnt protein is also found to be increased in the horsehair worm-infected hosts (Biron *et al*., 2005; 2006). This physical manipulation is long-term believed to be related to the suicide behavior in the infected hosts, although the function of the increased Wnt protein has never been known (Biron and Loxdale, 2013). It is worth to test the relationship in Wnt protein and the intersexual characteristic on horsehair worm-infected hosts. We hypothesize the mantid sexual differentiation is regulated by Wnt protein while the higher amount of Wnt protein expression creates the male-specific characteristics. By designing the primer set (Mantis_Wnt1-F: TGCCCAGCTTTAGAGTGGTC, Mantis_Wnt1-R: TATCTCCTGCGTCTTGTACCC) from mantid wingless partial gene (Genbank number: FJ802922, GU064802, FJ803121, KT732107, FJ803173, FJ803027), partial cDNA of the wingless gene from *H. patellifera* were sequenced. In

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the future experiment, we are going to compare the expression of wingless gene in both sexes of *H. patellifera* to test this hypothesis. Then investigating the development of these sex characteristics under the abnormal increase of the Wnt protein in non-infected mantids.

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四、科技部補助博士生/博士後國外研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

□ 達成目標

■ 未達成目標（請說明，以 100 字為限）

□ 實驗失敗

□ 因故實驗中斷

■ 其他原因

說明：雖然實驗顯示預期中的趨勢，然結果需要更大的樣本數來支持。分子機制的部分則已得到 Wnt 蛋白在螳螂體內的表現序列。目前飼養技術已經接近成熟，將先以此序列做為探針比較螳螂 Wnt 蛋白的表現量是否有雌雄間的差異。

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿■撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

鐵線蟲對其寄主的形態影響是純基礎研究議題。目前學界對鐵線蟲寄生關係的研究大多集中在對寄主的行為影響上。寄主的行為影響發生在鐵線蟲接近成熟時，但我們的研究顯示鐵線蟲進入寄主初期便開始對寄主的發育產生影響，且該影響牽涉到寄主的資源分配與昆蟲的雌雄二型性分化機制。目前昆蟲的雌雄二型性分化幾乎都以模式物種為材料，但至今仍然少有證據指出昆蟲雌雄二型性分化的調控機制，以至於過去數十年間昆蟲的性徵發育被認為是細胞自主的發育流程而不受外界因子的調控。然而近年陸續出現研究挑戰這個假說。我們對於鐵線蟲的研究意外的切入此議題並挑戰了傳統的假說。未來進一步的研究有機會挑戰現有的理論並找出昆蟲雌雄二型性分化的調控因子。

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