A new millipede-parasitizing horsehair worm, *Gordius chiashanus* sp. nov., at medium altitudes in Taiwan (Nematomorpha, Gordiida)

Ming-Chung Chiu¹,², Chin-Gi Huang³,⁴, Wen-Jer Wu⁵, Zhao-Hui Lin⁶, Hsuan-Wien Chen⁶, Shiuh-Feng Shiao⁶

¹ Department of Biology, Kobe University, Kobe 657-8501, Japan ² Department of Biology, National Changhua University of Education, Changhua City 55007, Taiwan ³ National Mosquito-Borne Diseases Control Research Center, National Health Research Institutes, Tainan City 704, Taiwan ⁴ Department of Earth and Life Science, University of Taipei, Taipei 100, Taiwan ⁵ Department of Entomology, National Taiwan University, Taipei 106, Taiwan ⁶ Department of Biological Resources, National Chiayi University, Chiayi 300, Taiwan

Corresponding author: Shiuh-Feng Shiao (sfshiao@ntu.edu.tw)

Academic editor: A. Schmidt-Rhaesa | Received 18 February 2020 | Accepted 8 April 2020 | Published 16 June 2020

http://zoobank.org/9095718C-8FB0-4618-8F9A-466CD3B0D3BF

Citation: Chiu M-C, Huang C-G, Wu W-J, Lin Z-H, Chen H-W, Shiao S-F (2020) A new millipede-parasitizing horsehair worm, *Gordius chiashanus* sp. nov., at medium altitudes in Taiwan (Nematomorpha, Gordiida). ZooKeys 941: 25–48. https://doi.org/10.3897/zookeys.941.49100

Abstract

*Gordius chiashanus* sp. nov., a newly described horsehair worm that parasitizes the *Spirobolus* millipede, is one of the three described horsehair worm species in Taiwan. It is morphologically similar to *G. helveticus* Schmidt-Rhaesa, 2010 because of the progressively broadening distribution of bristles concentrated on the male tail lobes, but it is distinguishable from *G. helveticus* because of the stout bristles on the mid-body. In addition, a vertical white stripe on the anterior ventral side and areoles on the inside wall of the cloacal opening are rarely mentioned in other *Gordius* species. Free-living adults emerged and mated on wet soil under the forest canopy in the winter (late November to early February) at medium altitudes (1100–1700 m). Mucus-like structure covering on the body surface, which creates a rainbow-like reflection, might endow the worm with high tolerance to dehydration. Although *Gordius chiashanus* sp. nov. seems to be more adaptive to the terrestrial environment than other horsehair worm species, cysts provisionally identified as belonging to this hairworm species found in the aquatic paratenic host, *Ephemera orientalis* McLachlan, 1875, suggest the life cycle of *Gordius chiashanus* sp. nov. could involve water and...
land. The free-living adults emerged from the definitive hosts might reproduce in the terrestrial environment or enter an aquatic habitat by moving or being washed away by heavy rain instead of manipulating the behavior of their terrestrial definitive hosts.

**Keywords**
definitive host, immature stage, parasitic life cycle, terrestrial adaptation

**Introduction**

In addition to the two previously described species of horsehair worm (Chiu et al. 2011, 2017), *Gordius chiashanus* sp. nov. is the third described species in Taiwan, and one among 90 valid *Gordius* species reported worldwide (Schmidt-Rhaesa 2010, 2014). *Gordius* horsehair worms are characterized by a cuticular fold, known as post-cloacal crescent, on the male tail (Schmidt-Rhaesa 2002). *Gordius* forms a monophyletic group (Gordiidae) with the genus *Acutogordius*, which bears the same characteristics; however, the phylogenetic relationship between these two genera is controversial (Schmidt-Rhaesa 2002). Although *Gordius* is the second most diverse genus, identification of species in the genus *Gordius* is difficult because of the lack of diagnostic characters and our limited understanding of its morphological variables (Schmidt-Rhaesa 2001, 2010). Phylogenetic comparison using DNA sequences with morphological descriptions has become increasingly crucial in detecting the cryptic species (Hanelt et al. 2015; Tobias et al. 2017).

The definitive hosts of *Gordius* cover a wide range of arthropod taxa. Although many host records might be questionable because the genus *Gordius* (*G. aquaticus* Linnaeus, 1758) had been used to represent the entire members of horsehair worms, *Gordius* species might parasitize several insect orders, Chilopoda, Diplopoda, and Araneae as their definitive hosts (Schmidt-Rhaesa 2012; Bolek et al. 2015). The *Gordius* life cycle is highly correlated with the definitive hosts. The freshwater horsehair worm typically exhibits a life cycle that involves aquatic and terrestrial environments; its life cycle comprises a reproduction and paratenic aquatic host phase and a terrestrial definitive host phase (Hanelt et al. 2005). The aforementioned complex life cycle has been reported in multiple *Gordius* species (e.g., *G. robustus* Leidy, 1851 and *G. difficilis* Smith, 1994) (Thorne 1940; Bolek and Coggins 2002); however, it has not been reported in some species that parasitize aquatic definitive hosts (e.g., *G. villoti* Rosa, 1882 and *G. albopunctatus* Müller, 1926) (Valvassori et al. 1988; Schmidt-Rhaesa and Kristensen 2006) or in species that reproduce in terrestrial environments (*G. terrestris* Anaya et al., 2019) (Anaya et al. 2019).

Free-living adults of *Gordius chiashanus* sp. nov. are frequently found in foggy forests situated at altitudes of 1100–1700 m in Taiwan. Their taxonomic status was first examined in the present study by using a description of morphology and phylogenetic comparison of partial mitochondrial DNA cytochrome oxidase subunit I (mtDNA-COI) genes. The definitive host was determined using worms with high sequence
A new species of *Gordius* from Taiwan

Materials and methods

Collection and preservation of horsehair worms

Horsehair worm samples were identified visually and collected from the ground. In total, 21 free-living adults (17 male and 4 female adults) were collected for morphological examination and DNA sequencing (detailed information provided in Table 1). All the living worms were killed by treatment with hot water (> 80 °C), fixed in a solution containing 75% alcohol with their hosts for a few days, and preserved in a solution of 95% alcohol. One mated female adult collected from Fenqihu, Zhuqi township, Chiayi county, Taiwan (23°30’12.70”N, 120°41’36.00”E) was placed in 800 mL of aerated tap water in the laboratory and maintained at 15 °C until it oviposited egg strings. The eggs were maintained in aerated water for 49 days until they hatched. One dead worm from a dead round-backed millipede (collected at 17-III-2019) and five immature worms from three of 50 round-backed millipedes (collected at 23-VII-2018 and 28-VII-2018) were collected to confirm the definitive host (detailed information provided in Table 1). All the hosts were preserved at −20 °C until dissection. The infected host and the harbored worms were preserved in a 95% alcohol solution for sequencing. Five cysts photographed from four mayfly naiads of *E. orientalis* collected from Lugu township, Nantou county, Taiwan (23°40’46.00”N, 120°47’18.50”E), where the free-living adult has ever been found in the upstream of less than 1 km, were putatively identified as belonging to this horsehair worm species. All the samples were preserved in a solution of 75% alcohol for morphological examination.

Morphological examination

Free-living adults. Fragments (approximately 0.5 cm in length) of the anterior end, mid-body, and posterior end of the preserved samples were examined and photographed using a stereomicroscope (Leica S8 APO, Leica, Wetzlar, Germany), dehydrated using 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), dried to the critical point, coated by being sputtered with gold, and examined using a scanning electronic microscope (SEM) (JEOL JSM-5600, Tokyo, Japan) at magnifications ranging from 100× to 15,000×.
### Table 1. *Gordius chiashanus* sp. nov. specimen information.

| Collection date | GenBank no. | Locality | Longitude and latitude | Collector | Depository | Sex | Status | Length (mm) |
|-----------------|-------------|----------|------------------------|-----------|------------|-----|--------|-------------|
| 20-XI-2017      | MN784831    | Dasyueshan (Heping, Taichung, Taiwan) | 24°14'47.90"N, 120°56'06.80"E | Ta-Chih Chen | NMNS | M | Free-living adult | 430 |
| 26-XI-2008      | MN784832    | Hongshi trail (Haituan, Taitung, Taiwan) | 23°04'14.50"N, 121°07'58.30"E | Po-Yen Chen | NMNS | M | Free-living adult | 744 |
| 22-I-2008       | MN784841    | Shihjhuo (Zhuqi, Chiayi, Taiwan) | 23°29'01.70"N, 120°42'05.90"E | Yu-Hsuan Tsai | NMNS | M | Free-living adult | 860 |
| 9-II-2007       | MN784833    | Shihjhuo (Zhuqi, Chiayi, Taiwan) | 23°29'01.70"N, 120°42'05.90"E | Yu-Hsuan Tsai | NMNS | F | Free-living adult | 707 |
| 17-XII-2013     | MN784820    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | LBM | M | Free-living adult | 771 |
| 17-XII-2013     | MN784821    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 734 |
| 17-XII-2013     | MN784822    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 726 |
| 17-XII-2013     | MN784823    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 594 |
| 17-XII-2013     | MN784824    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 383 |
| 18-XII-2013     | MN784825    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 474 |
| 18-XII-2013     | MN784826    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 666 |
| 18-XII-2013     | MN784827    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 717 |
| 18-XII-2013     | MN784828    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 498 |
| 18-XII-2013     | MN784829    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 572 |
| 18-XII-2013     | MN784830    | Dinghu (Alishan, Chiayi, Taiwan) | 23°29'29.10"N, 120°43'19.00"E | Ming-Chung Chiu | NMNS | M | Free-living adult | 502 |
| 17-III-2019     | MN784839    | Xitou (Lugu, Nantou, Taiwan) | 23°40'21.30"N, 120°47'27.50"E | Zhao-Hui Lin | NMNS | - | Dead worm in host | - |
| 23-VII-2018     | MN784834    | Shihjhuo (Zhuqi, Chiayi, Taiwan) | 23°29'01.70"N, 120°42'05.90"E | Yu-Wei Li | NMNS | - | Immature worm | 660 |
| 28-VII-2018     | MN784835    | Shihjhuo (Zhuqi, Chiayi, Taiwan) | 23°28'22.60"N, 120°41'42.80"E | Yu-Wei Li | NMNS | - | Immature worm | 894 |
| 28-VII-2018     | MN784836    | Shihjhuo (Zhuqi, Chiayi, Taiwan) | 23°28'22.60"N, 120°41'42.80"E | Yu-Wei Li | NMNS | - | Immature worm | 420 |
| 28-VII-2018     | MN784837    | Shihjhuo (Zhuqi, Chiayi, Taiwan) | 23°28'22.60"N, 120°41'42.80"E | Yu-Wei Li | NMNS | - | Immature worm | 442 |
| 28-VII-2018     | MN784838    | Shihjhuo (Zhuqi, Chiayi, Taiwan) | 23°28'22.60"N, 120°41'42.80"E | Yu-Wei Li | NMNS | - | Immature worm | 426 |

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

Holotype.
**Eggs and larvae.** Eggs and newly hatched larvae (living or treated with hot water (> 80 °C)) were examined and photographed on microslides by using a compound microscope (Olympus BH-2, PM-10AD, Olympus, Tokyo, Japan) at magnifications of 200× and 400×. The eggs examined using the SEM were first fixed using a solution of 75% alcohol, dehydrated, dried to the critical point, and coated with gold sputter. The eggs and larvae were examined at a magnification of 500×. ImageJ 1.47 was used for all morphological measurements (Abrámoff et al. 2004), and spatial calibration was conducted according to the scale included in each picture. The terminology for larval stages used in this study primarily followed that of Schmidt-Rhaesa (2014) and Szmygiel et al. (2014).

**Cysts in the paratenic host.** The mayflies preserved in 75% alcohol were first treated with Nesbitt’s fluid for 15–20 min at 40 °C and a 0.1% KOH solution for 5 min at 40 °C to ensure that the cuticle and muscles had become transparent (Walter and Krantz 2009; Chiu et al. 2016). One of the cysts was further treated with a 5% KOH solution for 6 h at room temperature to release the folded larva inside the cyst wall. The cysts were examined and photographed on microslides by using the compound microscope at 200× magnification.

**Phylogenetic analysis**

Genomic DNA from a 1-cm mid-body section of each worm was extracted using an ALS Tissue Genomic DNA Extraction Kit (Pharmigene, Kaohsiung, Taiwan). The partial cytochrome c oxidase subunit I (COI) sequence was amplified using universal primers (LCO1490 and HC02198) (Folmer et al. 1994) or a newly designed primer set (GoCOiF-1: TTAGGAACTGCTTTAAG, GoCOiR-1: ATAGGGTCAAAGAAGGAGG). PCR for both primer sets was initiated at 95 °C for 5 min, and amplification was conducted for 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 73 °C for 1 min, with a final extension at 73 °C for 5 min.

In addition to sequencing three free-living adult worms and six immature worms recovered from millipede hosts (242–457 high-quality base pairs), we obtained high-quality COI sequences (>500 base pairs) from 18 adult free-living individuals to be used in our phylogenetic analysis and estimates of intraspecific genetic distances. Pairwise distance matrices of COI sequence data were calculated using the Kimura 2-parameter model. A phylogenetic tree was reconstructed using the maximum likelihood method by using the General Time Reversible model with the addition of invariant sites and a gamma distribution of rates across sites. For phylogenetic analysis, the COI sequences were first aligned using CLUSTALX 2.0.10 (Thompson et al. 1997). A total of 422 base pairs shared by all the examined sequences, including for our 18 samples, *Gordius/Acutogordius* spp. (as reported by Sato et al. (2012), Hanelt et al. (2015), Chiu et al. (2017), and Tobias et al. (2017)) and *Chordodes formosanus* Chiu, 2011, *Euchordodes nigromaculatus* Poinar, 1991, and *Parachordodes diblastus* (Örley, 1881) (as reported by Chiu et al. (2011) and Tobias et al. (2017)), were analyzed using MEGA 7 (Kumar et al. 2016) (see detailed information in Table 2). One sequence of an un-
Table 2. List of COI sequences obtained from GenBank for phylogenetic analyses in this study.

| Accession number | Species/clade                      | Reference                  |
|------------------|-----------------------------------|----------------------------|
| KM382317         | Gordius/Acutogordius               | Hanelt et al. 2015         |
| KM382316         | "                                 | Hanelt et al. 2015         |
| KM382315         | "                                 | Hanelt et al. 2015         |
| KM382314         | "                                 | Hanelt et al. 2015         |
| KM382313         | "                                 | Hanelt et al. 2015         |
| KM382312         | "                                 | Hanelt et al. 2015         |
| KM382311         | "                                 | Hanelt et al. 2015         |
| KM382310         | G. terrestris                      | Hanelt et al. 2015, Anaya et al. 2019 |
| KM382309         | "                                 | Hanelt et al. 2015, Anaya et al. 2019 |
| KM382308         | "                                 | Hanelt et al. 2015, Anaya et al. 2019 |
| KM382307         | "                                 | Hanelt et al. 2015, Anaya et al. 2019 |
| KM382306         | G. cf. robustus (Clade 6)          | Hanelt et al. 2015         |
| KM382305         | "                                 | Hanelt et al. 2015         |
| KM382304         | "                                 | Hanelt et al. 2015         |
| KM382303         | "                                 | Hanelt et al. 2015         |
| KM382302         | "                                 | Hanelt et al. 2015         |
| KM382301         | "                                 | Hanelt et al. 2015         |
| KM382300         | "                                 | Hanelt et al. 2015         |
| KM382299         | "                                 | Hanelt et al. 2015         |
| KM382297         | "                                 | Hanelt et al. 2015         |
| KM382296         | G. cf. robustus (Clade 5)          | Hanelt et al. 2015         |
| KM382295         | "                                 | Hanelt et al. 2015         |
| KM382294         | G. cf. robustus (Clade 4)          | Hanelt et al. 2015         |
| KM382293         | "                                 | Hanelt et al. 2015         |
| KM382292         | "                                 | Hanelt et al. 2015         |
| KM382291         | "                                 | Hanelt et al. 2015         |
| KM382290         | "                                 | Hanelt et al. 2015         |
| KM382289         | "                                 | Hanelt et al. 2015         |
| KM382288         | "                                 | Hanelt et al. 2015         |
| KM382287         | "                                 | Hanelt et al. 2015         |
| KM382286         | "                                 | Hanelt et al. 2015         |
| KM382285         | "                                 | Hanelt et al. 2015         |
| KM382284         | "                                 | Hanelt et al. 2015         |
| KM382283         | "                                 | Hanelt et al. 2015         |
| KM382282         | "                                 | Hanelt et al. 2015         |
| KM382281         | G. cf. robustus (Clade 2)          | Hanelt et al. 2015         |
| KM382280         | "                                 | Hanelt et al. 2015         |
| KM382279         | "                                 | Hanelt et al. 2015         |
| KM382278         | "                                 | Hanelt et al. 2015         |
| KM382277         | "                                 | Hanelt et al. 2015         |
| KM382276         | G. attoni                          | Hanelt et al. 2015         |
| KM382275         | "                                 | Hanelt et al. 2015         |
| KM382274         | G. balicus                        | Hanelt et al. 2015         |
| KM382273         | Gordius sp. N178                   | Hanelt et al. 2015         |
| KM382272         | Gordius sp. N183                   | Hanelt et al. 2015         |
| KM382271         | Gordius sp. N297B                  | Hanelt et al. 2015         |
| KM382264         | Gordius sp. N357                   | Hanelt et al. 2015         |
| AB647235         | Gordius sp. KW-2011-A              | Sato et al. 2012           |
| AB647237         | Gordius sp. KW-2011-B              | Sato et al. 2012           |
| AB647241         | Gordius sp. KW-2011-D              | Sato et al. 2012           |
| KY172751         | Gordius sp. Tobias et al. 2017     | Tobias et al. 2017         |
| KY172750         | "                                 | Tobias et al. 2017         |
| KY172752         | "                                 | Tobias et al. 2017         |
| KY172759         | "                                 | Tobias et al. 2017         |
A new species of *Gordius* from Taiwan

| Accession number | Species/clade | Reference |
|------------------|---------------|-----------|
| KY172765         | ''            | Tobias et al. 2017 |
| KY172770*        | ''            | Tobias et al. 2017 |
| KY172777         | ''            | Tobias et al. 2017 |
| KY172749         | ''            | Tobias et al. 2017 |
| KY172792         | ''            | Tobias et al. 2017 |
| KY172789         | ''            | Tobias et al. 2017 |
| KY172791         | ''            | Tobias et al. 2017 |
| KY172799         | ''            | Tobias et al. 2017 |
| KY172801         | ''            | Tobias et al. 2017 |
| KY172802         | ''            | Tobias et al. 2017 |
| KY172804         | ''            | Tobias et al. 2017 |
| KY172753         | *G. paranensis* (Clade2) | Tobias et al. 2017 |
| KY172754         | ''            | Tobias et al. 2017 |
| KY172755         | ''            | Tobias et al. 2017 |
| KY172756         | ''            | Tobias et al. 2017 |
| KY172776         | ''            | Tobias et al. 2017 |
| KY172782         | ''            | Tobias et al. 2017 |
| KY172813         | ''            | Tobias et al. 2017 |
| KY172811         | *G. paranensis* (Clade1) | Tobias et al. 2017 |
| KY172812         | ''            | Tobias et al. 2017 |
| KX919148         | *Acutogordius taiwanensis* | Chiu et al. 2017 |
| KX919147         | ''            | Chiu et al. 2017 |
| KX919146         | ''            | Chiu et al. 2017 |
| KX919145         | ''            | Chiu et al. 2017 |
| KX919144         | ''            | Chiu et al. 2017 |
| KX919143         | ''            | Chiu et al. 2017 |
| KX919142         | ''            | Chiu et al. 2017 |
| KX919141         | ''            | Chiu et al. 2017 |
| KX919140         | ''            | Chiu et al. 2017 |
| KX919139         | ''            | Chiu et al. 2017 |
| KX919138         | ''            | Chiu et al. 2017 |
| KX919137         | ''            | Chiu et al. 2017 |
| KX919136         | ''            | Chiu et al. 2017 |
| KX919135         | ''            | Chiu et al. 2017 |
| KX919134         | ''            | Chiu et al. 2017 |
| KX919133         | ''            | Chiu et al. 2017 |
| KX919132         | ''            | Chiu et al. 2017 |
| KX919131         | ''            | Chiu et al. 2017 |
| KX919130         | ''            | Chiu et al. 2017 |
| KX919129         | ''            | Chiu et al. 2017 |
| KX919128         | ''            | Chiu et al. 2017 |
| KX919127         | ''            | Chiu et al. 2017 |
| KX919126         | ''            | Chiu et al. 2017 |
| KX919125         | ''            | Chiu et al. 2017 |
| KX919124         | ''            | Chiu et al. 2017 |
| KX919123         | ''            | Chiu et al. 2017 |
| KX919122         | ''            | Chiu et al. 2017 |
| MF983649         | *Myanmar nematomorph* |         |
| HM044105         | *Chordodes formosanus* | Chiu et al. 2011 |
| HM044124         | ''            | Chiu et al. 2011 |
| KY172780         | *Euchordodes nigromaculatus* | Tobias et al. 2017 |
| KY172803         | ''            | Tobias et al. 2017 |
| KY172747         | *Parachordodes dibrastus* | Tobias et al. 2017 |
| KY172778         | ''            | Tobias et al. 2017 |

* KY172770 was excluded from the analysis since its high difference from the member of *Gordius* and the high similarity with *Euchordodes nigromaculatus*. 
determined nematomorph (MF983649) was also included because it exhibited high similarity to *Acutogordius*. The bootstrap method (with 1000 replicates) was used to estimate branch support of the phylogenetic tree.

Seasonal occurrence of free-living adults

Seasonal occurrence of free-living adults was estimated by counting (and removing) free-living adults (living or dead) on the ground in Dinghu, Alishan township, Chiayi county, Taiwan (23°29’29.10"N, 120°43’19.00"E) between October 2017 and May 2018.

Results

*Gordius chiasphanus* Chiu, sp. nov.
http://zoobank.org/E904851F-6F48-423D-9AC2-5A7BB595FA7B

**Type locality.** Dinghu (23°29’29.10"N, 120°43’19.00"E), Alishan township, Chiayi county, Taiwan (holotype). Paratypes were collected from Dasyueshan (Heping district, Taichung city), Xitou (Lugu township, Nantou county), Shihjhuo, Fenqihu (Zhuqi township, Chiayi county), Dinghu (Alishan township, Chiayi county), and Hongshi forest road (Haituan township, Taitung county). Table 1 presents detailed information of the locality.

**Type material.** Partial bodies of the holotype and allotype were deposited at the National Museum of Natural Science, Taichung, Taiwan. Paratypes were deposited at the National Museum of Natural Science, Taichung, Taiwan and Lake Biwa Museum, Shiga, Japan (Table 1).

**Type hosts.** *Spirobolus* sp. nov. (Hsu and Chang, unpublished) (Diplopoda: Spirobolidae) (Fig. 5E, F)

**Etymology.** The specific name is the combination of *chia*, referring to the place (Chiayi county) where the first sample was found, and *shan*, referring to the Chinese word for “mountains.” The word *chia* is also in memory of our friend, Chia-Chih Lin, who died in an accident in a field experiment.

**Description.** *Male adults (N = 11) (Figs 1–3, 5).* Body length 627.94 ± 154.75 (383–860) mm, width (widest, after dehydration) 1.30 ± 0.31 (0.81–2.06) mm, light to dark brown, smooth, and covered with mucus-like structure (viscous liquid on live worms with rainbow-like reflection (Fig. 5C, Suppl. material 1: Video S1), and created haze that surrounded the body surface in hot water (Fig. 5A).

Anterior end columnar and spherical; anterior tip white (white cap) with a dark-brown collar and a vertical white stripe on the ventral side (Fig. 1A). Under SEM, surface of anterior end appeared smooth (Fig. 1B) or wrinkled (Fig. 1C) on the tip of one sample; scattered short bristles (11.24 ± 6.57 (4.92–22.24) µm in length) scattered except on tip in most samples (Fig. 1B, D).

Cuticle in mid-body ornamented with a dorsal and a ventral dark pigment line; white spots scattered across entire body surface (Figs 3C, D, 5A). Under SEM, cuticle
Figure 1. Anterior end of male *Gordius chiashanus* sp. nov. **A** stereomicroscopic image of the ventral side of the anterior end showing a white cap, dark-brown collar, and vertical white stripe on the ventral side **B**, **C** SEM images of the anterior end surface that is (B) smooth with scattered short bristles and (C) wrinkled **D** close-up view of the dotted square in C showing the short bristles (arrows) covered by a wrinkled structure. Scale bars: 2 mm (**A**), 200 µm (**B–D**).

Surface appeared smooth (Fig. 3A) with a few scattered short or cone-like bristles (6.75 ± 2.37 (2.31–10.34) µm in length) (Fig. 3A, B).

Posterior end divided into two tail lobes (Fig. 2A, B), each lobe 855.24 ± 100.89 (658.39–994.88) µm long and 458.55 ± 76.52 (365.95–643.00) µm wide with length-to-width ratio of 1.89 ± 0.26 (1.49–2.42). Inner side of lobe tips white (Fig. 2A). Under SEM, inner side of tail lobes concave in some samples; cuticle surface smooth, but one sample exhibited flat areoles on inner side of lobe tips; short bristles scattered across the surface and concentrated in most samples on lobe tips (Fig. 2C) and on in-
Figure 2. Posterior end of male *Gordius chiashanus* sp. nov. A stereomicroscopic image of the posterior end B–D SEM images of (B) overview of the posterior end with bristles concentrated on the (C) lobe tips (arrow), and (D) inner side of the lobe tips and the formation of a bristle field on each tail lobe posterior to the tips of the postcloacal crescent (arrows) E cloacal opening with areoles on the inside wall. Scale bars: 1 mm (A), 500 µm (B), 200 µm (C–D), 50 µm (E).

ner side of lobe tips forming a bristle field (322.67 ± 99.34 (187.60–412.75) µm long and 71.82 ± 35.49 (44.81–114.54) µm wide) on each of tail lobe posterior to tips of postcloacal crescent (Fig. 2D). Postcloacal crescent (Fig. 2A, B) 718.61 ± 118.77 (536.14–984.34) µm long and 86.7 ± 15.62 (54.73–118.65) µm wide and located on ventral side near base of tail lobes. Crescent generally semicircular or slightly angled,
A new species of *Gordius* from Taiwan

35

but a few samples exhibited a straightened form of crescent. Branches of postcloacal crescent usually ended at tail lobes. Cloacal opening circular ($40.5 \pm 21.87$ (27.41–56.14) µm) and anterior to postcloacal crescent (Fig. 2A, B). Wall inside cloacal opening exhibited areoles (Fig. 2E); no circumcloacal spine or bristles observed in region next to cloacal opening.

**Figure 3.** Mid-body of male *Gordius chiashanus* sp. nov. **A, B** SEM images of **(A)** cuticle in the mid-body with scattered short bristles (arrows) and **(B)** close-up view of a short bristle **C, D** white spots and dorsal and ventral dark pigmented line examined using **(C)** a compound microscope and **(D)** a stereomicroscope. Scale bars: 1 mm (**A, C, D**), 5 µm (**B**).
Female adults (N = 4) (Figs 4, 5). Body length 659.75 ± 77.06 (549–717) mm, width (widest, after dehydration) 1.54 ± 0.54 (1.00–2.03) mm, light to dark brown, smooth, and covered with mucus-like structure. White spots scattered on surface but relatively less obvious than those of male adults (Fig. 4F, G). Anterior end columnar and spherical. Anterior tip white (white cap) with a dark-brown collar and exhibited a vertical white stripe on the ventral side (Fig. 4A). Under SEM, surface of anterior end smooth and exhibited scattered short bristles (16.75 ± 4.60 (13.39–23.56) µm in length) except at tip (Fig. 4B). Cuticle in mid-body ornamented with a dorsal and a ventral dark pigment line (Fig. 4G). Under SEM, cuticle surface smooth with a few short or cone-like bristles (7.24 ± 2.01 (4.94–9.99) µm in length) scattered. Posterior end columnar and rounded at tip (Fig. 4E) and did not exhibit scattered bristles (Fig. 4D). Cloacal opening on terminal end (Fig. 4C, D) circular and 36.56 ± 23.23 (24.68–48.45) µm in diameter.

Eggs (N = 12) (Fig. 6C–E). Egg strings (Fig. 6E) 7.41 ± 3.46 (3.78–13.70) mm in length and 1.13 ± 0.12 (0.86–1.25) mm in width; white or light yellow in color, deposited in water as short pieces not adhering to substrate. Eggs round, 54.16 ± 242 2.89 (49.88–58.61) µm in diameter. Developing embryo surrounded by an inner membrane (Fig. 6C, D) separated by a distinct space from outer egg shell 14.35 ± 1.41 (12.43–17.33) µm).

Figure 4. Female Gordius chiashanus sp. nov. A, B anterior end examined using a (A) stereomicroscope and (B) SEM C–E posterior end with the terminal view examined using a (C) stereomicroscope and (D) SEM, and the (E) lateral view examined using a stereomicroscope F, G mid-body examined using a (F) stereomicroscope and (G) compound microscope. Co, cloacal opening. Scale bars: 1 mm (A, F, G), 200 µm (B–D).
A new species of *Gordius* from Taiwan

Living larvae (*N* = 10) (Fig. 6B). Eggs developed for approximately 49 days. Hatched larvae remained near egg strings or moved inside eggshells. Under light microscopy, living larvae appeared cylindrical with a single posterior spine. Preseptum length 32.33 ± 4.53 (27.06–40.04) μm, and the width 18.04 ± 0.86 (16.70–19.12) μm. Postseptum length 83.05 ± 8.31 (66.50–92.66) μm, width 15.05 ± 0.73 (14.21–16.10) μm; proboscis length 14.94 ± 1.99 (12.35–18.48) μm, width 4.11 ± 0.85 (2.77–5.34) μm; pseudointestine length 60.60 ± 5.40 (54.99–70.12) μm, width 11.66 ± 1.42 (8.84–13.56) μm, unequally subdivided, elongated oval with a depression in anterior end (Fig. 6B).

Larvae treated with hot water (*N* = 2) (Fig. 6A). Larvae treated with hot water similar in morphology but larger than living larvae. Preseptum length 44.57 ± 0.13 (44.48–44.66) μm, width 17.96 ± 0.16 (17.85–18.08) μm. Postseptum length 118.23 ± 1.91 (116.88–119.58) μm, width 15.36 ± 0.68 (14.88–15.84) μm. Proboscis length 12.63 ± 1.18 (11.80–13.47) μm, width 3.26 ± 0.05 (3.23–3.30) μm; pseudointestine length 77.99 ± 5.22 (74.30–81.68) μm, width 13.99 ± 0.81 (13.41–14.56) μm (Fig. 6A).

Field-collected cysts (*N* = 5) (Fig. 6F–H). Larvae in cysts unfolded (*N* = 4) (Fig. 6F) or exhibited a postseptum folded twice (*N* = 1) (Fig. 6G, H). Unfolded larvae morphologically similar to larvae but larger in size; preseptum length was 60.18 ± 6.72 (50.40–65.18) μm, width 20.87 ± 0.52 (20.28–21.33) μm; postseptum length

**Figure 5.** Field observation of *Gordius chiashanus* sp. nov. A hazy appearance (arrows) surrounding the body surface in hot water B spermatophore (arrow) on a female collected on the surface of the soil C rainbow-like reflection on the body surface D free-living adult collected in wet soil E, F infected host, *Spirobolus* sp. nov. (Hsu and Chang, unpublished), harboring (E) three immature worms (arrow) and (F) an adult worm. Photographs courtesy of (D) Fang, Hua-Te and (F) Hung, Ming-Chin. Scale bars: 1 cm (E).
Figure 6. Immature stages of *Gordius chiashanus* sp. nov. **A, B** free-living larva (**A**) treated with hot water and a living larva showing the depression in the anterior end of the pseudointestine (arrow) **C, D** eggs with the inner membrane examined using an (**C**) SEM and (**D**) compound microscope **E** egg strings **F–H** cysts in the paratenic host with (**F**) a unfolded larva and (**G**) a folded larva, showing (**H**) a single posterior spine (arrow) after treatment with a 5% KOH solution. Abbreviations: Ho, hooklet; PostS, postseptum; PreS, preseptum; Pro, proboscis; PsI, pseudointestine. Scale bars: 50 µm (**A–D, F–H**), 1 mm (**E**).

127.33 ± 20.05 (105.10–146.05) µm, width 19.82 ± 2.27 (17.61–22.91) µm; proboscis length 15.46 ± 1.67 (13.84–17.56) µm, width 4.10 ± 0.68 (3.09–4.52) µm; pseudointestine not visible (Fig. 6F). Folded larva (length 34.97 µm, width 30.47 µm) fold twice and surrounded by a clear cyst wall, 47.86 µm in total length and 42.40 µm in total width; proboscis length 15.57 µm, width 5.09 µm (Fig. 6G); a single posterior spine visible after treatment with a solution of 5% KOH (Fig. 6H).

**Phylogeny.** The partial COI sequences of the 18 free-living adults contained 15 haplotypes with 392 invariable sites, nine singletons, and 21 parsimoniously informative sites. The genetic distance among them was 0.0024 within the range of 0.0000–0.0510. The three living adults and six worms inside the hosts were considered conspecific with the 18 free-living adults because of their small genetic distances (0.0000–0.0719). The mean interspecific genetic distances between *Gordius chiashanus* sp. nov. and other *Gordius* species or clades were in the range of 0.2320–0.4242, and that between *Gordius chiashanus* sp. nov. and *Acutogordius taiwanensis* was 0.3648 (Table 3). In addition to short genetic distances, the conspecific status of the 18 free-living...
A new species of *Gordius* from Taiwan

**Table 3.** Intra- and interspecific mean COI genetic distances of *Gordius/Acutogordius* species or clades under K2P model.

| Species/Clade                                      | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---------------------------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1 Gordius chiashanus sp. nov.                      | 0.024 |
| 2 *G. cf. robustus* (Clade 1)                      | 0.285 | 0.009 |
| 3 *G. cf. robustus* (Clade 2)                      | 0.312 | 0.217 | 0.015 |
| 4 *G. cf. robustus* (Clade 3)                      | 0.293 | 0.297 | 0.275 | 0.007 |
| 5 *G. cf. robustus* (Clade 4)                      | 0.308 | 0.208 | 0.249 | 0.157 | 0.012 |
| 6 *G. cf. robustus* (Clade 5)                      | 0.272 | 0.165 | 0.211 | 0.227 | 0.222 | 0.003 |
| 7 *G. cf. robustus* (Clade 6)                      | 0.293 | 0.257 | 0.251 | 0.255 | 0.228 | 0.259 | 0.006 |
| 8 Gordius terrestris                                | 0.232 | 0.209 | 0.265 | 0.250 | 0.230 | 0.222 | 0.238 | 0.020 |
| 9 *G. cf. robustus* (Clade 8)                      | 0.265 | 0.203 | 0.307 | 0.338 | 0.253 | 0.244 | 0.251 | 0.122 | 0.026 |
| 10 G. attoni                                       | 0.277 | 0.229 | 0.288 | 0.337 | 0.274 | 0.238 | 0.289 | 0.231 | 0.249 | 0.010 |
| 11 G. balticus                                     | 0.316 | 0.260 | 0.298 | 0.288 | 0.269 | 0.274 | 0.304 | 0.264 | 0.323 | 0.337 | 0.065 |
| 12 Gordius sp. N178                                | 0.352 | 0.260 | 0.313 | 0.370 | 0.289 | 0.340 | 0.330 | 0.256 | 0.290 | 0.271 | 0.323 | 0.087 |
| 13 Gordius sp. N183                                | 0.329 | 0.302 | 0.290 | 0.373 | 0.317 | 0.344 | 0.365 | 0.294 | 0.336 | 0.277 | 0.301 | 0.246 | 0.075 |
| 14 Gordius sp. N297B                               | 0.424 | 0.416 | 0.462 | 0.547 | 0.441 | 0.443 | 0.478 | 0.375 | 0.412 | 0.348 | 0.455 | 0.343 | 0.414 | 0.074 |
| 15 Gordius sp. N357                                | 0.332 | 0.366 | 0.387 | 0.420 | 0.376 | 0.396 | 0.302 | 0.357 | 0.359 | 0.379 | 0.439 | 0.375 | 0.434 | 0.441 | 0.078 |
| 16 Gordius sp. KW-2011-A                           | 0.384 | 0.325 | 0.327 | 0.453 | 0.371 | 0.336 | 0.345 | 0.347 | 0.348 | 0.331 | 0.376 | 0.332 | 0.376 | 0.372 | 0.424 | 0.075 |
| 17 Gordius sp. KW-2011-B                           | 0.334 | 0.375 | 0.365 | 0.370 | 0.334 | 0.407 | 0.364 | 0.302 | 0.363 | 0.333 | 0.380 | 0.323 | 0.358 | 0.333 | 0.308 | 0.290 | 0.075 |
| 18 Gordius sp. KW-2011-D                           | 0.375 | 0.300 | 0.344 | 0.393 | 0.373 | 0.294 | 0.388 | 0.388 | 0.367 | 0.369 | 0.405 | 0.384 | 0.390 | 0.374 | 0.403 | 0.312 | 0.301 | 0.075 |
| 19 *G. paranensis* (Clade 1)                       | 0.369 | 0.405 | 0.381 | 0.450 | 0.381 | 0.410 | 0.359 | 0.373 | 0.395 | 0.409 | 0.398 | 0.408 | 0.466 | 0.426 | 0.453 | 0.415 | 0.386 | 0.440 | 0.049 |
| 20 *G. paranensis* (Clade 2)                       | 0.337 | 0.348 | 0.391 | 0.436 | 0.384 | 0.372 | 0.368 | 0.333 | 0.368 | 0.345 | 0.339 | 0.334 | 0.385 | 0.324 | 0.404 | 0.357 | 0.327 | 0.344 | 0.377 | 0.010 |
| 21 Gordius sp. Tobias et al. 2017                  | 0.335 | 0.283 | 0.293 | 0.436 | 0.355 | 0.311 | 0.366 | 0.287 | 0.337 | 0.347 | 0.358 | 0.254 | 0.308 | 0.343 | 0.353 | 0.304 | 0.335 | 0.321 | 0.354 | 0.337 | 0.012 |
| 22 Acutogordius taiwanensis                        | 0.365 | 0.343 | 0.327 | 0.401 | 0.386 | 0.368 | 0.345 | 0.322 | 0.375 | 0.304 | 0.336 | 0.270 | 0.210 | 0.462 | 0.469 | 0.432 | 0.376 | 0.366 | 0.435 | 0.389 | 0.311 | 0.002 |

- Indicates a single haplotype whose intraspecific distance could not be calculated.
Figure 7. Phylogenetic relationship of *Gordius/Acutogordius* spp. restructured using COI partial sequences compared with *C. formosanus*, *E. nigromaculatus*, and *P. diblastus* as out groups. Numbers at the nodes represent the percentage of 1000 bootstrap replicates.

Adults was also supported because all the samples were located in a single clade, as indicated by a high bootstrap value. No subgroup was detected because the polytomic topology exhibited low bootstrap values and short genetic distances. The *Gordius* species/clades in the present result were consistent with the results of Hanelt et al. (2015) and Tobias et al. (2017), despite slight differences in the relative relationships among species, which might be attributable to the differences in models used or the shorter sequence adopted in previous studies. The clade of *A. taiwanensis* was located within that of the *Gordius* species, and it did not behave as a sister group (Fig. 7).

**Reproductive season.** Free-living adult worms frequently aggregate and mate on wet ground (Fig. 5B, C) after rain or fog, and they are sometimes found in water or soil (Fig. 5D). They suddenly emerge in early December, and their number decreases within 1–2 months (Fig. 8). During the reproductive season, no infected host was found. The seasonality and pattern of *Gordius chiashanus* sp. nov. differed from the graph constructed using data from *C. formosanus* (Chiu et al. 2016).

**Diagnosis and comments.** The 21 free-living *Gordius* adults and six juvenile worms from round-backed millipedes were judged as belonging to the same species in accordance with the results that they all were located in the same clade in the phyloge-
A new species of *Gordius* from Taiwan

The concentration of bristles and spines on the male tail lobes has been previously described in species from the Palaearctic (Spiridonov 1984; Schmidt-Rhaesa 2010) and Nearctic realms (Anaya et al. 2019). In *Gordius chiashanus* sp. nov., this dense patch of bristles is a stable characteristic that was detected in all samples. The distribution pattern was similar to that of *G. helveticus* (Schmidt-Rhaesa 2010) because the bristles exhibited a progressively broader distribution instead of being concentrated along the row of the ventral border, such as in *G. karwendeli* Schmidt-Rhaesa, 2010 (Schmidt-Rhaesa 2010) and *G. terrestris* (Anaya et al. 2019), or in a circular patch of concentrated spines, such as in *G. spiridonovi* Schmidt-Rhaesa, 2010 (Spiridonov 1984).

Although the distribution pattern of the bristles is similar to that of *G. helveticus*, *G. chiashanus* sp. nov. is morphologically distinct because of the presence of stout bristles on the mid-body, a vertical white stripe on the anterior ventral side, and areoles on the inside wall of the cloacal opening. The vertical white stripe on the anterior ventral side can be easily observed by the naked eye, but it has rarely been mentioned thus far. The presence of a white stripe was previously reported in the terrestrial hairworm, *G. terrestris* (Anaya et al. 2019), which exhibits a broad white patch; however, the patch is likely to be the intensive aggregation of white spots in *Gordius chiashanus* sp. nov. The presence of areoles on the inside wall of the cloacal opening has only been reported in

![Seasonal occurrence of free-living adults of *Gordius chiashanus* sp. nov. Numbers at the bottom indicate the actual number of each bar.](image)
an unknown *Gordius* (Schmidt-Rhaesa 2012, fig. 3.2.2). Although cloacal openings are usually covered by contamination in many *Gordius* species, as was the case in most of our samples, the areole on the inside wall of cloacal opening might not be a general characteristic of the genus *Gordius* because it is absent in at least some species (e.g., *G. serratus* Schmidt-Rhaesa, 2010, *G. terrestris*, *G. spiridonovi*) (Schmidt-Rhaesa 2010; Schmidt-Rhaesa and Prous 2010; Anaya et al. 2019).

**Discussion**

*Gordius chiashanus* sp. nov. and the two previously described species, namely *A. taiwanensis* Chiu et al., 2017 and *C. formosanus* Chiu, 2011 (Chiu et al. 2011, 2017), are the three most frequently sighted horsehair worm species in Taiwan. Unlike the two low-altitude species, *Gordius chiashanus* sp. nov. inhabits medium altitude areas (1100–1700 m), which matches the distribution of its millipede host, *Spirobolus* sp. nov. (Hsu and Chang, unpublished), in Taiwan (1100–1600 m) (Hsu 2008).

**Morphology of *Gordius chiashanus* sp. nov.** With approximately 90 valid species, *Gordius* is the second most diverse genus of the phylum Nematomorpha (Schmidt-Rhaesa 2012). However, because of the lack of reliable diagnostic characteristics and non-hereditary morphological variation associated with methods of examination, environmental damage, mucus-like structure covering the surface, and different hosts, species identification within this genus is difficult (Schmidt-Rhaesa 2001, 2010; Chiu et al. 2011, 2017; Hanelt et al. 2015). Previously, the white spot has been only found on the male cuticle (Schmidt-Rhaesa 2010). However, we found it, but unexpectedly, in the female *Gordius chiashanus* sp. nov. by examination with a compound microscope. It is clearly necessary to reexamine other species since it might have been ignored especially in the female samples. The mucus-like structure is the structure covering the body surface which might also cause morphological variation. It was first reported in *A. taiwanensis* (Chiu et al. 2017) but not in our observations of *C. formosanus* (Chiu et al. 2011, 2017). In *Gordius chiashanus* sp. nov., it was more obvious than that of *A. taiwanensis* by the bright light reflection on the body surface and the hazy appearance surrounding the worms after treatment with hot water. The mucus-like structure appeared opaque under the SEM; this opacity might hamper the visibility of small structures (Fig. 1C, D), consequently, the reliability of such a diagnostic characteristic is low.

**Adult and larval size.** The body length of *Gordius* is variable and can be longer than 2 m (Schmidt-Rhaesa 2010). Relative to phylogeny, host size and intensity of infection play more crucial roles in determining worm size (Hanelt 2009; Chiu et al. 2017). Although the adult length is less likely to be a common feature shared among a species, larval size might have been overlooked. Hidden diversity due to large cysts in the paratenic host is often detected (Chiu et al. 2016). Larvae of *Gordius chiashanus* sp. nov. are morphologically similar to *A. taiwanensis* (Chiu et al. 2017) but significantly longer than *A. taiwanensis* larvae (preseptum + postseptum: 162.80 ± 1.78 µm vs. 112.00 ± 5.52 µm, larvae treated with hot water). In terms of comparison with other *Gordius* species, although the measurements varied considerably among the untreated larvae, the larval
lengths of *Gordius chiashanus* sp. nov. (115.38 ± 12.08 µm) were similar to those of *G. cf. robustus* #1 (110.0 µm in Szmygiel et al. 2014) but longer than the unfolded larva of a *Gordius* species (80.02 µm in Fig. 1D, Harkins et al. 2016) and shorter than those of *G. cf. robustus* #2 (140.2 µm in Szmygiel et al. 2014). The fine structures of larvae are potential to be adopted in distinguishing the close species. By examining with SEM, Anaya et al. (2019) found differences in the number of spines on the proboscis, while *G. terrestris* has seven spines on the distal end of the left lateral and right lateral sides, whereas there are nine in *G. cf. robustus* #1 (Szmygiel et al. 2014). Similarly, the pattern of spines on the proboscis is also different in *C. formosanus* (nine on the distal end of the dorsal and ventral sides (Chiu et al. 2011)) and *C. morgani, C. kenyaensis, and C. janovyi* (5 on the each side) (Bolek et al. 2010, 2013; Szmygiel et al. 2014). In this study, larvae of *Gordius chiashanus* sp. nov. are failed to be examined by SEM, but it is worth to compare the larval morphology through the horsehair worm species in future studies.

**Phylogenetic relationship of *Gordius* and *Acutogordius***. Molecular comparisons have been rarely conducted in the 19 nematomorph genera (Bleidorn et al. 2002; Efeykin et al. 2016), and the present study is the first examination of the phylogenetic relationship of *Acutogordius* and *Gordius* belonging to the family Gordiidae. Because of the shared characteristic of the postcloacal crescent, *Acutogordius* was considered to be phylogenetically close to *Gordius* but distinct because of its pointed tail lobes (Schmidt-Rhaesa 2002). Two hypotheses have suggested that *Acutogordius* might act as a sister group or a subtaxon of *Gordius* (Schmidt-Rhaesa 2002). Our results indicate that the genus *Acutogordius* is a subtaxon of *Gordius* species, although including only one *Acutogordius* species in analysis is insufficient to support a monophyly of the genera *Gordius* and *Acutogordius*. Moreover, our results suggest that *Acutogordius* might be a group of *Gordius* that adapts to tropical habitats. The three clades of tropical horsehair worms are grouped together with the sequences for *A. taiwanensis* from Taiwan, one sequence from Myanmar (Myanmar nematomorph, MF983649), *Gordius* sp. M178 (KM382321) from Nicaragua, and *Gordius* sp. M178 (KM382322) from Malaysia. The adaptation to the tropical habitat of these two genera corresponds with the global distribution. *Acutogordius* species are mostly distributed in the lower latitude regions; by contrast, the *Gordius* species mainly inhabits the Palaearctic realm (Schmidt-Rhaesa 2002, 2014; Schmidt-Rhaesa and Geraci 2006; Schmidt-Rhaesa and Schwarz 2016; Chiu et al. 2017). In addition, similar patterns were observed in the altitudinal distribution of these two genera in Taiwan. *Acutogordius taiwanensis* mainly inhabits low-altitude rivers (Chiu et al. 2017), whereas *Gordius chiashanus* sp. nov. is only found in mountains at 1000 m. It is worth to note that *Gordius chiashanus* sp. nov. is in the same clade with *G. terrestris* and *G. cf. robustus* (clade 8). Despite not highly supported by the bootstrap method, these three species show a distinct similarity in biology. The definitive host of *G. cf. robustus* (clade 8) is the millipede, whereas that of most of *G. cf. robustus* (clade 2, 3, 4, 6) are orthopterans (Hanelt et al. 2015). For *Gordius chiashanus* sp. nov. and *G. terrestris*, the egg with a distinct membrane around the larva and the free-living adapting to terrestrial environment have never mentioned in other species. This clade of *Gordius* might represent a unique life history of the horsehair worm.
Definitive host and route of transmission. The millipede has been known to be the host of horsehair worms, including the genera *Gordius* and *Gordionus* (Schmidt-Rhaesa et al. 2009; Schmidt-Rhaesa 2012; Hanelt et al. 2015). As a detritivore, it is less likely to ingest horsehair worm cysts from the paratenic host. In 1930, Dorier suggested water and vegetation possible route of transmission after observing the formation of horsehair worm cysts in the external environment instead of inside the paratenic host (reviewed in Schmidt-Rhaesa et al. 2009). Recent observations of free-living cysts support this hypothesis (Bolek et al. 2015; Chiu et al. 2017). However, a detritivore definitive host can also be infected by ingesting corpses of the infected paratenic hosts. The cysts, which were putatively identified as *Gordius chiashanus* sp. nov., found in the mayfly naiads suggest that this is a possible route of transmission. However, the prevalence was low (3.85 and 8.33% from 26 and 24 hosts collected in Shihjhuo in the end of July). It might suggest the less efficiency in transmission or the under estimation of the prevalence since the samples were collected 4 months before the worm appeared on the soil surface.

Host and host manipulation of horsehair worms. The host and biological characteristics of *Gordius chiashanus* sp. nov. suggest an atypical life history. In general, freshwater horsehair worms (gordiids) develop in terrestrial definitive hosts and reproduce in aquatic environments (Hanelt et al. 2005). Adult worms maturing in terrestrial hosts have long been observed and confirmed through experimentation to manipulate host behavior to facilitate host falling into water, which enables them to reproduce in water (Thomas et al. 2002; Sanchez et al. 2008; Ponton et al. 2011). However, these observations are confined to the gordiids parasitizing a few host taxa (mantids and orthopterans) (Schmidt-Rhaesa and Ehrmann 2001; Thomas et al. 2002), whereas that parasitizing other hosts, crossing several arthropod taxa (Schmidt-Rhaesa 2010; Bolek et al. 2015), is likely to exhibit the different reproductive strategy. The alternative non-manipulative hypotheses include the “chance hypothesis” suggested by observations of adult *C. ferganensis* Kirjanova & Spiridonov, 1989 emerging from mantids that drowned in small puddles formed by heavy rains (Kirjanova and Spiridonov (1989), reviewed by Schmidt-Rhaesa and Ehrmann (2001)). The “aquatic life cycle hypothesis” is suggested by the Gordius spp. parasitizing aquatic caddisfly larvae as definitive hosts (Valvassori et al. 1988; Schmidt-Rhaesa and Kristensen 2006), and the “terrestrial life cycle hypothesis” suggested by *G. terrestris* laying eggs in wet soil (Anaya et al. 2019).

In this study, the female adult oviposited in the water. The cysts found in the aquatic paratenic hosts and the eggs developing in water also suggest the life cycle of *Gordius chiashanus* sp. nov. could occur in water and on land. However, the current evidence did not exclude the oviposition in the terrestrial environment because no terrestrial paratenic host was examined for cysts. In addition, the double membraned egg (Anaya et al. 2019) and the mating on the ground both suggest *Gordius chiashanus* sp. nov. might be able to reproduce in the terrestrial environment. Regardless of the scenarios, the adult worm might not be carried to water by manipulating behavior of its millipede host. Alternatively, they may emerge in the terrestrial environment, and move into the water or reproduce in the soil. Free-living adults of *Gordius chiashanus* sp. nov. are frequently found moving and mating on the surface of wet soil during periods of fog and rain. The mucus-like structure, which causes a rainbow-like reflec-
A new species of *Gordius* from Taiwan

might endow the worm with a high tolerance to dehydration. In the winter (late November to early February), the number of free-living adults sampled from the surface of the soil, suddenly increased and then steadily diminished. The adult *C. formosanus* has a pattern that differs from the bell curve in terms of its presence inside a manipulated host (Chiu et al. 2016, fig. 8) and free-living adults of *G. difficilis* in the water (Bolek and Coggins 2002). This difference suggests that the seasonal occurrence of *Gordius chiashanus* sp. nov. does not represent the time when the worm matures but the time of reproduction after the free-living adult has waited for suitable soil conditions. That worms emerging from the hosts in the soil might explain why infected millipedes are rarely found on the ground.

**Acknowledgments**

We deeply appreciate the assistance provided by Hua-Te Fang, Po-Yen Chen, Yu-Hsun Tsai, Ta-Chih Chen, and Yu-Wei Li during sample collection, Wei-Chun Chien during SEM examination, and Masato Nitta, Rui Ueda, Takuya Sato, and Scotty Yang during DNA sequencing. We specially thank Ming-Chin Hung for providing host information. This manuscript was edited by Wallace Academic Editing.

**References**

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics International 11: 36–42. http://imagej.nih.gov/ij/

Anaya C, Schmidt-Rhaesa A, Hanelt B, Bolek MG (2019) A new species of *Gordius* (Phylum Nematomorpha) from terrestrial habitats in North America. ZooKeys 892: 59–75. https://doi.org/10.3897/zookeys.892.38868

Bleidorn C, Schmidt‐Rhaesa A, Garey JR (2002) Systematic relationships of Nematomorpha based on molecular and morphological data. Invertebrate Biology 121: 357–364. https://doi.org/10.1111/j.1744-7410.2002.tb00136.x

Bolek MG, Coggins JR (2002) Seasonal occurrence, morphology, and observations on the life history of *Gordius difficilis* (Nematomorpha: Gordioidea) from southeastern Wisconsin, United States. Journal of Parasitology 88: 287–294. https://doi.org/10.1645/0022-3395(2002)088[0287:SOMAOO]2.0.CO;2

Bolek MG, Schmidt-Rhaesa A, Hanelt B, Richardson DJ (2010) Redescription of the African *Chordodes albibarbatis* Montgomery 1898, and description of *Chordodes janovyi* n. sp. (Gordiida, Nematomorpha) and its non-adult stages from Cameroon, Africa. Zootaxa 2631: 36–54. https://doi.org/10.11646/zootaxa.2631.1.3

Bolek MG, Szmygiel C, Kubat A, Schmidt-Rhaesa A, Hanelt B (2013) Novel techniques for biodiversity studies of gordids and description of a new species of *Chordodes* (Gordiida, Nematomorpha) from Kenya, Africa. Zootaxa 3717: 23–38. https://doi.org/10.11646/zootaxa.3717.1.2

Bolek MG, Schmidt-Rhaesa A, De Villalobos LC, Hanelt B (2015) Phylum Nematomorpha. In: Thorp J, Rogers DC (Eds) Ecology and General Biology: Thorp and Covich’s Fresh-
water Invertebrates (4th ed.). Academic Press, London, 303–326. https://doi.org/10.1016/B978-0-12-385026-3.00015-2
Chiu MC, Huang CG, Wu WJ, Shiao SF (2011) 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*. ZooKeys 160: 1–22. https://doi.org/10.3897/zookeys.160.2290
Chiu MC, Huang CG, Wu WJ, Shiao SF (2016) Annual survey of horsehair worm cysts in northern Taiwan, with notes on a single seasonal infection peak in chironomid larvae (Diptera: Chironomidae). Journal of Parasitology 102: 319–326. https://doi.org/10.1645/15-907
Chiu MC, Huang CG, Wu WJ, Shiao SF (2017) A new orthopteran-parasitizing horsehair worm, *Acutogordius taiwanensis* sp. n., with a redescription of *Chordodes formosanus* and novel host records from Taiwan (Nematomorpha, Gordiida). ZooKeys 683: 1–23. https://doi.org/10.3897/zookeys.683.12673
Dorier A (1930) Recherches Biologiques et Systématiques sur les Gordiacés. Travaux du Laboratoire d’Hydrobiologie et de Pisciculture de l’Université de Grenoble 22: 1–183.
Efeykin BD, Schmatko VY, Spiridonov SE (2016) Comparative phylogenetic informativity of single ribosomal cluster regions in freshwater horsehair worms (Gordiacea, Nematomorpha). Biology Bulletin 43: 34–41. https://doi.org/10.1134/S1062359016010040
Folmer O, Black M, Hoen W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299. https://doi.org/10.10371/journal.pone.0013102
Hanelt B (2009) An anomaly against a current paradigm—Extremely low rates of individual fecundity variability of the Gordian worm (Nematomorpha: Gordiida). Parasitology 136: 211–218. https://doi.org/10.1017/S0031182008005337
Hanelt B, Thomas F, Schmidt-Rhaesa A (2005) Biology of the phylum Nematomorpha. Advances in Parasitology 59: 243–305. https://doi.org/10.1016/S0065-308X(05)59004-3
Hanelt B, Schmidt-Rhaesa A, Bolek MG (2015) Cryptic species of hairworm parasites revealed by molecular data and crowdsourcing of specimen collections. Molecular Phylogenetics and Evolution 82: 211–218. https://doi.org/10.1016/j.ympev.2014.09.010
Harkins C, Shannon R, Papeş M, Schmidt-Rhaesa A, Hanelt B, Bolek MG (2016) Using Gordiid cysts to discover the hidden diversity, potential distribution, and new species of Gordiids (Phylum Nematomorpha). Zootaxa 4088: 515–530. https://doi.org/10.11646/zootaxa.4088.4.3
Hsu MH (2008) Taxonomic study of the millipede order Spirobolida (Class Diplopoda) of Taiwan. Master thesis, Kaohsiung, Taiwan: National Sun Yat-sen University. [in Chinese with English abstract]
Kirjanova ES, Spiridonov SE (1989) Two new species of the nematomorph genus *Chordodes* from mantids. Parazitologiya 23: 358–362. [in Russian with English abstract]
Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33: 1870–1874. https://doi.org/10.1093/molbev/msw054
Ponton F, Otálora-Luna F, Lefèvre T, Guerin PM, Lebarbenchon C, Duneau D, Biron DG, Thomas F (2011) Water-seeking behavior in worm-infected crickets and reversibility of parasitic manipulation. Behavioral Ecology 22: 392–400. https://doi.org/10.1093/beheco/arq215
A new species of *Gordius* from Taiwan

Sanchez MI, Ponton F, Schmidt-Rhaesa A, Hughes DP, Misse D, Thomas F (2008) Two steps to suicide in crickets harbouring hairworms. Animal Behaviour 76: 1621–1624. https://doi.org/10.1016/j.anbehav.2008.07.018

Sato T, Watanabe K, Tamotsu S, Ichikawa A, Schmidt-Rhaesa A (2012) Diversity of nematomorph and cohabiting nematode parasites in riparian ecosystems around the Kii Peninsula, Japan. Canadian Journal of Zoology 90: 829–838. https://doi.org/10.1139/z2012-048

Schmidt-Rhaesa A (2001) Problems and perspectives in the systematics of Nematomorpha. Organisms Diversity & Evolution 1: 161–163. https://doi.org/10.1078/1439-6092-00013

Schmidt-Rhaesa A (2002) Are the genera of Nematomorpha monophyletic taxa? Zoologica Scripta 31: 185–200. https://doi.org/10.1046/j.1463-6409.2002.00073.x

Schmidt-Rhaesa A (2010) Considerations on the genus *Gordius* (Nematomorpha, horsehair worms), with the description of seven new species. Zootaxa 2533: 1–35. https://doi.org/10.11646/zootaxa.2533.1.1

Schmidt-Rhaesa A (2012) Nematomorpha. In: Schmidt-Rhaesa A (Ed.) Handbook of Zoology. Gastrotricha, Cycloneuralia and Gnathifera (Vol. 1). Nematomorpha, Priapulida, Kinorhyncha and Loricifera. De Gruyter, Berlin, 29–145. https://doi.org/10.1515/9783110272536

Schmidt-Rhaesa A, Ehrmann R (2001) Horsehair worms (Nematomorpha) as parasites of praying mantids with a discussion of their life cycle. Zoologischer Anzeiger 240: 167–179. https://doi.org/10.1078/0044-5231-00014

Schmidt-Rhaesa A, Geraci CJ (2006) Two new species of *Acutogordius* (Nematomorpha), with a brief review of literature data of this genus. Systematics and Biodiversity 4: 427–433. https://doi.org/10.1017/S14772006001964

Schmidt-Rhaesa A, Kristensen P (2006) Horschair worms (Nematomorpha) from the Baltic island Bornholm (Denmark), with notes on the biology of *Gordius albopunctatus*. Journal of Natural History 40: 9–10. https://doi.org/10.1080/00222930600761803

Schmidt-Rhaesa A, Prous M (2010) Records of horsehair worms (Nematomorpha) in Estonia, with description of three new species from the genus *Gordius*. Estonian Journal of Ecology 59: 39–51. https://doi.org/10.3176/eco.2010.1.03

Schmidt-Rhaesa A, Schwarz CJ (2016) Nematomorpha from the Philippines, with description of two new species. Zootaxa 4158: 246–260. https://doi.org/10.11646/zootaxa.4158.2.6

Schmidt-Rhaesa A, Farfan MA, Bernard EC (2009) First record of millipeds as hosts for horsehair worms (Nematomorpha) in North America. Northeastern Naturalist 16: 125–130. https://doi.org/10.1656/045.016.0110

Spiridonov SE (1984) Two new Nematomorpha species of the family Gordiidae. Proceedings of the Zoological Institute USSR Academy of Sciences 126: 97–101.

Szmygiel C, Schmidt-Rhaesa A, Hanelt B, Bolek MG (2014) Comparative descriptions of non-adult stages of four genera of Gordiids (Phylum: Nematomorpha). Zootaxa 3768: 101–118. https://doi.org/10.11646/zootaxa.3768.2.1

Thomas F, Schmidt-Rhaesa A, Martin G, Manu C, Durand P, Renaud F (2002) Do hairworms (Nematomorpha) manipulate the water seeking behaviour of their terrestrial hosts? Evolutionary Biology 15: 356–361. https://doi.org/10.1046/j.1420-9101.2002.00410.x

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876–4882. https://doi.org/10.1093/nar/25.24.4876
Thorne G (1940) The hairworm *Gordius robustus* Leidy, as a parasite of the Mormon cricket, *Anabrus simplex* Haldeman. Journal of the Washington Academy of Sciences 30: 219–231. https://www.jstor.org/stable/24529727

Tobias ZJC, Yadav AK, Schmidt-Rhaesa A, Poulin R (2017) Intra- and interspecific genetic diversity of New Zealand hairworms (Nematomorpha). Parasitology 144: 1026–1040. https://doi.org/10.1017/S0031182017000233

Valvassori R, Scari G, De Eguileor M, Lernia LD, Magneto P, Melone G (1988) *Gordius villoti* (Nematomorpha) life cycle in relation with caddis fly larvae. Bollettino di Zoologia 55: 1–4. https://doi.org/10.1080/11250008809386624

Walter DE, Krantz GW (2009) Collecting, rearing, and preparing specimens. In: Walter DE, Krantz GW (Eds) A Manual of Acarology (3rd ed.). Texas Tech University Press, Lubbock, 83–96.

**Supplementary material 1**

**Video S1**
Authors: Ming-Chung Chiu, Chin-Gi Huang, Wen-Jer Wu, Zhao-Hui Lin, Hsuan-Wien Chen, Shiu-Feng Shiao
Data type: multimedia
Explanation note: Free-living adult of *Gordius chiashanus* sp. nov. moving on the ground at night, with a rainbow-like reflection on the body surface.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/zookeys.941.49100.suppl1

**Supplementary material 2**

**Fasta 2**
Authors: Ming-Chung Chiu, Chin-Gi Huang, Wen-Jer Wu, Zhao-Hui Lin, Hsuan-Wien Chen, Shiu-Feng Shiao
Data type: molecular data
Explanation note: COI sequences adopted in the phylogenetic analysis, including for *Gordius/Acutogordius* spp. with *Chordodes formosanus*, *Euchordodes nigromaculatus*, and *Parachordodes diblastus* as out groups.
Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
Link: https://doi.org/10.3897/zookeys.941.49100.suppl2