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六胸遠環蚓 *Amynthas sexpectatus* 複合種群（Crassiclitellata: Megascolecidae）的系統分類、親緣關係與生物地理

The systematics, phylogenetic relationships and biogeography of the earthworms in the *Amynthas sexpectatus* species complex (Crassiclitellata: Megascolecidae)

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中文摘要

六胸遠環蚓（*Amynthas sexpectatus* Tsai et al., 1999）與其近緣種隸屬於蚯蚓中物種數最多的巨蚓科（Megascolecidae）。牠們是一群形態上極為相似的台灣特有大型蚯蚓，分布在雪山山脈與中央山脈以西低海拔丘陵到山區，從新北市的三峽到屏東瑪家皆有採集紀錄。前人的研究將這群蚯蚓分成五個物種，但現有的辨識特徵無法有效鑑定既有典藏樣本，顯示這群蚯蚓的分類有重新檢討的必要。我們以分子與形態資料探討這群蚯蚓的分類，結果顯示雖然粒線體基因樹呈現明顯的地理分群，核基因單倍型網路卻顯示群間的分化尚未完成，以三種不同物種界定方法（Assemble Species by Automatic Partitioning、Bayesian implementation of the Poisson tree processes、Bayesian Phylogenetics and Phylogeography）分析的結果也高度的不一致。此外，在多變量分析中也證實了辨識特徵在鑑定上的不足。因此，我們認為六胸遠環蚓 *Amynthas sexpectatus* 應為一外形多變的物種，而 *A. binoculatus*、*A. fusing*、*A. lioujia*，和 *A. majia* 皆為其同物異名。同時，在大量檢視樣本後，我們依據交配腔的特徵將牠們重新歸到腔環蚓（*Metaphire*）。

關鍵詞：六胸遠環蚓、環帶綱、巨蚓科、物種界定、生物地理

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Abstract

Amynthas sexpectatus Tsai et al., 1999 (Crassiclitellata: Megascolecidae) and several closely related species are large-bodied earthworms endemic to Taiwan. They live in hills and low altitude mountains on the west side of Mt. Sylvia and Central Mountain Range, ranging from Sanxia, New Taipei City to Majia, Pingtung County. Currently, there are five species in this species complex, but diagnostic characters documented in the literature have largely failed to provide reliable species identification. Thus, the taxonomic status of this species complex requires further re-evaluation. Integrative taxonomywas conducted using both molecular and morphological data. The phylogeny based on mitochondrial genes showed that there are seven geographically isolated clades, but nuclear haplotype networks indicated incomplete lineage sorting. The results of three species delimitation methods (Assemble Species by Automatic Partitioning, Bayesian implementation of the Poisson tree processes, and Bayesian Phylogenetics and Phylogeography) were inconsistent with each other. Furthermore, diagnostic characters previously documented in the literature and other characters commonly used in earthworm taxonomy failed to separate different lineages in FAMD (factor analysis of mixed data). We concluded that all our samples belong to a single species, and *A. binoculatus*, *A. fusing*, *A. lioujia*, and *A. majia* are all junior synonyms of *A. sexpectatus*.

Additionally, after extensively examining all of the specimens collected throughout the known distribution of this species, we reassigned it to the genus *Metaphire* based on the presence of copulatory pouches in the male pore area.

Keywords: *Amynthas sexpectatus*, Crassiclitellata, Megascolecidae, Species delimitation, Biogeography

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Contents

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Introduction

The pheretimoid is a clade of earthworms within the family Megascolecidae. Comprised of 12 genera with over one thousand species (Csuzdi 2012, Aspe 2016, DriloBASE 2022), the pheretimoid is the dominant earthworm group in Southeast Asia and the eastern part of East Asia. They are found not only in the tropical lowlands but also at high elevations up to around 3000 m (Shen et al. 2021), where air temperature can drop to around freezing point during winter. Among them, *Amynthas* and *Metaphire* are the most species-rich genera. They are both polyphyletic (James et al. 2009), and can only be differentiated using morphology: presence of a structure called copulatory pouch in the male pore area will lead to a *Metaphire* assignment.

Earthworm diversity in Taiwan has been rapidly uncovered in the last two decades. Before 1999, only 26 earthworm species were described (Shih et al. 1999). This number increased to 73 in 2009 (Chang et al. 2009, Tsai et al. 2009) and then to 119 in 2021 (Shen 2018, Shen et al. 2019, Shen and Fang 2020). Among the 119 species, 111 are in the order Crassiclitellata, and 81 are endemic. Comprising 97 species, the family Megascolecidae is the largest earthworm family in Taiwan, and is mostly dominated by the genera *Amynthas* (69 species) and *Metaphire* (22 species).

Amynthas sexpectatus Tsai, Shen & Tsai, 1999 is a large (~30 cm long) earthworm species endemic to Taiwan. It can be easily distinguished by its brownish yellow or dark green appearance, as well as three pairs of spermathecal pores on 6/7-8/9. In Taiwan, four other species share similar morphological characters with *A. sexpectatus*, including *A. binoculatus* Tsai, Shen & Tsai, 1999, *A. fusing* Wang & Shih, 2017, *A. lioujia* Wang & Shih, 2017, and *A. majia* Wang & Shih, 2017. In the original descriptions, these five species can be distinguished by the shape, position, and number of genital pads and papillae around spermathecal pores and male pore regions (Table 1, Tsai et al. 1999, Wang and Shih 2017). Collectively, the five species inhabit primarily the low elevation, foothill regions in western Taiwan, occurring from north to south on the west side of the island.

However, after preliminary sampling covering the known distribution of the five species, we found that the morphological "boundary" among these species became ambiguous. For instance, there are specimens in our collection possessing six genital pads near spermathecal pores, the diagnostic character of *A. sexpectatus*, and two pairs of round papillae around male pores, the diagnostic character of *A. lioujia*. In addition, DNA barcode analysis showed that the interspecific genetic distance among these species can be as low as 4.3% (Wang and Shih 2017), much lower than previously suggested for

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interspecific distances in earthworms (10% or higher, Chang and James 2011). Thus, it appears that the five species may belong to a species complex, their taxonomic statuses need re-evaluation, and the diagnostic characters may require further revision.

Species boundaries are traditionally defined by morphological traits, along with behavioral or ecological traits (Harrison and Larson 2014). In the last two decades, more and more researchers incorporated molecular data in species delimitation, and our understanding of species boundaries has been improved by the rapid development of molecular techniques and sophisticated algorithms.

The concept of cryptic species refers to species that are morphologically indistinguishable and thus classified as the same nominal species, but are distinguishable using molecular approaches (Martinsson and Erséus 2017, Korshunova et al. 2019). Although widely used in evolutionary and ecological studies, some researchers argued that if we look deeper into finer morphological details, we may still find morphological differences (Korshunova et al. 2019). In practice, the use of cryptic species in taxonomically ambiguous groups, or species complexes, is considered temporary, and robust morphological evidence is required to meaningfully revise their taxonomic statuses (Korshunova et al. 2017). Most importantly, by integrating multiple data types, including, but not limited to, morphology and DNA sequences, researchers can either find additional support for their hypothesis or detect conflicts between different data sets (Fišer et al. 2018), and avoid making inaccurate inference of species.

As more molecular markers are incorporated into phylogenetic studies, cytonuclear discordance may be detected more frequently. Such situations have been found in many groups of animals (Marshall et al. 2021, Taylor et al. 2021), including earthworms (Martinsson and Erséus 2017). Cytonuclear discordance can be caused by hybridization or incomplete lineage sorting (ILS) in recently-diverged groups (Harrison and Larson 2014, Martinsson and Erséus 2017, Maresova et al. 2020, Marshall et al. 2021, Taylor et al. 2021). As lineages coalesce, they tend to persist into deeper parts of the species tree when the branches are short. Thus, coalescence can occur between lineages that are not closely related, leading to the discordance between gene trees (Degnan and Rosenberg 2009). The occurrence of cytonuclear discordance may also be caused by hybridization or introgression (introgressive hybridization) that occurred after a speciation event. Both nuclear and mitochondrial genomes may introgress independently (Harrison and Larson 2014, Taylor et al. 2021).

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Currently, there is not a standard protocol for species delimitation, and all nominal species should be treated as hypotheses, which can be tested using different data types and methods (Fišer et al. 2018). Such methods using multiple criteria including morphology, physiology, ecology or behavior, are called integrative taxonomy (Dayrat 2005). Integrative taxonomic approaches have been routinely conducted in many groups of animals, such as treefrogs (Wang et al. 2017), bullfinches (Dong et al. 2020), and earthworms (Marchan et al. 2020). Analyzing reproductively-associated characters, such as acoustic sign (Wang et al. 2017, Dong et al. 2020) and morphologies on genital organs (Marchan et al. 2020), have been proven useful in species delimitation among closely related taxa.

The objectives of this study were to delimit species within the *Amynthas sexpectatus* species complex and revisit its taxonomic status. We analyzed five molecular markers and 22 morphological characters based on 235 specimens that were either newly collected or were archived at the Museum of Zoology, National Taiwan University (NTUZM) or the Taiwan Endemic Species Research Institute (TESRI). The distribution of the 235 specimens covered the known geographical range of the species complex and included representatives of all five nominal species.

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Material and methods

Sample collection

A total of 235 earthworm specimens morphologically similar to *Amynthas sexpectatus*, were collected throughout Taiwan. The sampling sites were located in hills and low altitude mountains on the west side of Mt. Sylvia and Central Mountain Range, stretching from Sanxia, New Taipei City to Majia, Pingtung County. Some of the 235 specimens were collected from type localities of *A. fusing* and *A. binoculatus* and near the type localities of *A. sexpectatus*, *A. lioujia*, and *A. majia*. Specimens collected were washed in fresh water, anaesthetized in 10% ethanol, fixed in 95% ethanol, and preserved in 75% ethanol. Specimens examined in this study are deposited at National Taiwan University Museum of Zoology, Taipei, Taiwan (NTUZM) and Taiwan Endemic Species Research Institute, Jiji, Nantou, Taiwan (TESRI). One *Amynthas lini* (NTUM-EW-09225) and two *Amynthas aspergillum* (NTUM-EW-09178 and NTUM-EW-09190) were included as outgroup.

A one-year sampling was conducted to access life history of this species complex. Samplings took place on three localities at Fuxing Dist., Taoyuan City during the first two weeks in each month during 2021, and on eight localities across Yunlin to Pingtung during the first two weeks in each month starting from April, 2021. Number of mature and immature earthworms were recorded respectively.

DNA extraction, PCR, and sequencing

Total genomic DNA was extracted using the EasyPure Genomic DNA Spin Kit (Bioman, New Taipei City, Taiwan) from a small piece of muscle tissue taken from the posterior part of each specimen following manufacturer’s instruction. Three mitochondrial markers (cytochrome *c* oxidase subunit 1 (COI), 12S rDNA (12S), and 16S rDNA(16S)) and two nuclear markers (internal transcribed spacers 1 and 2 (ITS) and introns of elongation factor 1-α (EF1-α)) were amplified and sequenced. Among them, EF1-α has never been used in earthworms or any other oligochaetes, but has been shown to exhibit both suitable variability and low homoplasy for closely-related species in other invertebrate groups, such as flightless weevils (Tseng et al. 2018) and amphipods (Fišer et al. 2018).

Polymerase chain reaction (PCR) was conducted in 20 μL volume (comprising 12.3 μL of ddH$_2$O, 2μL of 10X buffer, 2μL of 12.8 mg/ml BSA, 1.6μL of 2.5μM dNTP, 0.5μL of 10μM forward primer, 0.5μL of 10μM reverse primer, 0.1 μL of super-therm Taq polymerase, and 1μL of DNA template) for COI, 12S, 16S, and ITS and 40μL volume for

EF1-α (comprising double volume of each of the above material).

PCR for COI was conducted using primers LCO1490 (5’-GGT CAA CAA ATC ATA AAG ATA TTG G-3’) and HCO2198 (5’-TAA ACT TCA GGG TGA CCA AAA AAT CA-3’) (Folmer et al. 1994), started with an initial denaturation for 1 min at 94 ◦C, followed by 35 cycles of 30 s at 94 ◦C, annealing for 30 s at 60 ◦C, extension for 90 s at 72 ◦C, and then final extension for 10 min at 72 ◦C. An alternative cycle using primers PontoF (5’-CTA GGA GTG TGG GCT GGA AT-3’) and PontoR (5’-AGC AGG ATC AAA GAA GGA GGT-3’) (Taheri et al. 2018) started with an initial denaturation for 1 min at 94 ◦C, followed by 6 cycles of 30 s at 94 ◦C, annealing for 30 s at 45 ◦C, extension for 50 s at 72 ◦C, and final extension for 10 min at 72 ◦C, and 30 cycles of 30 s at 94 ◦C, annealing for 30 s at 54 ◦C, extension for 50 s at 72 ◦C, and then final extension for 10 min at 72 ◦C.

PCR for 12S was conducted using primers 12SE1 (5’-AAA ACA TGG ATT AGA TAC CCR YCT AT-3’) and 12SH (5’-ACC TAC TTT GTT ACG ACT TAT CT-3’) (Jamieson et al. 2002) started with an initial denaturation for 1 min at 94 ◦C, followed by 35 cycles of 30 s at 94 ◦C, annealing for 30 s at 51 ◦C, extension for 50 s at 72 ◦C, and then final extension for 10 min at 72 ◦C.

PCR for 16S was conducted using primers 16sar (5’-CGC CTG TTT ATC AAA AAC AT-3’) and 16sbr (5’-CCG GTY TGA ACT CAG ATC AYG T-3’) (Palumbi S et al. 1991) started with an initial denaturation for 1 min at 94 ◦C, followed by 35 cycles of 30 s at 94 ◦C, annealing for 30 s at 51 ◦C, extension for 50 s at 72 ◦C, and then final extension for 10 min at 72 ◦C.

PCR for ITS was conducted using primers ITSF (5’-CGC CCG TCG CTA CTA CCG ATT-3’) and ITSR (5’-GTC CCG AAC ACC ACA GTT CCC-3’) started with an initial denaturation for 1 min at 94 ◦C, followed by 35 cycles of 30 s at 94 ◦C, annealing for 30 s at 58.3 ◦C, extension for 50 s at 72 ◦C, and then final extension for 10 min at 72 ◦C.

PCR for EF1-α was conducted using primers ewEF1a-F (5’-CTC GCT TTC ACT CTT GGT GT-3’) and ewEF1a-R (5’-TCA GTS GTC ARG TTS ACT GG-3’) (designed in this study) started with an initial denaturation for 1 min at 94 ◦C, followed by 35 cycles of 30 s at 94 ◦C, annealing for 30 s at 52.8 ◦C, extension for 40 s at 72 ◦C, and then final extension for 10 min at 72 ◦C. PCR products underwent gel extracting using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). A second PCR with only 15 cycles was conducted after gel extraction before the final PCR products were ready for sequencing.

DNA sequencing was done by Genomics (Taipei, Taiwan), using an ABI 3730X Genetic Analyzer (Applied Biosystems, US). Sequences were edited and assembled using Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI USA) or Geneious (Biomatters Ltd., Auckland, New Zealand) and then aligned using CLUSTALX (Thompson et al. 1997) with default settings.

Phylogenetic analyses and haplotype networks

We first conducted a DNA barcode analysis based on all 235 COI sequences acquired in this study. The analysis resulted in 155 haplotypes in seven lineages representing seven geographical clades whose distributions did not overlap. Among the 235 sequences, 190 sequences were acquired from newly collected specimens, whereas 45 sequences were from specimens without DNA samples (Permanently lost in 2016 due to freezer malfunction). Thus, from the 190 specimens whose DNA samples were available, one individual was selected from each COI haplotype for the amplification of 12S and 16S sequences, leading to a mitochondrial dataset (COI, 12S, and 16S) with 119 samples. For ITS and EF1-α, PCR was attempted for all 190 specimens, and was successful for 182 and 143 samples, respectively.

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A DNA barcode tree was constructed based on all of the 235 specimens using the neighbor-joining method in MEGA 10.1.7 (Kumar et al. 2018) with uncorrected *p*-distance and 1,000 bootstrap pseudo-replicates. The analysis resulted in seven clades representing seven populations that do not overlap in their distributions. Clades 1 to 5 corresponded to the five nominal species, and the other two clades were assigned lineage 6 and lineage 7, respectively. Details of the division of these specimens were explained in the Results. These specimens were mapped and color-coded based on their clade affiliation using ggmap v3.0.0 package (Kahle and Wickham 2013) in R v4.0.5 (R core Team 2021).

Sequences of COI, 12S, and 16S were concatenated using DAMBE 7.2.136 (Xia 2018) Maximum likelihood analysis of concatenated mitochondrial sequences was conducted using RAxML v8.2.12 (Stamatakis 2014) with 1,000 bootstraps. Bayesian inference of phylogeny was performed in MrBayes v3.2.7a (Ronquist et al. 2012). Two independent MCMC runs with four parallel chains (one cold and three heated) were conducted for 1×10^7 generations with a sampling frequency of 1000 generations. The substitution rate of each marker and codon were estimated using PartitionFinder 2 v2.1.1 (Lanfear et al. 2017). All these analyses above were conducted via CIPRES Science Gateway (Miller et al. 2011), except for concatenation of sequences. Phylogeny trees

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were edited using the R package ggtree v2.4.2 (Yu 2020).

ITS and EF1-α sequences were used to generate haplotype networks. Phasing was done by PHASE algorithm (Stephens et al. 2001, Stephens and Donnelly 2003, Rozas et al. 2017) implemented in DnaSP v6 (Rozas et al. 2017) to deal with heterozygotes. Two independent MCMC runs were conducted with 1,000 iterations, thinning interval of 10, and burn-in of 100. Haplotype networks for two nuclear markers were produced with PopART (Leigh and Bryant 2015) using the TCS network mode (Clement et al. 2002).

Molecular species delimitation

Three species delimitation method were used to examining different hypotheses for species clustering, including a distance-based method—Assemble Species by Automatic Partitioning, ASAP (Puillandre et al. 2021), a tree-based method—the Bayesian implementation of the Poisson tree processes, bPTP (Zhang et al. 2013), and a coalescent model-based approach implemented in the program BPP (Rannala and Yang 2003, Yang and Rannala 2010, Rannala and Yang 2013).

We used ASAP for single locus analysis with the three mitochondrial genes, which were concatenated and seen as a ‘locus’. We selected Kimura’s 2-parameter distance as our model, other parameters were set by default, and we asked for keeping 10 best scores. bPTP was conducted on a concatenated mitochondrial phylogeny with the three mitochondrial genes. bPTP used directly the number of substitutions to model speciation instead of the time and to search for the transition points between among-species branching pattern and within-species pattern (Zhang et al. 2013). The analysis was conducted with 5 × 10^5 generations, thinning of 100, and burn-in of 0.2. The third species delimitation method, joint Bayesian multi-locus species delimitation and species tree estimation algorithm (A11), was performed using two nuclear markers, ITS and introns of EF1-α with a total of 182 samples. Prior was set to 1, which means the probability of rooted trees are all equal. The putative species were assigned based on preliminary grouping by the unrooted neighbor-joining tree. Priors of population size (θ) and divergence time (τ) were set as invgamma IG_θ (3, 0.02) and IG_τ (3, 0.13) calculated by Minimalist BPP (Minimalist bpp (brannala.github.io)). We ran three independent MCMC chain for 5 × 10^5, sampling every 5, with a 10% burn-in.

Morphological analysis

In addition to the 235 samples with COI sequences, additional 88 samples collected during 1998-2016 and archived at TESRI were also included for morphological analysis. Thus, the morphology dataset contained 323 specimens. The samples without COI

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sequences were assigned into the seven mitochondrial lineages based on their geographic origins. We assume that we have sufficient knowledge about the distributions of the seven lineages, which appear to be allopatric (see Results), and therefore do so would not introduce unexpected errors. Additionally, specimens that had incomplete records or that were immature or amputated were excluded from the analyses. Thus, a total of 159 specimens were included in the morphological analysis, among which 108 had COI sequences.

Several external characters were chosen based on the original descriptions (Tsai et al. 1999, Wang and Shih 2017) and were further categorized into different character states, which were scored for each individual for analysis. Qualitative characters include the positions and shapes of (1) genital pads near spermathecal pores in segments VI-VIII, (2) papillae on the ventral side of segments VII and VIII, and (3) papillae around male pores. Quantitative characters include body length, number of segments, length and width of clitellum, and number of setae on segments VIII and XX and between male pores.

Since the dataset contained both qualitative and quantitative traits, we conducted factor analysis of mixed data (FAMD) (Pagès 2004) to allow simultaneous analysis of both data types in one analysis and to balance their influence (Ben Krima et al. 2020) using FactomineR and factoextra package (Lê et al. 2008) in R v4.0.5 (R core Team 2021). In FAMD, qualitative and quantitative data went through different transformation processes. For qualitative data, a disjunctive table was produced to make objects with the same categories close and with different categories apart, and then scaled as in multiple correspondence analysis (MCA). For quantitative data, variables are scaled to unit variance to be incorporated into FAMD.

Two preliminary FAMDs were conducted with all variables, one included all specimens, while the other included only specimens with DNA sequences. After the preliminary analyses, variables whose contributions to dimensions 1 and 2 were lower than expected average contribution were filtered out. The remaining variables, which were considered as important, were used to conduct another round of FAMD.

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Results

A total of 235 samples were sequenced for COI, 119 for 12S, 119 for 16S, 182 for ITS, and 143 for introns of EF1-α. The aligned sequences were 658 bp for COI, 389 bp for 12S, 487 bp for 16S, 787 bp for ITS, and 243 bp for introns of EF1-α.

Mitochondrial phylogeny

The COI neighbor-joining tree (Fig. 1) resulted in seven lineages with an allopatric distribution (Fig. 2). Five of these lineages can be unambiguously assigned to the five nominal species based on specimens from or near the type localities: (from north to south) *A. fusing*, *A. binoculatus*, *A. sexpectatus*, *A. lioujia*, and *A. majia*, and the remaining two lineages are hereafter called Lineage 6 (L6) and Lineage 7 (L7). The minimum uncorrected *p*-distances between lineages ranged from 8.57% between *A. sexpectatus* and L7 to 4.53% between *A. fusing* and *A. binoculatus* (Table 2).

The distribution of each lineage may be restricted to a certain area in Taiwan without contacts, resulting an allopatric distribution (Fig. 2). *A. fusing* can be found from Sanxia Dist. (三峽區), New Taipei City, to Zhuolan Township (卓蘭鎮), Miaoli County; *A. binoculatus* can be found from Fengyuan Dist. (豐原區), Taichung City to Guoxing

Township (國姓鄉), Nantou County; *A. sexpectatus* can be found from Guoxing Township (國姓鄉), Nantou County to Zhongliao Township (中寮鄉), Nantou County; *A. lioujia* can be found from Zhuqi Township (竹崎鄉), Chiayi County to Shanlin Dist. (杉林區), Kaohsiung City; *A. majia* can be found from Meinong Dist. (美濃區), Kaohsiung City to Majia, Pingtung County; L6 can be found from Zhushan Township (竹山鎮), Nantou County to Fanlu Township (番路鄉), Chiayi County; L7 can be found at Jiaxian Dist. (甲仙區), Kaohsiung City.

Maximum likelihood analysis and Bayesian inference using COI, 12S, and 16S resulted in phylogenies (Fig. 3) that are congruent with each other and are similar to the COI tree. In both analyses, the *Amynthas sexpectatus* species complex formed a monophyletic group and recovered the same seven lineages. All of the seven lineages were supported by bootstrap values over 80 and posterior probabilities over 0.95. Among these, L6 and *A. sexpectatus* were sister groups, which also inhabit in adjacent regions. Five lineages, *A. sexpectatus*, *A. binoculatus*, *A. fusing*, *A. lioujia* formed a monophyletic group with bootstrap value being 70 and posterior probability over 0.95. As for lineage 7 and *A. majia*, although they were grouped together in COI neighbor-joining tree, the node comprising them did not receive high bootstrap support in mitochondrial phylogeny.

Haplotype networks

The ITS and EF1-α sequences contained 63 and 57 haplotypes, respectively. In the ITS haplotype network (Fig. 4a), the only shared haplotype was found between L6 and *A. lioujia*. However, in the EF1-α haplotype network (Fig. 4b), seven shared haplotypes were found and two haplotypes were shared among three lineages. In contrast to mitochondrial phylogeny, it showed no distinct, grouping in both nuclear haplotype networks.

Species delimitations

In ASAP, 10 species were recovered under the best asap-score with a threshold Kimura's 2-parameter distance of 0.0243 (Fig. 3). *Amynthas fusing* was divided into three species, *A. lioujia* was divided into two, and the other five mitochondrial lineages were recovered as separate species. However, no distinct barcode gap was observed. If we chose other thresholds in the top 10 best asap-scores between the interval of the lowest distance distribution, 9 (thresholds value 0.0333) or 8 (threshold value 0.0415) species would be recognized. The bPTP resulted in 32 species (Fig. 3). None of the seven putative lineages was recovered as a single species. In addition, the posterior probabilities of nearly all clades were far below 0.95, and many clades were composed of only one sequence. Finally, we test the support for mitochondrial lineages by a multi-locus species method, analyzing two nuclear sequences. And all seven species received high posterior probabilities (over 0.99, Fig. 3)).

FAMD of morphological characters

The two FAMDs showed similar results (Fig. 5). There were in general no clear patterns regarding groupings among the seven mitochondrial lineages. None of the putative species was recovered. The distribution of *A. fusing* scatters across four quadrants and overlaps with all other mitochondrial lineages. Other lineages may occupy some dominant places, but some points may scatter sparsely apart and make the boundaries between lineages blurred.

Life history inferred from one-year sampling

Due to the COVID-19 pandemic, we did not conduct field sampling in central and southern Taiwan in June. Our results showed that *A. sexpectatus* is an annual species (Fig. 6). Individuals started to be found in April in northern Taiwan and in July in southern Taiwan. Clitellates were observed in August – October in northern Taiwan and in August – November in southern Taiwan (Table 3).

Discussion

Although seven distinct lineages were recovered in the COI neighbor-joining tree and the mitochondrial ML and BI phylogenies, no such pattern was found in the haplotype networks based on ITS and EF1-α. Similarly, different species delimitation approaches, namely ASAP, bPTP, and BPP, also resulted in different estimated numbers of species. These inconsistent results are clear evidence that the seven lineages are populations of the same species, namely *A. sexpectatus*, and *A. binoculatus*, *A. fusing*, *A. lioujia*, and *A. majia* are all its junior synonyms.

This is the first time that discordance between mitochondrial and nuclear genes is reported in the family Megascolecidae, the largest earthworm family in the world. However, cytonuclear discordance is common in earthworms, as similar situations have been found in several earthworm genera in the families Lumbricidae and Hormogastridae, including *Aporrectodea* (Martinsson et al. 2015), *Lumbricus* (Giska et al. 2015, Martinsson and Erséus 2017), and *Hormogaster* (Marchan et al. 2017). This may be due to different mutation rates and lineage sorting among genes, especially when mitochondrial genes are often regarded as having higher mutation rates than nuclear genes (Martinsson and Erséus 2017).

The allopatric distribution of the seven mitochondrial lineages indicated that the genetic differentiation was likely resulted from geographical isolation and high mutation rates of mitochondrial genes. Rivers are often one of the main causes of geographic isolation (Klabacka et al. 2020). In this study, the most likely boundaries between neighboring lineages are rivers, including, from north to south, Daan River (大安溪) or Dajia River (大甲溪), Wu River (烏溪), Zhuoshui River (濁水溪), Bazhang River (八掌溪), and Qishan River (旗山溪), as shown in the map.

In the ITS network, we can still find clues of potential groupings, as two lineages, *A. sexpectatus* and *A. binoculatus*, had no shared haplotypes with other groups. In the haplotype network based on the introns of EF1-α, there are seven shared haplotypes in lineages that, in most cases, do not seem to have opportunities of contacting each other, and no clear grouping can be inferred. These patterns may be resulted from the retention of ancestral polymorphism and, in the case of EF1-α, strong ILS.

The discordance can also be found in species numbers delimited by different methods. As an advanced version of automatic barcode gap discovery (ABGD), ASAP ranks its results based on “scores”, and it is reasonable to accept the delimitation with the highest scores as the best result. However, in this study, there is no obvious barcode ‘gap’ in the histogram based on genetic distances. Eight (10th ranking) or nine (2nd ranking) species may also be recovered if different threshold values are chosen in the lowest distance distribution. However, regardless of the number of species determined using ASAP, it is inconsistent with the seven lineages in the barcode analysis and leads to oversplitting. The situation of oversplitting observed among species delimitations has become more and more common in recent studies, and usually, a conserved and robust delimitation is preferred (Martinsson and Erséus 2017, Pozzi et al. 2020, Xu et al. 2020). Although exactly seven species were recovered in BPP, recent studies have shown that BPP consistently fails to distinguish speciation from population structure (Sukumaran and Knowles 2017, Chambers and Hillis 2020), especially in the delimitation on short-range endemic species, since high levels of genetic structure may mislead the analysis (Xu et al. 2020). *Amynthas sexpectatus* is apparently another example of this situation.

The presence of copulatory pouch is the main difference between *Metaphire* and *Amynthas*. However, these two genera are both polyphyletic (James et al. 2009), and thus, classification can only be achieved by morphology. *Amynthas sexpectatus* (and its four junior synonyms) was classified in the genus *Amynthas* by Tsai et al. (1999) and by Wang and Shih (2017) with no mentioning of copulatory pouches. However, in the 235 specimens we examined, it is clear that the “male pores” that previous studies referred to were “secondary” male pores, and the real male pores usually cannot be seen as they were hidden in a small chamber. This chamber, based on the definitions by Sims and Easton (1972) and by Shen et al. (2019), is indeed a copulatory pouch. Given the presence of this character, we followed Shen et al.’s (2019) opinion on assigning species to either *Amynthas* or *Metaphire* and transferred this species from *Amynthas* to *Metaphire*.

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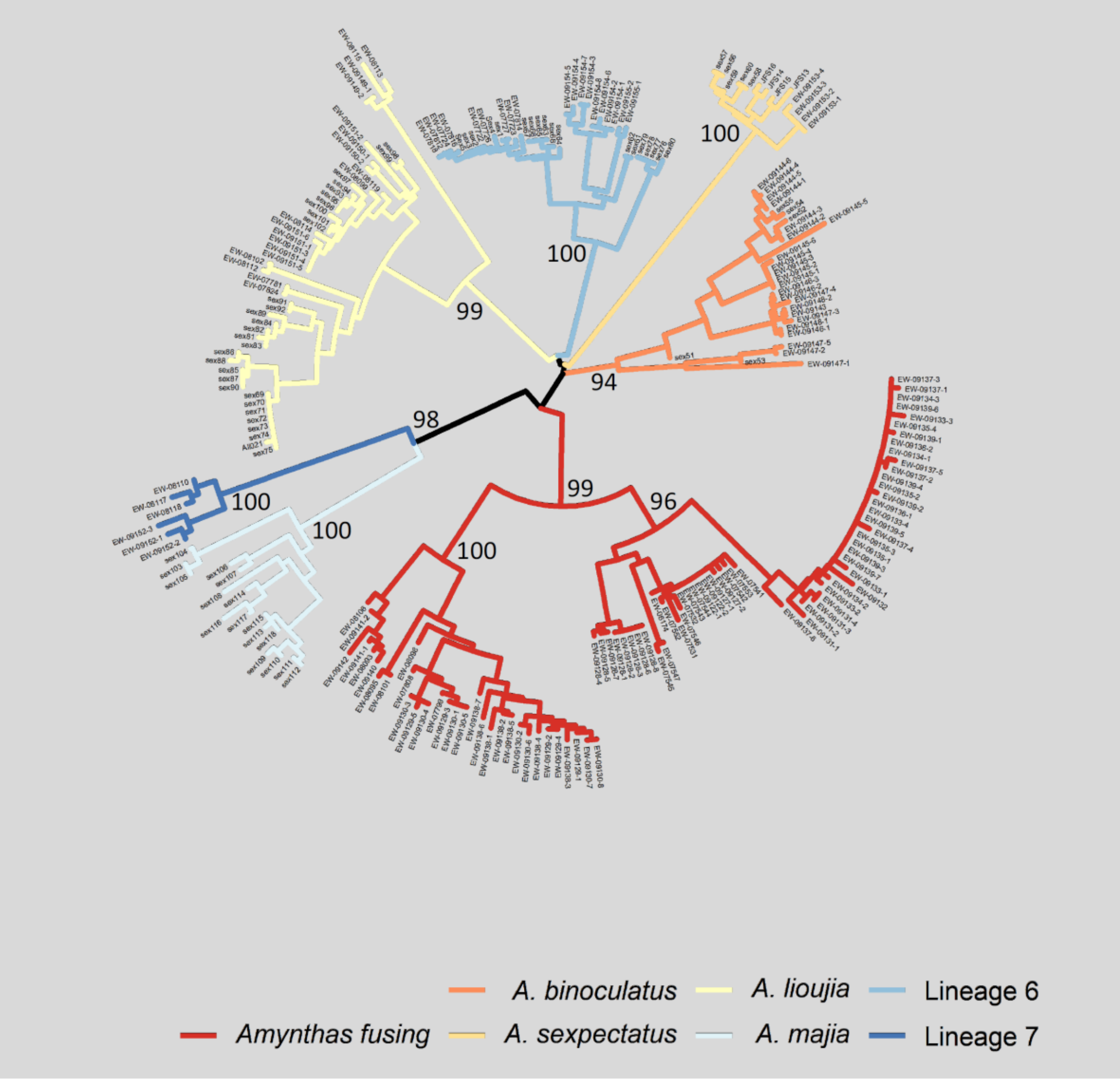

Fig. 1 Unrooted COI neighbor-joining tree of the *Amynthas sexpectatus* species complex. Seven monophyletic groups, including *A. fusing*, *A. binoculatus*, *A. sexpectatus*, *A. lioujia*, *A. majia*, lineage 6, and lineage 7, are marked with different colors. The numbers on the nodes are bootstrap support values.

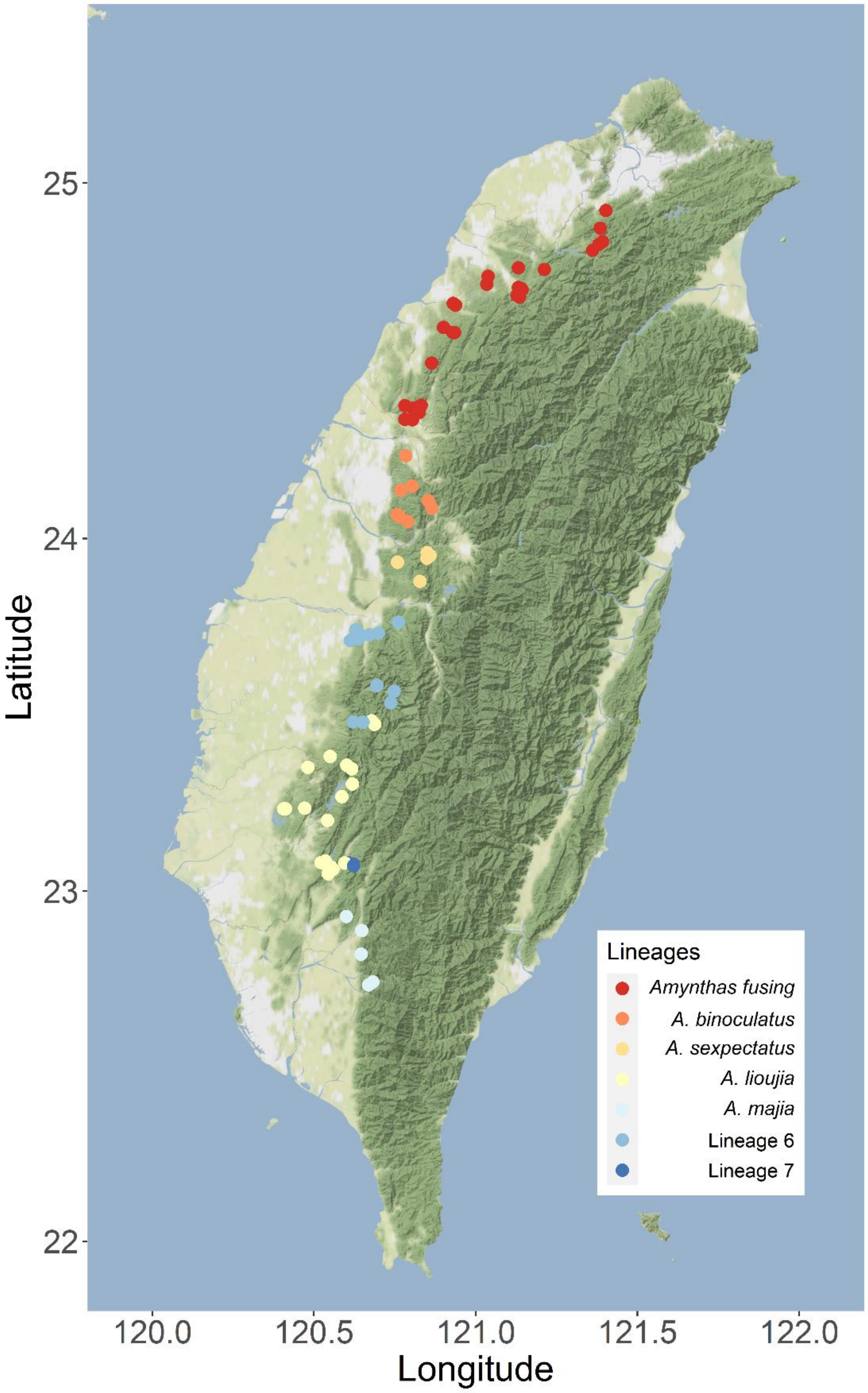

Fig. 2 Sampling sites of the 235 specimens of *Amynthas sexpectatus* species complex. Different colors represent different lineages in DNA barcode analysis and the mitochondrial phylogeny.

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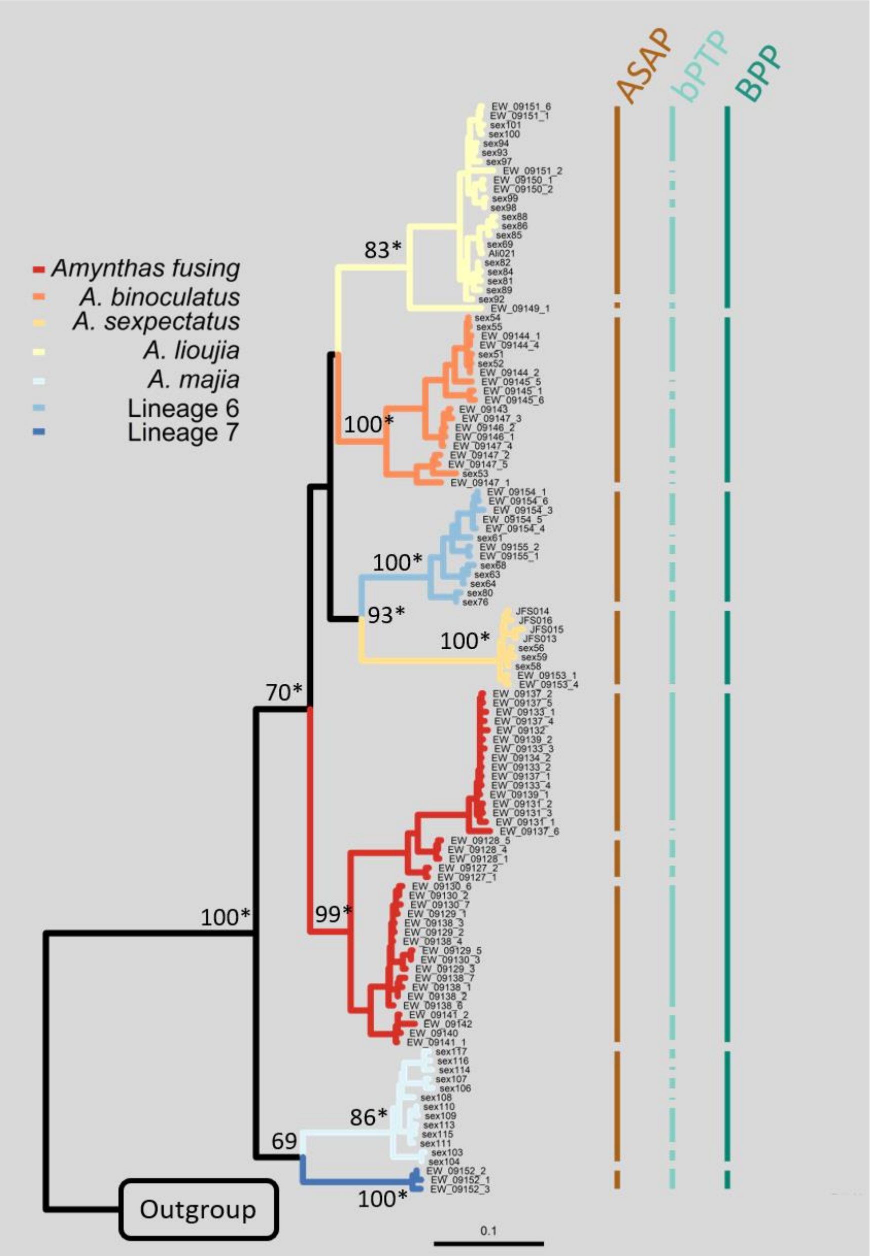

Fig. 3 Maximum likelihood tree based on the mitochondrial COI, 12S rDNA, and 16S rDNA and results of species delimitation from ASAP, bPTP, and BPP. The values on the nodes are bootstrap values in the ML analysis and the asterisks indicate supported nodes with a posterior probability > 0.95 in the Bayesian Inference. Seven monophyletic groups are recovered, including *A. fusing*, *A. binoculatus*, *A. sexpectatus*, *A. lioujia*, *A. majia*, lineage 6, and lineage 7.

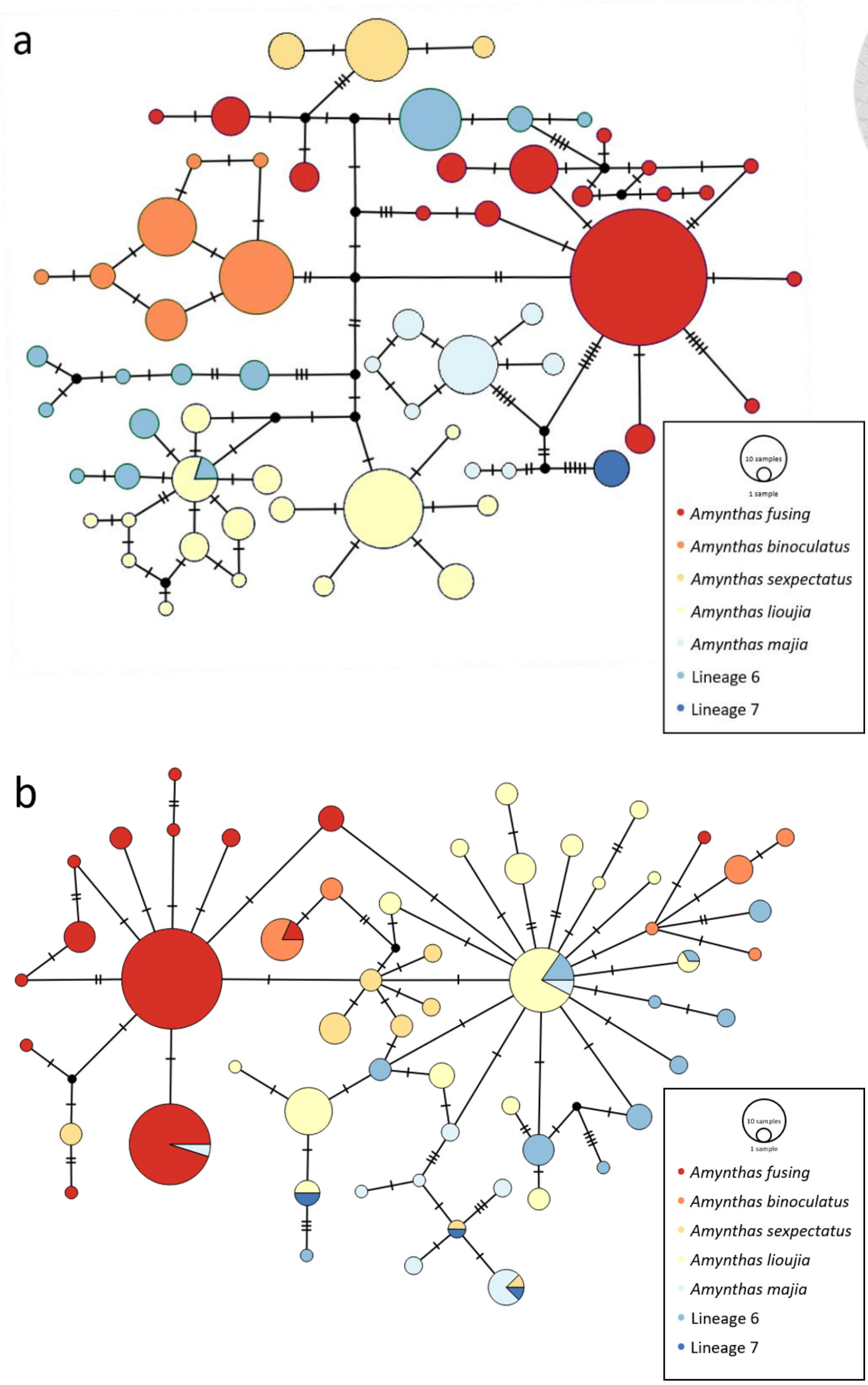

Fig. 4 TCS network based on (a) ITS and (b) EF1-α. Different colors represent different mitochondrial lineages: *A. fusing*, *A. binoculatus*, *A. sexpectatus*, *A. lioujia*, *A. majia*, lineage 6, and lineage 7. The size of circles reflects number of sequences possessing that haplotype.

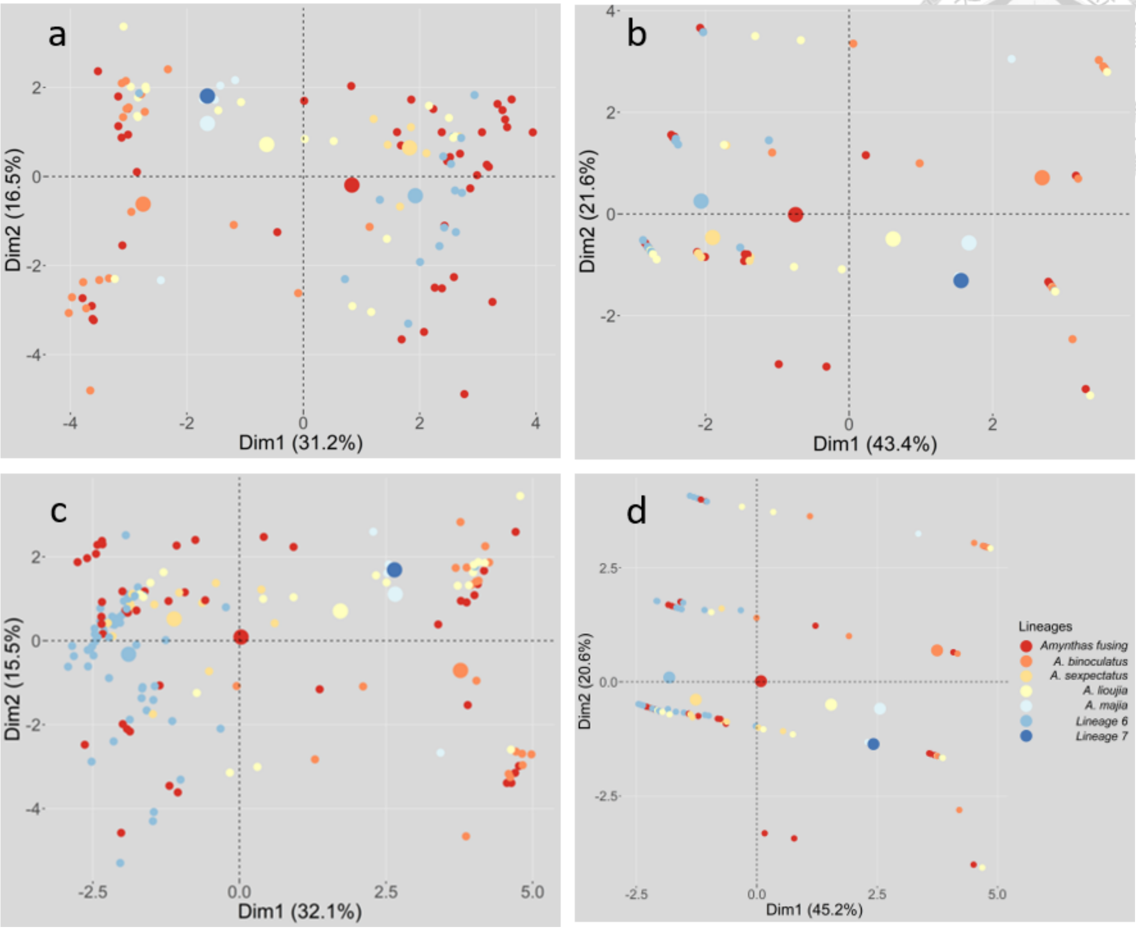

Fig. 5 FAMD results of the *Amynthas sexpectatus* species complex. (a) Specimens with DNA and all characters; (b) specimens with DNA and characters with high contribution to dimensions 1 and 2; (c) all specimens and all characters; (d) all specimens and characters with high contribution to dimensions 1 and 2. Different colors represent different mitochondrial lineages: *A. fusing*, *A. binoculatus*, *A. sexpectatus*, *A. lioujia*, *A. majia*, lineage 6, and lineage 7.

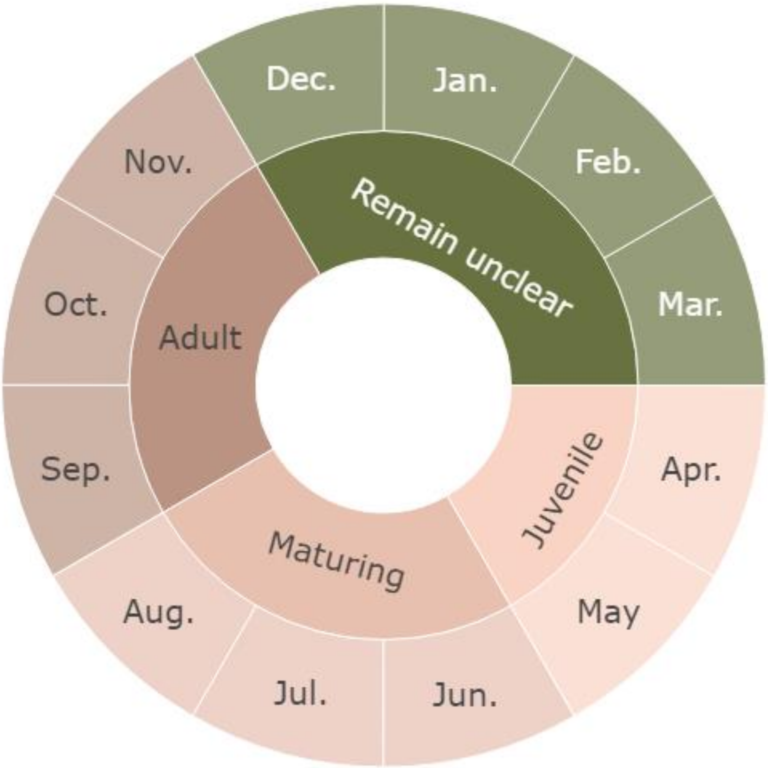

Fig. 6 Life history of *Amynthas sexpectatus* species complex.

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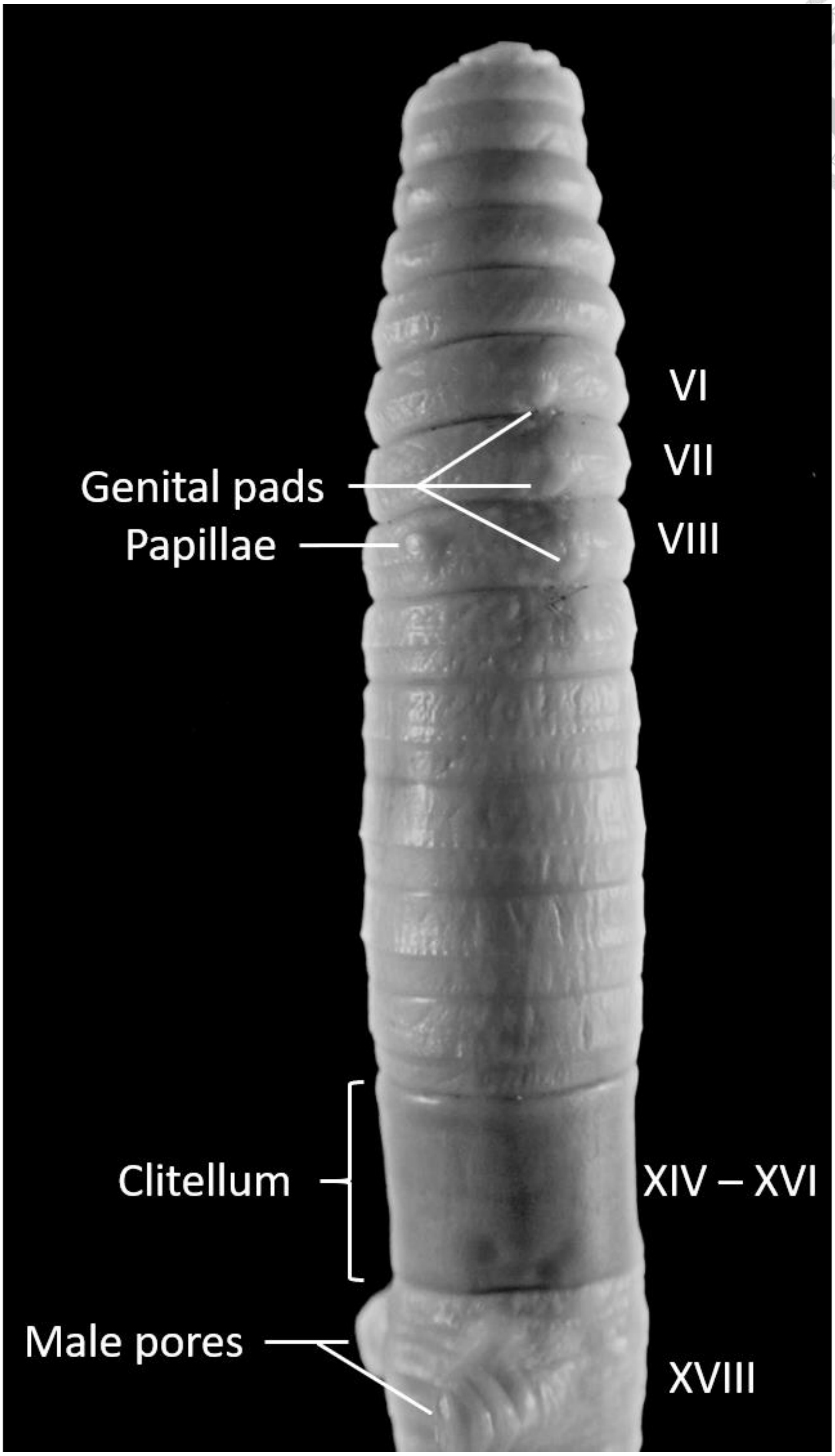

Fig. 7 Photo viewing from the left vento-lateral side of earthworm before segment XVIII.

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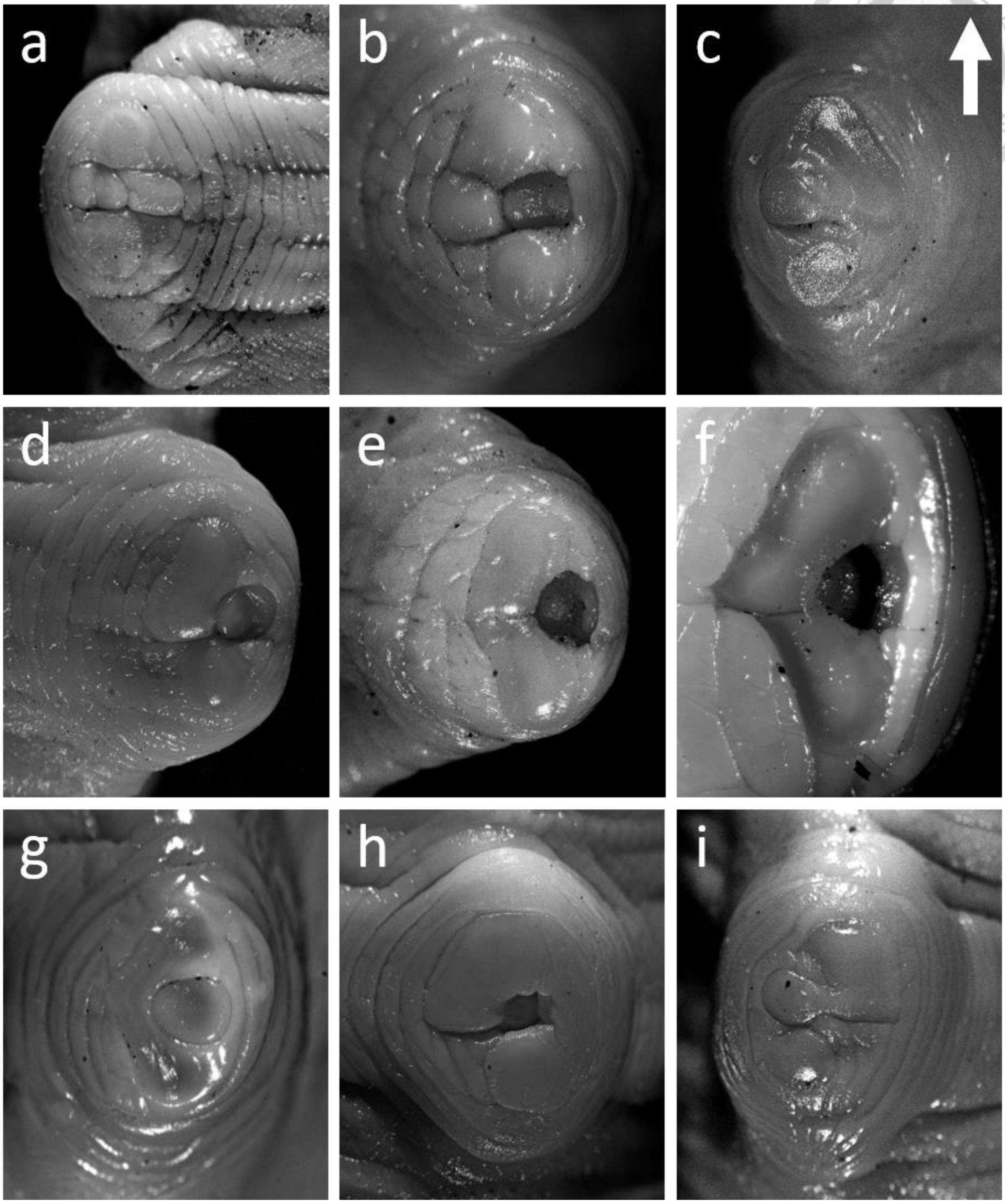

Fig. 8 Photos of papillae around male pores. (a) and (b) three seperated papillae; (c), (d), (e) and (f) two papillae with various shapes; (g) one boomerang-shaped papilla; (h) and (i) untypical papillae shapes. The arrow indicates the rostral way.

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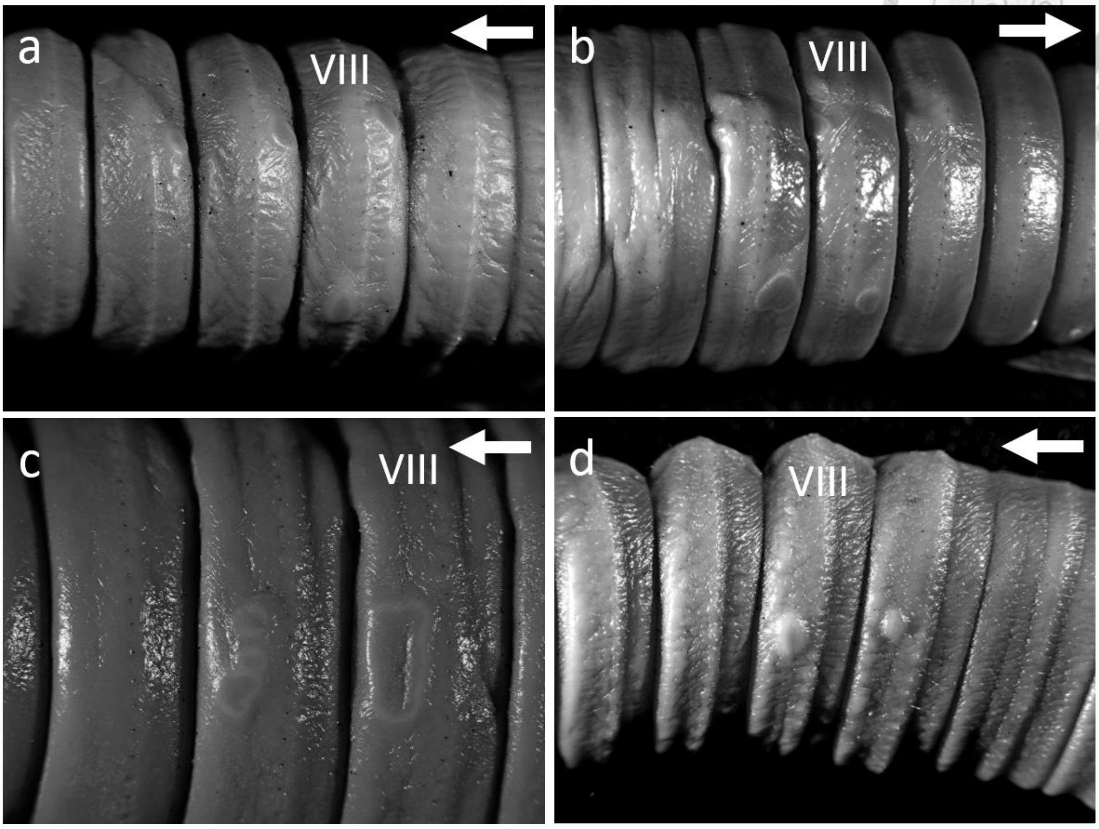

Fig. 9 Photos of genital markings near spermathecal pores. (a) A papilla on VIII; (b) two papillae on VII and VIII; (c) three papilla on one side of VII; (d) two papillae on VIII and IX. The arrows indicate the rostral way.

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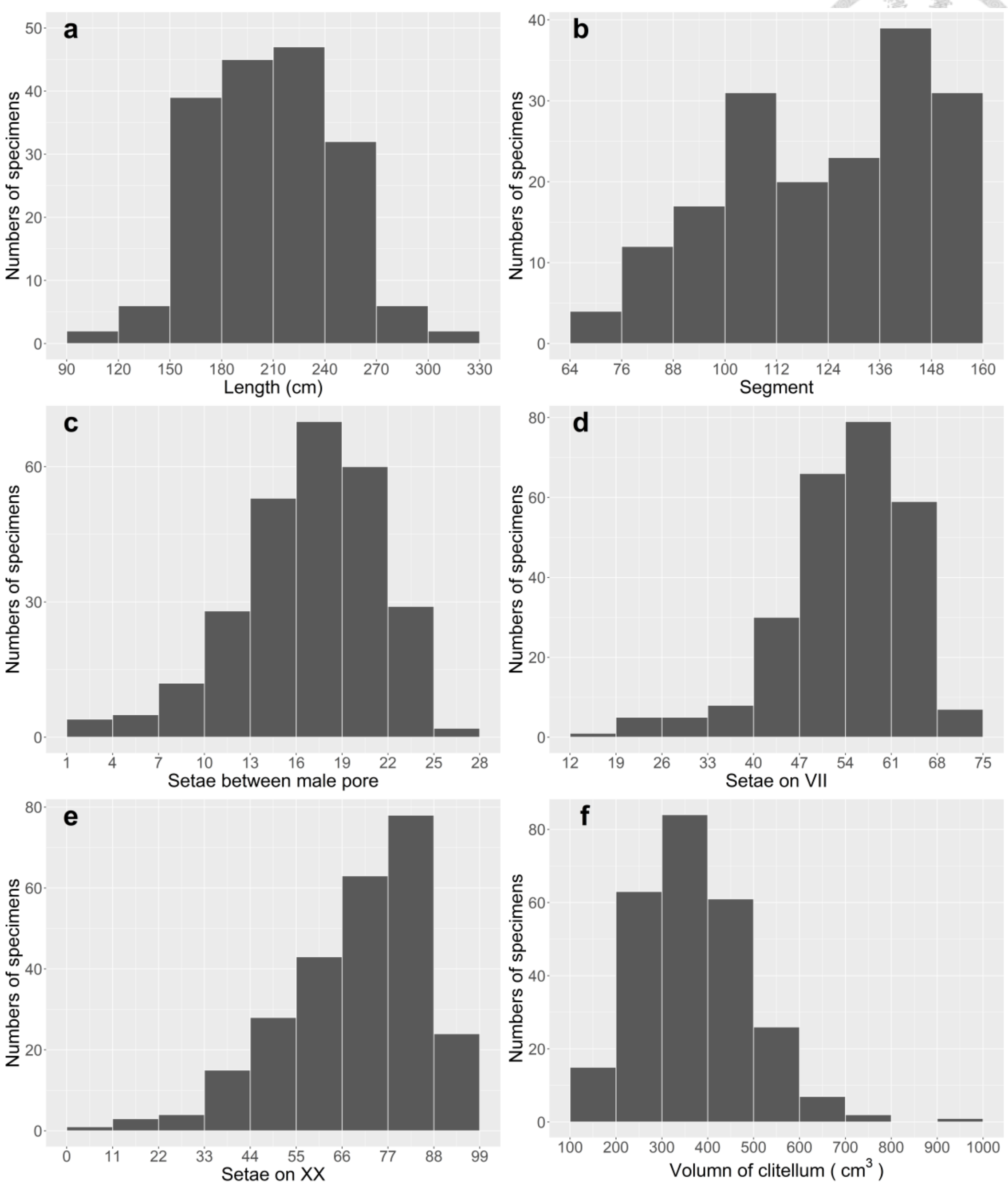

Fig. 10 Histograms of quantitative characters of the *Amynthas sexpectatus* species complex. (a) Length; (b) segments; (c) setae on VII; (d) setae on XX; (e) setae between male pores; (f) volume of clitellum.

Fig. 11 Individuals collected in one-year sampling from District way TYN No.119, Fuxing Dist., Taoyuan City.

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Table 1. Morphological comparisons among nominal species in *Amynthas sexpectatus* species complex.

Species	Genital pads around spermathecal pores	Presetal papillae on the ventral site of VII and VIII	Papillae around male pores (on XVIII)	Reference
A. fusing	3 pairs on VI – VIII	absent	1 pair, C-shaped	Wang and Shih 2017
A. binoculatus	absent	1 pair on VIII, round	3 pairs, triangular- and sandal-shaped	Tsai et al. 1999
A. sexpectatus	3 pairs on VI – VIII, oval-shaped or absent	absent	2 or 3 pairs, round or sandal-shaped	Tsai et al. 1999
A. lioujia	3 pairs on VI – VIII or absent	1 pair on VIII	2 pairs, round	Wang and Shih 2017
A.majia	absent	absent	1 pair, V-shaped	Wang and Shih 2017

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Table 2. Minimum uncorrected *p*-distances between lineages and maximum uncorrected *p*-distances within lineages

%	*A. fusing*	*A. binoculatus*	*A. sexpectatus*	*A. lioujia*	*A. majia*	Lineage 6	Lineage 7
A. fusing	**8.98** **5.24**	4.86	10.49	8.91	9.72	8.41	8.89
A. binoculatus	4.53	**8.51** **4.58**	7.03	6.05	7.52	4.90	8.66
A. sexpectatus	6.68	5.87	**1.98** **1.37**	10.03	11.40	9.42	12.92
A. lioujia	6.75	4.93	7.14	**8.70** **5.18**	10.94	7.75	12.40
A.majia	6.75	6.97	7.92	7.67	**4.10** **2.55**	10.64	6.74
Lineage 6	6.43	5.20	6.37	5.98	7.41	**4.41** **2.69**	12.31
Lineage 7	6.83	6.21	8.57	8.25	6.02	8.46	**2.33** **0.72**

* Upper right: distances based on COI marker; lower left: distances based on COI, 12S rDNA, and 16S rDNA.

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Table 3. One-year collecting data of *Amynthas sexpectatus* species complex.

	January	February	March	April	May	June	July	August		September		October		November	December
Locality				Immature	Immature	Immature	Immature	Immature	Mature	Immature	Mature	Immature	Mature	Mature	
a	0	0	0	3	2	4	1	0	0	0	0	0	2	0	0
b	0	0	0	8	3	0	1	0	0	0	2	0	0	0	0
c	0	0	0	0	0	1	2	4	8	0	8	0	0	0	0
d	-	-	-	-	-	-	-	20	0	9	11	1	20	0	0
e	-	-	-	-	-	-	6	-	-	-	-	-	-	-	-
f	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-
g	-	-	-	-	-	-	-	15	0	4	16	0	21	0	0
h	-	-	-	0	0	-	3	-	-	-	-	-	-	-	-
i	-	-	-	0	0	-	2	19	1	18	2	0	20	18	0
j	-	-	-	0	0	-	-	15	0	4	0	1	20	6	0
k	-	-	-	0	0	-	2	10	0	3	4	3	10	3	0

* Locations a – k were arranged from north to south. (a) District way TYN No.119, Fuxing Dist., Taoyuan City (24.836, 121.392); (b) District way TYN No.119, Fuxing Dist., Taoyuan City (24.828, 121.381); (c) District way TYN No.119, Fuxing Dist., Taoyuan City (24.812 N, 121.362 E); (d) 149A county road, Caoling, Gukeng Township, Yunlin County (23.589, 120.699); (e) Caoling, Gukeng Township, Yunlin County (23.588, 120.687); (f) Caoling, Gukeng Township, Yunlin County (23.587, 120.687); (g) between telephone pole 83 and 84 on 149A county road at Laiji Village (elevation 730m), Alishan Township, Chiayi County, (23.533, 120.733); (h) Dahua Track 42.5K, Zhonghe Village, Zhuqi Township, Chiayi County (23.474, 120.687); (i) near Xianlin Bridge, Shanlin Dist., Kaohsiung City (23.050, 120.544); (j) near a telephone pole on Provincial Highways No.24, Wutai, Sandimen Township, Pingtung County (22.742, 120.682); (k) Provincial Highways No.24 26.2K, Wutai, Sandimen Township, Pingtung County (22.735, 120.660).

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Appendix 1. List of samples used in this study

Samples were collected from roadside slopes and ditches on the road if not mentioned in the context. A clitellate collected 20 July 1999 from roadside slopes and ditches on the road from dormitory to working place of Forestry Bureau (elevation 1000m), Wushihkeng, Heping Dist., Taichung City by C.F. Tsai, S.C. Tsai, H.P. Shen, J.C. Chang and C.I. Chang (coll. no. 1999-12-Shen); a clitellate collected 24 October 2000 on Laonongxi Forest-Road 4.5k (elevation 557m), Liugui Dist., Kaohsiung City by S.T. Chang, H.P. Yang and C.I. Chang (coll. no. 2000-69-Shen); an aclitellate collected 26 April 2003 at Baoshan Reservoir, Baoshan Township, Hsinchu County by I.H. Chen (coll. no. NTUM-EW-06174) (voucher numbers: sp61-11); two clitellates collected 17 July 2007 at Dakeng (elevation 235m), Huben Village, Linnei Township, Yunlin County by C.H. Chang, T.J. Lin and Y.H. Lin (coll. no. 2007-39-Shen); a clitellate collected 17 July 2007 from ditches on the road from Tiansheng Temple, Huben Village, to Douliu City (elevation 201m), Linnei Township, Yunlin County by C.H. Chang, T.J. Lin and Y.H. Lin (coll. no. 2007-41-Shen); two clitellates collected 18 July 2007 at Huangdekeng river (elevation 139m), Gukeng Township, Yunlin County by C.H. Chang, T.J. Lin and Y.H. Lin (coll. no. 2007-48-Shen); three clitellates collected 14 August 2007 from Tiansheng Temple, Huben Village (elevation 194m), Linnei Township, Yunlin County by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-52-Shen); five clitellates collected 14 August 2007 on the road to zhongguping , Huben Village (elevation 265m), Linnei Township, Yunlin County by C.H. Chang, Y.H. Lin and

Y.P. Li (coll. no. 2007-54-Shen); a clitellate collected 14 August 2007 from roadside of pavilion on Pingding Rd. (elevation 237m), Linnei Township, Yunlin County by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-55-Shen); a clitellate collected 15 August 2007 outside Youqinggu (elevation 228m), Douliu City, Yunlin County, by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-57-Shen); a clitellate collected 15 August 2007 in Youqinggu (elevation 178-230m), Douliu City, Yunlin County, by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-58-Shen); two clitellates collected 15 August 2007 at Waihu river, (elevation 200m) on the boundary between Linnei Township and Douliu City, Yunlin County, by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-60-Shen); three clitellates collected 16 August 2007 at Dakeng (elevation 235m), Huben Village, Linnei Township, Yunlin County by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-62-Shen); two clitellates collected 16 August 2007 at Qiedongkeng (elevation 208m), Huben Village, Linnei Township, Yunlin County by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-63-Shen); two clitellates collected 16 August 2007 from ditches on the road from Tiansheng Temple, Huben Village, to Douliu City (elevation 201m), Linnei Township, Yunlin County by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-64-Shen); a clitellate collected 16 August 2007 at FengshuHunan River (elevation 150-210m), Douliu City, Yunlin County by C.H. Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-65-Shen); two clitellates collected 17 August 2007 at Chamourenkeng (elevation 172-216m), Linnei Township, Yunlin County by C.H.

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Chang, Y.H. Lin and Y.P. Li (coll. no. 2007-67-Shen); a clitellate collected 11 September 2007 outside Youqinggu (elevation 228m), Douliu City, Yunlin County, by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-71-Shen); a clitellate collected 11 September 2007 in Youqinggu (elevation 178-230m), Douliu City, Yunlin County, by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-72-Shen); a clitellate collected 11 September 2007 at Waihu river, (elevation 200m) on the boundary between Linnei Township and Douliu City, Yunlin County, by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-74-Shen); two clitellates collected 12 September 2007 at Fairy Pitta Birdwatching Trail (elevation 153m), Linnei Township, Yunlin County, by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-77-Shen); seven clitellates collected 12 September on the road to zhongguping , Huben Village (elevation 265m), Linnei Township, Yunlin County by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-78-Shen); a clitellate collected 12 September 2007 from roadside of pavilion on Pingding Rd. (elevation 237m), Linnei Township, Yunlin County by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-79-Shen); three clitellates collected 13 September 2007 at Dakeng (elevation 235m), Huben Village, Linnei Township, Yunlin County by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-81-Shen); a clitellate collected 13 September 2007 at Qiedongkeng (elevation 208m), Huben Village, Linnei Township, Yunlin County by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-82-Shen); four clitellates collected 13 September 2007 at FengshuHunan

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River (elevation 150-210m), Douliu City, Yunlin County by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-83-Shen); seven clitellates collected 14 September 2007 at Chamourenkeng (elevation 172-216m), Linnei Township, Yunlin County by T.J. Lin, Y.P. Li, H.Y. Tsai and L.H. Chen (coll. no. 2007-86-Shen); two clitellates collected 23 October 2007 outside Youqinggu (elevation 228m), Douliu City, Yunlin County, by T.J. Lin, Y.P. Li, C.H. Chang and K.C. Lin (coll. no. 2007-91-Shen); a clitellate collected 24 October 2007 at Fairy Pitta Birdwatching Trail (elevation 153m), Linnei Township, Yunlin County, by T.J. Lin, Y.P. Li, C.H. Chang and K.C. Lin (coll. no. 2007-97-Shen); a clitellate collected 24 October 2007 from roadside of pavilion on Pingding Rd. (elevation 237m), Linnei Township, Yunlin County by T.J. Lin, Y.P. Li, C.H. Chang and K.C. Lin (coll. no. 2007-99-Shen); two clitellates collected 30 October 2007 at Qiedongkeng (elevation 208m), Huben Village, Linnei Township, Yunlin County by T.J. Lin, Y.P. Li, C.H. Chang and K.C. Lin (coll. no. 2007-102-Shen); a clitellate collected 3 July 2010 at Hengshan Township, Hsinchu County by W.J. Chih (coll. no. NTUM-EW-07531) (voucher numbers: sp61-12); a clitellate collected 3 July 2010 at Hengshan Township, Hsinchu County by W.J. Chih (coll. no. NTUM-EW-07532) (voucher numbers: sp61-13); a clitellate collected 3 July 2010 at Hengshan Township, Hsinchu County by W.J. Chih (coll. no. NTUM-EW-07541) (voucher numbers: sp61-14); a clitellate collected 3 July 2010 at Hengshan Township, Hsinchu County by W.J. Chih (coll. no. NTUM-EW-07542)

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(voucher numbers: sp61-15); a clitellate collected 3 July 2010 at Hengshan Township, Hsinchu County by W.J. Chih (coll. no. NTUM-EW-07543) (voucher numbers: sp61-16); a clitellate collected 3 July 2010 at Hengshan Township, Hsinchu County by W.J. Chih (coll. no. NTUM-EW-07544) (voucher numbers: sp61-17); a clitellate collected 3 July 2010 at Yongheshan Reservoir, Toufen City, Miaoli County by W.J. Chih (coll. no. NTUM-EW-07545) (voucher numbers: sp61-18); a clitellate collected 3 July 2010 at Yongheshan Reservoir, Toufen City, Miaoli County by W.J. Chih (coll. no. NTUM-EW-07546) (voucher numbers: sp61-19); a clitellate collected 3 July 2010 at Yongheshan Reservoir, Toufen City, Miaoli County by W.J. Chih (coll. no. NTUM-EW-07547) (voucher numbers: sp61-20); a clitellate collected 3 July 2010 at Baoshan Reservoir, Baoshan Township, Hsinchu County by W.J. Chih (coll. no. NTUM-EW-07552) (voucher numbers: sp61-25); a clitellate collected 3 July 2010 at Baoshan Reservoir, Baoshan Township, Hsinchu County by W.J. Chih (coll. no. NTUM-EW-07553) (voucher numbers: sp61-26); two clitellates collected 10 August 2010 at Linmao Village, Linnei Township, Yunlin County by W.J. Chih (coll. no. NTUM-EW-07722~07723) (voucher numbers: Asex-1~2); an aclitellate collected 10 August 2010 at Huben Village, Linnei Township, Yunlin County by W.J. Chih (coll. no. NTUM-EW-07724) (voucher numbers: Asex-3); a clitellate collected 10 August 2010 at Huben Village, Linnei Township, Yunlin County by W.J. Chih (coll. no. NTUM-EW-07726) (voucher numbers: Asex-5); a clitellate collected 8 September 2010 at

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Huben Village, Linnei Township, Yunlin County by W.J. Chih (coll. no. NTUM-EW-07727) (voucher numbers: Asex-6); two clitellates collected 8 September 2010 at Baihe Reservoir, Baihe Dist., Tainan City by W.J. Chih (coll. no. NTUM-EW-07781, 07824) (voucher numbers: Asex-7, 13); a clitellate collected 9 September 2010 at Mingde Reservoir, Touwu Township, Miaoli County by W.J. Chih (coll. no. NTUM-EW-07799) (voucher numbers: sp61-34); a clitellate collected 9 September 2010 at Mingde Reservoir, Touwu Township, Miaoli County by W.J. Chih (coll. no. NTUM-EW-07808) (voucher numbers: sp61-35); three clitellates collected 9 September 2010 in Douliu City, Yunlin County by W.J. Chih (coll. no. NTUM-EW-07812, 07814, 07818) (voucher numbers: Asex-8, 9, 12); a clitellate collected 9 September 2010 in Douliu City, Yunlin County by W.J. Chih (coll. no. NTUM-EW-07816) (voucher numbers: Asex-10); a clitellate collected 18 September 2010 at Liyutan Reservoir, Dahu Township, Miaoli County by W.J. Chih (coll. no. NTUM-EW-08093) (voucher numbers: sp61-21); a clitellate collected 18 September 2010 at Liyutan Reservoir, Dahu Township, Miaoli County by W.J. Chih (coll. no. NTUM-EW-08095) (voucher numbers: sp61-38); a clitellate collected 18 September 2010 at Mingde Reservoir, Touwu Township, Miaoli County by W.J. Chih (coll. no. NTUM-EW-08098) (voucher numbers: sp61-41); a clitellate collected 18 September 2010 at Liyutan Reservoir, Dahu Township, Miaoli County by W.J. Chih (coll. no. NTUM-EW-08106) (voucher numbers: sp61-49); a clitellate collected 2 October 2010 under

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white stone on Township way ML No.56, Sanyi Township, Miaoli County by W.J. Chih (coll. no. NTUM-EW-08101) (voucher numbers: sp61-44); a clitellate collected 22 October 2010 on the road from Jiaxian Dist. to Nanhua Dist., Kaohsiung City by W.J. Chih (coll. no. NTUM-EW-08099) (voucher numbers: sp61-42); a clitellate collected 22 October 2010 at Nanhua reservoir, Nanhua Dist., Tainan City by W.J. Chih (coll. no. NTUM-EW-08102) (voucher numbers: sp61-45); a clitellate collected 22 October 2010 on Provincial Highway No.21, Jiaxian Dist., Kaohsiung City by W.J. Chih (coll. no. NTUM-EW-08110) (voucher numbers: sp61-53); a clitellate collected 22 October 2010 at Nanhua reservoir, Nanhua Dist., Tainan City by W.J. Chih (coll. no. NTUM-EW-08112) (voucher numbers: sp61-55); a clitellate collected 22 October 2010 in Dongshan Dist., Tainan City by W.J. Chih (coll. no. NTUM-EW-08113) (voucher numbers: sp61-56); a clitellate collected 22 October 2010 on the road from Jiaxian Dist. to Nanhua Dist., Kaohsiung City by W.J. Chih (coll. no. NTUM-EW-08114) (voucher numbers: sp61-57); a clitellate collected 22 October 2010 on County way No.174, Liujia Dist., Tainan City by W.J. Chih (coll. no. NTUM-EW-08115) (voucher numbers: sp61-58); a clitellate collected 22 October 2010 on Provincial Highway No.21, Jiaxian Dist., Kaohsiung City by W.J. Chih (coll. no. NTUM-EW-08117) (voucher numbers: sp61-60); a clitellate collected 22 October 2010 on Provincial Highway No.21, Jiaxian Dist., Kaohsiung City by W.J. Chih (coll. no. NTUM-EW-08118) (voucher numbers: sp61-61); a clitellate collected 22 October 2010 on

Provincial Highway No.21, Jiaxian Dist., Kaohsiung City by W.J. Chih (coll. no. NTUM-EW-08119) (voucher numbers: sp61-62); two clitellates collected 26 July 2013 at Niu Mountain (elevation 1024m), Guanghua Village, Zhuqi Township, Chiayi County by H.D. Fang (coll. no. 2013-6) (voucher numbers: Ali021); two clitellates collected 25 May 2016 on Leek Lake Bridge (elevation 647m), Jiufen'er Mountain, Guoxing Township, Nantou County by P.H. Lin and C.S. Huang (coll. no. 2016-13) (voucher numbers: JFS13); a clitellate collected 22 June 2016 at Eastern toppled house (elevation 730m), Jiufen'er Mountain, Guoxing Township, Nantou County by P.H. Lin and C.S. Huang (coll. no. 2016-30) (voucher numbers: JFS14); two clitellates collected 19 July 2016 on Zhongxing Bridge Side Slope (elevation 450m), Jiufen'er Mountain, Guoxing Township, Nantou County by P.H. Lin and C.S. Huang (coll. no. 2016-35) (voucher numbers: JFS15); three clitellates collected 20 July 2016 on Eastern toppled house (elevation 730m), Jiufen'er Mountain, Guoxing Township, Nantou County by P.H. Lin and C.S. Huang (coll. no. 2016-40); four clitellates collected 16 August 2016 on Zhongxing Bridge Side Slope (elevation 450m), Jiufen'er Mountain, Guoxing Township, Nantou County by P.H. Lin and C.S. Huang (coll. no. 2016-44) (voucher numbers: JFS16); a clitellate collected 16 August 2016 at Eastern toppled house (elevation 730m), Jiufen'er Mountain, Guoxing Township, Nantou County by P.H. Lin and C.S. Huang (coll. no. 2016-49); two clitellates collected 29 July 2019 in banana orchard (elevation 233m), Tonglin, Wufeng Dist., Taichung City by W.J. Chih and T.L. Ai (coll. no. 2019-1) (voucher numbers: sex51~52); two clitellates and an aclitellate collected 29 July 2019 at side slope near Lingyin Temple (elevation 233m), Minsheng Rd., Wufeng Dist., Taichung City by W.J. Chih and T.L. Ai (coll. no. 2019-1) (voucher numbers: sex53~55); four clitellates and an aclitellate collected 30 July 2019 on Mazhu Ln. (elevation 367m), Zhongliao Township, Nantou County by W.J. Chih and T.L. Ai (coll. no. 2019-2) (voucher numbers: sex56~60); a clitellate and an aclitellate collected 30 July 2019 on Guoxi Forest Trail (elevation 209m), Tiandong Rd., Zhushan Township, Nantou County by W.J. Chih and T.L. Ai (coll. no. 2019-3) (voucher numbers: sex61~62); two clitellates and two aclitellates collected 30 July 2019 at Caoling (elevation 790m), Gukeng Township, Yunlin County by W.J. Chih and T.L. Ai (coll. no. 2019-4) (voucher numbers: sex63~66); a clitellate collected 30 July 2019 at Fengshan Village (elevation 737m), Alishan Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-5) (voucher numbers: sex67); a clitellate collected 30 July 2019 at Laiji Village (elevation 730m), Alishan Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-6) (voucher numbers: sex68); four clitellates and three aclitellates collected 31 July 2019 on Dahua Track 42.5K (elevation 1082m), Zhonghe Village, Zhuqi Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-7) (voucher numbers: sex69~75); a clitellate collected 31 July 2019 on County way No.159A (elevation 610m), Jiadongzi, Guanghua Village, Zhuqi Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-8) (voucher numbers:

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sex76); four clitellates collected 31 July 2019 on County way No.159A (elevation 785m), Dahu, Fanlu Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-9) (voucher numbers: sex77~80); three clitellates and a aclitellate collected 31 July 2019 near Zhonglun No.1 Bridge (elevation 268m), Provincial Highway No.3 298K, at Zhongpu Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-10) (voucher numbers: sex81~84); four clitellates and two aclitellates collected 31 July 2019 near Yongxing Fu'an Temple (elevation 841m), Fanlu Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-11) (voucher numbers: sex85~90); two clitellates collected 31 July 2019 at Shanyangkan (elevation 367m), Caoshan Village, Fanlu Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-12) (voucher numbers: sex91~92); four clitellates collected 31 July 2019 on Provincial Highway No.3, Heping No.2 Bridge (elevation 258m), Dapu Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-13) (voucher numbers: sex93~96); a clitellate and two aclitellates collected 31 July 2019 on Provincial Highway No.3, near Heping No.2 Bridge (elevation 297m), Dapu Township, Chiayi County by W.J. Chih and T.L. Ai (coll. no. 2019-14) (voucher numbers: sex97~99); two clitellates and a aclitellate collected 1 August 2019 near Xianlin Bridge (elevation 270m), Shanlin Dist., Kaohsiung City by W.J. Chih and T.L. Ai (coll. no. 2019-15) (voucher numbers: sex100~102); three aclitellates collected 1 August 2019 in Yellow Butterfly Valley (elevation 107m), Meinong Dist., Kaohsiung City by W.J. Chih and T.L. Ai (coll. no.

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2019-16) (voucher numbers: sex103~105); two aclitellates collected 1 August 2019 on Provincial Highways No.27 23.5K (elevation 139m), Liugui Dist., Kaohsiung City by W.J. Chih and T.L. Ai (coll. no. 2019-17) (voucher numbers: sex106~107); a tail only sample collected 1 August 2019 near Ocean God's Pools (elevation 140m), Qingshan Village, Sandimen Township, Pingtung County by W.J. Chih and T.L. Ai (coll. no. 2019-18) (voucher numbers: sex108); five clitellates collected 15 October 2019 on Provincial Highways No.24 26.2K (elevation 526m), Dewen, Sandimen Township, Pingtung County by T.L. Ai and C.C. Li (coll. no. 2019-19) (voucher numbers: sex109~113); five clitellates collected 15 October 2019 near a telephone pole on Provincial Highways No.24 (elevation 482m), Wutai, Sandimen Township, Pingtung County by T.L. Ai and C.C. Li (coll. no. 2019-20) (voucher numbers: sex114~118); two aclitellates collected 3 June 2020 on Township way HC No.35, Hengshan Township, Hsinchu County by C. Kao (coll. no. NTUM-EW-09122-1~2) (voucher numbers: NTUM-EW-09122-1~2); a clitellate and an aclitellates collected 7 July 2020 on Township way HC No.35, Hengshan Township, Hsinchu County by C. Kao (coll. no. NTUM-EW-09127-1~2) (voucher numbers: NTUM-EW-09127-1~2); six clitellates and two aclitellates collected 7 July 2020 on Township way HC No.36, Qionglin Township, Hsinchu County by C. Kao (coll. no. NTUM-EW-09128-1~8) (voucher numbers: NTUM-EW-09128-1~8); five clitellates collected 15 July 2020 on County way No.36, Touwu Township, Miaoli County by C. Kao (coll. no. NTUM-EW-

09129-1~5) (voucher numbers: NTUM-EW-09129-1~5); seven clitellates and a aclitellate collected 15 July 2020 on County way No.36, Touwu Township, Miaoli County by C. Kao (coll. no. NTUM-EW-09130-1~8) (voucher numbers: NTUM-EW-09130-1~8); four clitellates collected 16 July 2020 at Aly. 1, Ln. 138, Sec. 2, Jieshou Rd., Sanxia Dist., New Taipei City by C. Kao (coll. no. NTUM-EW-09131-1~4) (voucher numbers: NTUM-EW-09131-1~4); a clitellate collected 16 July 2020 on Jinmin Track, Sanxia Dist., New Taipei City by C. Kao (coll. no. NTUM-EW-09132) (voucher numbers: NTUM-EW-09132); four clitellates collected 29 July 2020 on District way TYN No.119, Fuxing Dist., Taoyuan City by C. Kao (coll. no. NTUM-EW-09133-1~4) (voucher numbers: NTUM-EW-09133-1~4); three clitellates collected 29 July 2020 on District way TYN No.119, Fuxing Dist., Taoyuan City by C. Kao (coll. no. NTUM-EW-09134-1~3) (voucher numbers: NTUM-EW-09134-1~3); four clitellates collected 29 July 2020 on District way TYN No.119, Fuxing Dist., Taoyuan City by C. Kao (coll. no. NTUM-EW-09135-1~4) (voucher numbers: NTUM-EW-09135-1~4); two clitellates collected 29 July 2020 on District way TYN No.119, Fuxing Dist., Taoyuan City by C. Kao (coll. no. NTUM-EW-09136-1~2) (voucher numbers: NTUM-EW-09136-1~2); six clitellates collected 29 July 2020 on District way TYN No.119, Fuxing Dist., Taoyuan City by C. Kao (coll. no. NTUM-EW-09137-1~6) (voucher numbers: NTUM-EW-09137-1~6); six clitellates and a aclitellate collected 4 August 2020 on Township way ML No.24-1, Gongguan Township, Miaoli

County by C. Kao (coll. no. NTUM-EW-09138-1~7) (voucher numbers: NTUM-EW-09138-1~7); seven clitellates collected 6 August 2020 at Chikeshan, Yushan Village, Guanxi Township, Hsinchu County by C. Kao (coll. no. NTUM-EW-09139-1~7) (voucher numbers: NTUM-EW-09139-1~7); a clitellate collected 18 August 2020 on Fuxing Track, Dahu Township, Miaoli County by C. Kao (coll. no. NTUM-EW-09140) (voucher numbers: NTUM-EW-09140); a clitellate and an aclitellate collected 18 August 2020 on Yihu Road, Sanyi Township, Miaoli County by C. Kao (coll. no. NTUM-EW-09141-1~2) (voucher numbers: NTUM-EW-09141-1~2); a clitellate collected 18 August 2020 on Shenshui Track, Sanyi Township, Miaoli County by C. Kao (coll. no. NTUM-EW-09142) (voucher numbers: NTUM-EW-09142); a clitellate collected 19 August 2020 on Nankeng Ln., Shuiyuan Rd., Fengyuan Dist., Taichung City by C. Kao (coll. no. NTUM-EW-09143) (voucher numbers: NTUM-EW-09143); three clitellates and three aclitellates collected 24 August 2020 on Minsheng Rd., Wufeng Dist., Taichung City by C. Kao (coll. no. NTUM-EW-09144-1~6) (voucher numbers: NTUM-EW-09144-1~6); six clitellates collected 25 August 2020 on Township way NT No.97, Guoxing Township, Nantou County by C. Kao (coll. no. NTUM-EW-09145-1~6) (voucher numbers: NTUM-EW-09145-1~6); three clitellates collected 25 August 2020 on Township way NT No.97, Guoxing Township, Nantou County by C. Kao (coll. no. NTUM-EW-09146-1~3) (voucher numbers: NTUM-EW-09146-1~3); five clitellates collected 25 August 2020 on Township way NT No.97,

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Guoxing Township, Nantou County by C. Kao (coll. no. NTUM-EW-09147-1~5) (voucher numbers: NTUM-EW-09147-1~5); two clitellates collected 25 August 2020 on Township way NT No.97, Guoxing Township, Nantou County by C. Kao (coll. no. NTUM-EW-09148-1~2) (voucher numbers: NTUM-EW-09148-1~2); two clitellates collected 1 September 2020 on County way No.174, Liujia Dist., Tainan City by C. Kao (coll. no. NTUM-EW-09149-1~2) (voucher numbers: NTUM-EW-09149-1~2); two aclitellates collected 1 September 2020 on District way TNN No.192, Nanxi Dist., Tainan City by C. Kao (coll. no. NTUM-EW-09150-1~2) (voucher numbers: NTUM-EW-09150-1~2); three clitellates and three aclitellates collected 2 September 2020 on Provincial Highway No.20, Nanhua Dist., Tainan City by C. Kao (coll. no. NTUM-EW-09151-1~6) (voucher numbers: NTUM-EW-09151-1~6); a clitellate and two aclitellates collected 2 September 2020 on Provincial Highway No.20, Jiaxian Dist., Kaohsiung City by C. Kao (coll. no. NTUM-EW-09152-1~3) (voucher numbers: NTUM-EW-09152-1~3); four clitellates collected 8 September 2020 on County way NT No.17, Zhongliao Township, Nantou County by C. Kao (coll. no. NTUM-EW-09153-1~4) (voucher numbers: NTUM-EW-09153-1~4); eight clitellates collected 9 September 2020 on Zhongxinlun Forest Trail, Zhushan Township, Nantou County by C. Kao (coll. no. NTUM-EW-09154-1~8) (voucher numbers: NTUM-EW-09154-1~8); two clitellates collected 9 September 2020 on Shuitou Ln., Lugu Township, Nantou County by C. Kao (coll. no. NTUM-EW-09155-1~2) (voucher numbers: NTUM-EW-09155-1~2).