DNA barcoding and phylogenetic analysis of midges belonging to Culicoides (Diptera: Ceratopogonidae) subgenus Hoffmania in Yunnan, China

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Abstract:	DNA barcodes obtained from cytochrome c oxidase subunit 1 (cox1) offer a fast and easy way to identify a range of biological organisms. Culicoides (Diptera: Ceratopogonidae) are a group of small, blood sucking midges whose species are the vectors for some arboviruses, such as bluetongue virus, African horse sickness virus, epizootic hemorrhagic disease virus and equine encephalosis virus. Identification of these small insects is difficult so constructing DNA barcode libraries for species present in certain areas is helpful to clarify the taxonomy and assist non-specialist workers to identify species. In this study, we analysed specimens belonging to C. subgenus Hoffmania collected from 12 towns of Yunnan Province, China. Specimens were identified by morphology and processed to construct DNA barcodes. A total of 185 specimens referable to 6 morphological species were processed for cox1 and 28S rRNA sequencing. The resulting 185 cox1 sequences were assigned to 13 barcode index numbers (BINs) which include 9 novel BINs. Molecular and morphological evidence was used to support the transfer of 4 species previously assigned to C. subg. Avaritia into C. subg. Hoffmania . Molecular analysis revealed the presence of 7 potential cryptic species within C. innoxius, three within C. liui and two within C. insignipennis .
Suggested Reviewers:	Lara Harrup lara.harrup@pirbright.ac.uk Culicoides researcher
	David Gopurenko david.gopurenko@dpi.nsw.gov.au major in Culicoides species and phylogenetic analysis

Dear editor:

Culicoides are a category of small biting midges, some of which are the insect vectors of arboviruses. In this study, we collected and identified the *Culicoides* species (belong to subgenus *Hoffmania* according to Wirth system) in Yunnan Province, China, and made the *cox1* barcode for them.

1) So far, approximately 1347 *Culicoides* species are identified all over the world, but the DNA sequences registered on public data base are rare for species. So it is meaningful to publish more *cox1* barcode of *Culicoides* species, since these small midges are very difficult to identify by morphology for virologists. Here, we made the *cox1* barcodes for the species in Yunnan belonging to subgenus *Hoffmania*.
2) So far, 33 subgenera and 38 unplaced groups were reported all over the world. However, *Culicoides* species in China (totally about 350 species) were placed to 12 subgenera and some unplaced groups in China according to the taxonomy system of Yu YX (2005). Here, we advise to replace some species in Yunnan Province from subgenera *Avaritia* and *Culicoides* to *Hoffmania* (a new subgenus for Chinese system), according to the phylogenetic analysis.
3) Also, in this study, three species *C. bubalus*, *C. insignipennis*, and *C. spiculae* were suspected to be the same species with *C. gaponus* (Hainan, China), *C. elongatus* (Yunnan, China) and *C. yunanensis* (Yunnan, China) respectively.

Best regards

Duan YL

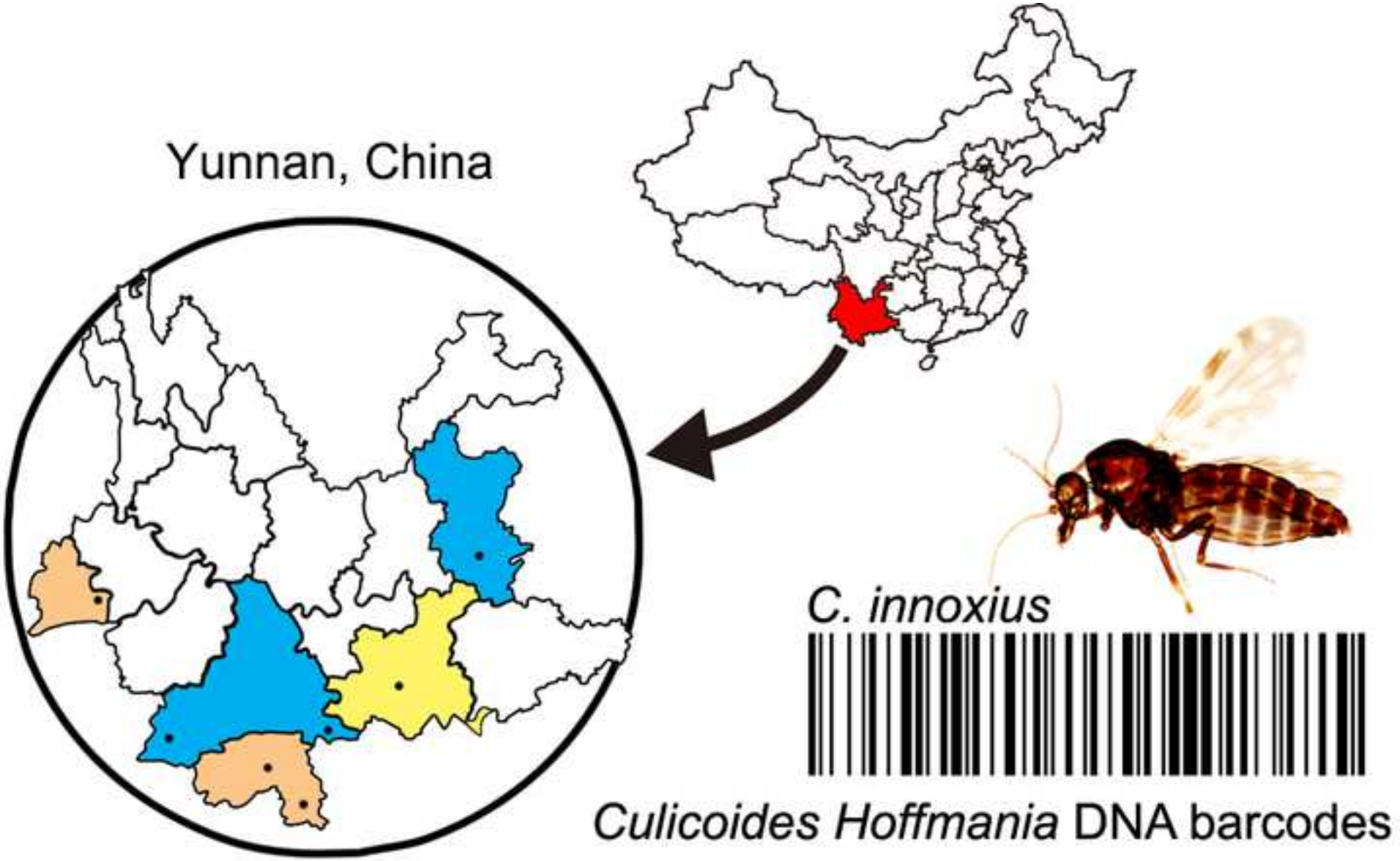
High lights:

1) We made 9 *cox1* barcodes (BIN) for three *Culicoides* species belonging to subgenus *Hoffmania* in Yunnan, China.

2) *Culicoides* species in China (totally about 350 species) were placed to 12 subgenera and some unplaced groups in China according to the taxonomy system of Yu YX (2005). Here, we advise to replace some species in Yunnan Province from subgenera *Avaritia* and *Culicoides* to *Hoffmania* (a new subgenus for Chinese system), according to the phylogenetic analysis.

3) Three species *C. bubalus*, *C. insignipennis*, and *C. spiculae* were suspected to be the same species with *C. gaponus* (Hainan, China), *C. elongatus* (Yunnan, China) and *C. yunanensis* (Yunnan, China) respectively.

Graphical Abstract



DNA barcoding and phylogenetic analysis of midges belonging to *Culicoides* (Diptera: Ceratopogonidae) subgenus *Hoffmania* in Yunnan, China

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Abstract DNA barcodes obtained from cytochrome c oxidase subunit 1 (*cox1*) offer a fast and easy way to identify a range of biological organisms. *Culicoides* (Diptera: Ceratopogonidae) are a group of small, blood sucking midges whose species are the vectors for some arboviruses, such as bluetongue virus, African horse sickness virus, epizootic hemorrhagic disease virus and equine encephalosis virus. Identification of these small insects is difficult so constructing DNA barcode libraries for species present in certain areas is helpful to clarify the taxonomy and assist non-specialist workers to identify species. In this study, we analysed specimens belonging to *C.* subgenus *Hoffmania* collected from 12 towns of Yunnan Province, China. Specimens were identified by morphology and processed to construct DNA barcodes. A total of 185 specimens referable to 6 morphological species were processed for *cox1* and *28S rRNA* sequencing. The resulting 185 *cox1* sequences were assigned to 13 barcode index numbers (BINs) which include 9 novel BINs. Molecular and morphological evidence was used to

support the transfer of 4 species previously assigned to *C.* subg. *Avaritia* into *C.* subg. *Hoffmania*. Molecular analysis revealed the presence of 7 potential cryptic species within *C. innoxius*, three within *C. liui* and two within *C. insignipennis*.

Key words *Culicoides*; *Hoffmania*; *C. innoxius*; *cox1*; *28S rRNA*; DNA barcode

[#]Ying Liang Duan and Glenn Bellis contributed equally in this study.

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Introduction

DNA analysis became a powerful tool for taxonomy following the development of molecular biology during the latter half of the 20th century. The term DNA barcode was first coined by Arnot *et al* in 1993 (Arnot et al., 1993; Fiser Pecnikar and Buzan, 2014) and the 5' end of the cytochrome c oxidase subunit 1 (*cox1*) of the mitochondrial DNA (mtDNA) has since become the most widely used molecular marker for animal taxonomy (Hebert et al., 2003). In addition to *cox1* barcodes, a range of other loci are used to explore the genetic diversity of eukaryotes. Amongst these are ribosomal RNA (rRNA) which ~~have~~ become a common locus for nucleic acid based investigation of microbial diversity since 1980s (Glockner et al., 2017).

The proliferation of DNA barcodes quickly created a need for storage of sequence data in

publically available databases. Four major databases are currently used for this purpose, namely the Barcode of Life Data (BOLD) system (Ratnasingham and Hebert, 2013; Sarkar and Trizna, 2011), the National Center for Biotechnology Information (NCBI) (Anonymous, 2018), the European Bioinformatics Institute (EMBL-EBI)(Cook et al., 2020), and the DNA Data Bank of Japan (DDJB) (Kodama et al., 2018). The volume of *cox1* data stored in these databases has increased exponentially during the past two decades. For example, the number of *cox1* records on NCBI increased from 8 137 in 2003 to approximately 2.5 million in 2017 which is an average increase of 51% per year (Porter and Hajibabaei, 2018). Sequence data sources are global with the top 6 countries contributing data to NCBI being Canada, USA, Costa Rica, Australia, China and Germany (Porter and Hajibabaei, 2018). The collation of data from disparate sources has, however, led to problems with data integrity, particularly the retention of vouchers for subsequent confirmation of identifications.

This problem is evident amongst datasets for *Culicoides* Latreille (Diptera: Ceratopogonidae) which are small blood-sucking midges, some species of which act as vectors for arboviruses of economic importance, such as bluetongue virus, African horse sickness virus, epizootic hemorrhagic disease virus and equine encephalosis virus (Desvars et al., 2015; Meiswinkel et al., 2004). There are currently 1347 described species of *Culicoides* placed into 33 subgenera and 38 species groups (Borkent and Dominiak, 2020) although DNA barcodes are only available for a small proportion of species and these are mostly from Europe and Africa. Producing barcodes from the fauna of other regions, including Asia, is however increasing particularly in countries such as China where the vector potential of local species is actively being investigated. Ensuring these records are verifiable by retaining specimens for examination or alternatively providing an independent means of confirming identifications, for example

photographs of wing patterns, is however, yet to be adopted as standard procedure.

The *Culicoides* fauna of China is well documented and includes approximately 348 species (Chang et al., 2017), most of which were placed by Yu et al ~~(Liu et al., 2005)~~ into 12 subgenera: *Avaritia* Fox, *Beltranmyia* Vargas, *Culicoides* Latreille, *Fastus* Liu, *Haemophoructus* Macfie, *Jilinocoides* Chu, *Monoculicoides* Khalaf, *Nullicella* Lee, *Oecacta* Poey, *Pontoculicoides* Remm, *Sinocoides* Chu, and *Trithecoides* Wirth & Hubert, although several other species were not able to be placed into a formal subgenus. However, the diagnoses provided by Yu et al (L~~iu et al., 2005)~~ for some of these subgenera differ~~ed~~ to that used by authors in most other parts of the world leading to confusion in the placement of several species. This confusion was compounded by the transfer of the type species of some subgenera to other subgenera which effectively synonymised these subgenera. Yu et al (~~Liu et al., 2005)~~ did not action these synonymies and the transfer of these species has subsequently been largely ignored (Borkent and Dominiak, 2020).

One of the subgenera entangled in this confusion is *C.* subg. *Hoffmania* Fox. Wirth & Hubert (~~Wirth and Hubert, 1989)~~ placed a number of species from southeast Asia into *C.* subg. *Hoffmania* but Yu et al (~~Liu et al., 2005)~~ did not recognize the presence of C. subg *Hoffmania* in China and placed all of those species which occur in China into other subgenera such as *C.* subg. *Avaritia* and *C.* subg. *Culicoides.* ~~Dyce et al~~ (Dyce et al., 2007), who followed Wirth & Hubert's diagnosis for this subgenus, subsequently transferred all of the species present in Australasia back into *C.* subg. *Hoffmania* resulting in a confused situation where some species present in China are placed following the system of Wirth & Hubert (Wirth and Hubert, 1989) and others are placed following the system of Yu et al (~~Liu et al., 2005).~~

The rich diversity of *Culicoides* in China is a reflection of its wide range of ecological zones. Yunnan province, situated in the southwestern corner of China, is one of the most ecologically diverse provinces ranging from tropical lowland rainforest which is contiguous with that found in southeast Asia, to temperate, high altitude regions. The biodiversity of the province is equally varied containing southeast Asian elements in the south and Palaearctic influences in the north (Huang et al., 2016; Liu et al., 2021; Liu et al., 2017). The *Culicoides* fauna of Yunnan has been relatively well studied in relation to vectors of arboviruses (Duan et al., 2019; Duan et al., 2021a; Duan et al., 2021b; Meng et al., 2020) and taxonomic surveys however few species have been subjected to molecular analysis.

The first taxonomic investigation of *Culicoides* in Yunnan was conducted between 1963 and 1967 producing a checklist of 25 species, including 5 new species (Chu and Liu, 1978). An additional 14 species were recorded by Lee (Lee, 1980), then 70 by Chen et al (Chen et al., 2014) then to 85 by Liu et al (Liu et al., 2016) and Meng et al (Meng et al., 2021). This diversity is significant and represents about one fifth of the total *Culicoides* species in China and indicates elements from southeast Asia, the Oriental and Palaeartic regions. Although barcodes are available for several of these species, all but 7 of these originate from specimens from other areas and while these barcodes may be relevant to Yunnan populations, they are yet to be tested.

This study reports the species referable to *Culicoides* subgenus *Hoffmania sensu* Wirth & Hubert (Wirth and Hubert, 1989) collected during a province-wide survey of cattle and goat farms in Yunnan between 2018 and 2021. Species identifications are supported by DNA barcodes and *28S rDNA* sequences.

Materials and methods

Culicoides collection

Midges were collected from livestock farms from 17 separate sites in 12 cities/counties in Yunnan Province, China between August 2018 and June, 2021 (**Fig. 1**, **Table 1**). UV light traps (LTS-M02; Wuhan Lucky Star Medical Treatment Technology Co., Wuhan, China) were set inside livestock farms, 5–10 meters from penned cattle, buffalo, chickens or goats. Two traps were set at each farm for a single night from 5 pm to 9 am next day. At most sites, midges were collected dry into mesh bags and transferred to 90% ethanol immediately after clearing the trap. Collections from Mengla and from Jinghong in November 2019 and July 2021 were however collected directly into 75% ethanol and transferred to fresh 75% ethanol after clearing the trap. At Elephant Valley in Jinghong, collections were made near elephants instead of livestock.

DNA extraction

Specimens were sorted in ethanol into morphospecies based primarily on wing patterns. Representative specimens of each morphospecies from each site were submitted for individual DNA extraction by a nondestructive method described by Duan et al (Duan et al., 2021a; Duan et al., 2021b). Briefly, each midge was placed in a clean 200 µl tube and digested in 50 µl tissue lysis buffer containing 0.2 mg/ml proteinase K from Genomic DNA Extraction kit #DP304 (TIANGEN, Tiangen, Beijing, China) at 30 °C for 16 h. DNA was extracted from a 30 µl aliquot of lysate using a MagMAX™M-96 Viral RNA Isolation kit (Ambion®, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's directions and a MagMAX™ Express-96 machine (Ambion®, Thermo Fisher Scientific). Extracted

DNA was eluted with 50 µl of elution buffer and stored at − 20 °C until use.

Morphological identification

The specimens subjected to DNA extraction were digested further in tissue lysis buffer containing 0.2 mg/ml proteinase K at 50 °C for 1 – 3 hours until completely cleared. Specimens were subsequently mounted following the methods described by Bellis et al (Bellis et al., 2013) and identified using keys and descriptions of Wirth & Hubert (Wirth and Hubert, 1989) and Yu et al (Liu et al., 2005) and comparison with reference material. Species were placed into subgenera following the system proposed by Wirth & Hubert (Wirth and Hubert, 1989).

Gene sequencing

All specimens processed were submitted for *cox1* amplification. Following placement of *cox1* sequences into BOLD Barcode Index Numbers (BINs) (Ratnasingham and Hebert, 2013) by BOLD, representatives of each BIN were submitted for *28S rDNA* amplification. DNA fragments of *cox1* and *28S rDNA* were amplified by polymerase chain reaction (PCR) using high-fidelity DNA polymerase. Briefly, primers BC1culicFm and JerR2m (Bellis et al., 2013) were used to amplify *cox1*; while the primer used by Bakhoum et al (Bakhoum et al., 2018) (28S_A335, 5-TCGGAAGGAA CCAGCTACTA) and a novel improved primer 28DF (5-GAGAGTCAAA AAGTACGTGA AAC) were used to amplify *28S rDNA*. 5 µl of DNA was added to 15 µl of reaction solution prepared using the PrimeSTAR® GXL kit (Takara) and one pair of the primers described above. PCR cycling conditions were: 95 °C, 2 min; then 95 °C/10 s, 45 °C (for *cox1*) or 50 °C (for *28S rDNA*)/10 s, 68 °C/50 s for 30 cycles; with a final

extension at 68 ℃ for 30 s, followed by incubation at 4 ℃. Subsequently, 10 μl of fresh 1× PCR solution was replenished to each tube and a second round of PCR was run as described above for 22 cycles. PCR products were sent to Kunming Shuoqing Biological Technology Company (China) for Sanger sequencing with an ABI3739XL machine (Applied Biosystems).

The fragments of *cox1* between the 3' ends of the forward and reverse primers and the fragments of *28S rDNA* between given conservative 5' sequence (5-TATCTGAATG AGGAAATTCA) and 3' sequence (5-CGATTGTCAG AGTTGGGTAT) were assembled using the bidirectional readings of sequencing. All sequences produced are saved in the BOLD dataset DS-HOFFYUNN.

Phylogenetic analysis

All the *Culicoides* sequenced by us and registered on BOLD or GenBank in this study were listed in **Table S1**. *Cox1* and *28S rRNA* ribosomal RNA (rRNA) sequences were queried for best matched specimens on NCBI & BOLD sequence databases and *cox1* and *28S rRNA* sequences relevant to our analysis were downloaded (**Table S2**, **Table S3**). *Cox1* sequences with lengths of 515 bp and *28S rRNA* sequences with the same conservative terminal sequences (5-AATTCA……AGTTGGGTAT-3) from conspecific specimens or species relevant to the analysis were aligned by Muscle (Edgar, 2004) using default parameters respectively after their extrusive terminal bases were truncated. For comprehensive analysis, a sequence comprising concatenated *cox1* segment (498 bp) and *28S rRNA* segment (about 610 bp) from the same *Culicoides* specimens were used. Phylogenetic trees were constructed by the Neighbor-Joining (NJ) method (model = Kimura 2, rates = T+T, bootstrap = 1000) or the Maximum Likelihood (ML) method (model = Kimura 2, rates = G+I, gaps treatment = use all sites, bootstrap =

1000) for *cox1* and *28S rRNA* respectively (**Table S4**). Bootstrap values less than 50% were omitted from the trees. These works were finished by MEGA-11 software (Hall, 2013).

Results

Detailed results of trapping will be published elsewhere but specimens referable to species belonging to *C.* subg. *Hoffmania sensu* Wirth & Hubert (Wirth and Hubert, 1989) were only collected from sites below about 1100 m altitude and not from higher altitude sites and were placed into 6 species by morphological analysis (**Table 2, Fig. 2**). Inconsistency between the keys and descriptions of Yu et al (Liu et al., 2005) and those of Wirth & Hubert (Wirth and Hubert, 1989) caused difficulties in the identification of 3 taxa. Specimens that keyed to *C. insignipennis* Macfie, *C. spiculae* Howarth and *C. bubalus* Wirth & Hubert in Wirth & Hubert's key keyed to *C. elongatus* Chu & Liu, *C. yunanensis* Chu & Liu and *C. gaponus* Yu respectively in Yu et al's key. Because of difficulties encountered using the key of Yu et al (Liu et al., 2005) and a lack of reference specimens of the latter three species, we have more confidence in the identification provided from the key of Wirth & Hubert (Wirth and Hubert, 1989) and so defer to the identifications offered by that key until such times as reference specimens of the Chinese species are available for comparison.

DNA amplification success rate

Of 194 specimens processed for *cox1,* 185 (95.4%) produced barcode-compliant sequences containing 500-646 bp while the success rate to get intact *cox1* sequences of 646 bp in length was lower at 89.2% (173/194). Of the 45 specimens processed for *28S rRNA* amplification, 36 (80.0%) successfully

produced sequences based on the complete match of known conservative sequences on both the 5’ end (5-TATCTGAATG AGGAAATTCA-3) and the 3’ end (5-CGATTGTCAG AGTTGGGTAT-3) in addition to clean chromatogram peaks.

DNA barcodes

Analysis of *cox1* sequences revealed 13 BINs from specimens referable to 6 species (**Table 2**). These included 7 BINs referable to *C. innoxius* Sen & Das Gupta, 2 BINs referable to *C. liui* Wirth & Hubert and a single BIN referable to each of *C. insignipennis*, *C. spiculae*, *C. sumatrae* Macfie, and *C. bubalus*.

Comparison of these *cox1* sequences with publically available data revealed that our data for *C. sumatrae, C. insignipennis* and *C. spiculae* and some specimens of *C. innoxius* were placed into the same BIN as specimens identified as these species respectively, thus confirming our identifications (**Fig. 3, Table 2**). Data from the remaining specimens, however were placed into novel BINs and included 6 BINs (AAZ4971, ABX8508, AEA9245, AEA9246, AEB1023, and AEB5981) for *C. innoxius*, 2 BINs (AEB2690 and AEB2691) for *C. liui*, and 1 BIN (BOLD: AEB4620) for *C. bubalus*(**Table 2**). Each of these differed from published BINs for their respective species by more than 0.03 (**Fig. 3**).

Phylogenetic analysis

The NJ tree of *cox1* sequences (**Fig. 3**) showed complete agreement with the BIN system of BOLD by placing all BINs into monophyletic clades. Our *C. innoxius* specimens were placed into two publically available clades (ACG0386 and ADV2561) and 5 novel clades but there was little support for the monophyly of this complex as the 5 novel BINs grouped more closely to *C. sumatrae* and *C. bubalus*

than to the two publicly available BINs for *C. innoxius*. Similarly, the phylogenetic tree of *28S rRNA* (**Fig. S1**) indicated support for a close relationship of 4 of these BINs but these BINs showed a closer relationship to *C. liui*, *C. insignipennis*, *C. bubalus* and *C sumatrae* than to the remaining clade of *C. innoxius* (BIN ADV2561). The *cox1* genetic distance between these clades of *C. innoxius* ranged from 0.030-0.114 (**Table S5**). Each of these BINs was supported by *28S rRNA* analysis with genetic distances ranging from 0.004-0.042 (**Table S6**).

There were two clades of *C. liui* which both had strong support from both *cox1* and *28S rRNA* with genetic distances of 0.099 and 0.023 respectively (**Fig. 3 and Fig. S1, Table S5 and Table S6**). The minimum genetic distance between these clades and the *cox1* sequence from a specimen from Thailand was 0.057. The specimens of *C. insignipennis* collected by us belonged to a different BIN with a *cox1* genetic distance of 0.074 to that from 4 conspecific specimens including 1 from Fujian, China and 3 specimens of unknown origin. However our specimens were placed into the same BIN (ACT1912) as specimens from Thailand (~~Bellis~~ et al in press) (**Fig. 3**, **Table 2 and Table S3**). The single public sequence available for *C. bubalus* was too short (406 bp) to allow meaningful comparison with our data. A ML tree constructed by *28S rRNA* was generally concordant with the topology of the *cox1* tree excepting for the placement by *cox1* of *C. sumatrae* within the large clade containing specimens from the *C. innoxius* complex and *C. bubalus* (**Fig. 3, Fig. S1)**.

The genetic distance within *cox1* BINs were always less than 0.002 while distances between BINs ranged from 0.030 to 0.286 (**Fig. 3, Table S5**). The relative *28S rRNA* genetic distances between specimens within a BIN were no more than 0.004 except for BIN AEA9246 with distances ~~no~~ more than 0.006, distances between BINs ranged from to 0.004 to 0.082 (**Fig. S1, Table S6**). The minimum

genetic distance of concatenated *cox1* and *28S rRNA* sequences between any of our specimens to species from subgenera other than *C.* subg. *Hoffmania* was 0.136 (**Fig. 4**).

Status of C. subg. Hoffmania in China

Several species collected during this study that are currently placed into *C.* subg. *Avaritia* by Yu et al (Liu et al., 2005) and more recently by Chang et al (Chang et al., 2017) were found to conform to the diagnosis for *C.* subg. *Hoffmania* proposed by Wirth & Hubert (Wirth and Hubert, 1989). Similarly, the phylogenetic tree of concatenated *cox1 + 28S rRNA* sequence data of our specimens and a range of specimens from other subgenera indicates that all of the species reported here, excepting *C. spiculae* and *C. insignipennis*, form a monophyletic clade which is separated from all species of *C.* subg. *Avaritia*, including the type species, *C. obsoletus* (Meigen), by species from several other subgeneric groups including *C.* subg. *Synhelea* Kieffer and *C.* subg. *Remmia* Glukhova (**Fig 5**). *C. spiculae* and *C. insignipennis* did not group with the other species of *C.* subg. *Hoffmania* but instead formed their own, poorly supported clade which is sister to *C. moreli* Clastrier and a large clade containing species from *C.* subg. *Avaritia*, *C.* subg. *Synhelea* and *C.* subg. *Remmia*.

Of the six species we have analysed here, *C. spiculae* was placed into *C.* subg. *Hoffmania* by Howarth (Howarth, 1985) and *C. sumatrae* was transferred into *C.* subg. *Hoffmania* by Dyce et al (Dyce et al., 2007) so we consequently propose to formally transfer the remaining four species (listed below) to *C.* subg. *Hoffmania*.

C. innoxius Sen & Das Gupta 1959

C. insignipennis Macfie 1937

C. liui Wirth & Hubert 1961

C. bubalus Wirth & Hubert 1989

Additionally, both Xiang et al (Xiang et al., 1989) and Liu et al (Liu et al., 2016) considered that *C. elongatus* and *C. insignipennis* were probably synonymous. Comparison of *cox1* sequence data of specimens from Mengla, the type locality of *C. elongatus*, and of *C. insignipennis* from Thailand supports their suggestion so we propose to formally synonymise these 2 species. NEW SYNONYMY.

Discussion

Of the six morphologically recognised species collected during this study, 4 were confirmed by DNA analysis; the remaining 2 species, *C. liui* and *C. bubalus*, formed unique clades which were genetically distant from existing conspecifics. One species, *C. bubalus*, appears to be a new record for Yunnan Province bringing the total number of species reported from the province to 86 although the sinking of *C. elongatus* under *C. insignipennis* reduces this total to 85.

The subgeneric system proposed by Yu et al (Liu et al., 2005) and followed by subsequent Chinese workers including Chen et al (Chen et al., 2014), does not record *C.* subg. *Hoffmania* from China. Borkent & Dominiak (Borkent and Dominiak, 2020) noted the state of confusion of many subgenera due largely to disagreement on morphological limits of these subgenera and this confusion has led to different interpretations of several of the subgenera represented in China with all six of the species collected in our study being bounced from one subgenus to another, depending on the system adopted by the most recent publication. Employing an independent means of testing these placements, such as the molecular analysis used here, may assist with clarifying relationships between species within the

genus (Mathieu et al., 2020). The six species examined by us conform morphologically to *C.* subg. *Hoffmania* sensu Wirth & Hubert and this, combined with the molecular analysis which does not support the placement of any of these six species into *C.* subg. *Avaritia*, leads us to propose the formal transfer of these species into *C.* subg. *Hoffmania*.

This study has raised some issues with the taxonomy of several species. Difficulties in distinguishing *C. insignipennis* from *C. elongatus* was also reported by Xiang et al (Xiang et al., 1989) and Liu et al (Liu et al., 2016) and populations from Mengla, the type locality of *C. elongatus*, share the same BIN as a specimen of *C. insignipennis* from Thailand. This result supports the suggestion from Xiang et al (Xiang et al., 1989) and Liu et al (Liu et al., 2016) that these species are synonymous and we propose to formally sink *C. elongatus* under *C. insignipennis*. We have been unable to locate the holotype specimen of *C. elongatus* for comparison with *C. insignipennis* but should this specimen become available for study, a comparison can confirm this proposal.

Meng et al (Meng et al., 2021) recently reported the presence of *C. spiculae* in Yunnan and our comparison with molecular data of *C. spiculae* from Thailand supports the presence of this species in Yunnan. Meng et al (Meng et al., 2021) asserted that this species is different to *C. yunanensis* but we were unable to confidently distinguish female *C. spiculae* from *C. yunanensis* using the characters they proposed. Meng et al (Meng et al., 2021) also suggested that differences exist between the male genitalia of these two species but did not provide details of the difference. Comparison of the illustrations of male *C. yunanensis* from Chu & Liu (Chu and Liu, 1978) and that of *C. spiculae* from Wirth & Hubert (Wirth and Hubert, 1989) suggests that the relative lengths of the basal arms and distal process of the aedeagus may differ between these two species but this requires further study to confirm

its significance. The aedeagus of our male specimens from Yunnan appear closer to the illustration of *C. spiculae* than to that of *C. yunanensis* but examination of more specimens is required to lend confidence to this character. Until these comparisons are complete we hesitate to synonymise these two species.

The status of *C. bubalus* and *C. gaponus* also requires clarification as we were unable to distinguish these two species using available information. We have been unable to locate the type specimen or even reference specimens of *C. gaponus* for comparison with specimens of *C. bubalus*. In the absence of type specimens, comparison of the DNA of specimens from the type locality of these species may help to clarify the status of these species however we have been unable to secure any material of *C. gaponus* from the type locality of Hainan Island for analysis or comparison with our specimen. Sequence data from our specimen does not match that of a specimen of *C. bubalus* from Thailand although the Thai sequence is short and this may explain the poor match. Until reference material of *C. gaponus* becomes available, the status of these 2 species remains unresolved.

The status of the various haplotypes of *C. innoxius* and *C. liui* also requires clarification. The differences in both *cox1* and *28S rRNA* data for several of these haplotypes supports their potential as cryptic species but further research is required to detect the consistent morphological differences required to describe these as new species (Gopurenko et al., 2015). An additional problem with these cryptic species is confidently assigning one of them to the nominate species and the process of examining type specimens or sampling DNA from the type locality should assist with this process as well. The existence of a further BIN from Thailand suggests that there may be more cryptic species within *C. liui.*

The distribution of *C.* subg. *Hoffmania* in Yunnan appears to be largely subtropical at altitudes

316 below 1100m. Yu et al (Liu et al., 2005) however, reported some of the species reported here from
317 provinces such as Tibet which indicates that these species can survive in temperate environments.
318 Several species belonging to *C.* subg. *Hoffmania sensu* Wirth & Hubert (Wirth and Hubert, 1989) which
319 were previously reported from Yunnan by Yu et al (Liu et al., 2005), Chen et al (Chen et al., 2014), Liu
320 et al (Liu et al., 2019) and Di et al (Di et al., 2021) were not collected during this study for example *C.*
321 *atrinervosa, C. indianus, C. lungchiensis, C. nipponensis, C. peregrinus* and *C. recurvus*. Confusion
322 over the status of some species, as reported here for *C. elongatus, C. yunanensis C. bubalus, C. gaponus,*
323 *C. insignipennis and C. spiculae* may explain some of this discrepancy but it is also likely that
324 collecting in different habitats and in different seasons may reveal the presence of some of the species
325 not reported herein. Additionally, several of the haplotypes reported in this study were represented by
326 single specimens indicating the need for more surveys to more comprehensively sample the fauna.
327 The assignment of *cox1* sequences into BINs in this study was strongly supported by the
328 phylogenetic trees of both *cox1* and *28S rRNA* (**Fig. 3, Fig. S1 and Fig. 4**). However, the 7 cryptic
329 species (AAZ4971, ABX8508, ADV2561, AEB5981, AEA9245, AEB1023, and AEA9246) from the *C.*
330 *innoxius* complex were paraphyletic in *cox1* (**Fig. 3**), *28S rRNA* (**Fig S1**) and concatenated *cox1* and *28S*
331 *rRNA* analyses (**Fig. 4**) with *C. bubalus* consistently placed within a clade containing haplotypes of *C.*
332 *innoxius*. The bootstrap value at the nodes between this *C. innoxius/C. bubalus* complex and *C.*
333 *sumatrae* on the concatenated tree was less than 50% (**Fig. 4**) suggesting that the evolutionary
334 relationships between the *C. innoxius* complex and *C. sumatrae* are uncertain. Wirth & Hubert (Wirth
335 and Hubert, 1989) noted a close morphological relationship between *C. innoxius* and *C. sumatrae* so a
336 close genetic relationship is probably not surprising. At present there has been no detailed

337 morphological or cladistic analysis of any these species and such analyses may help clarify the
338 evolutionary relationships therein.

339 Gopurenko et al (Gopurenko et al., 2015) observed that clades with an interclade *cox1* distance less
340 than 0.03 were likely to be conspecific while those with a genetic distance greater than 0.06 were likely
341 to represent cryptic species, and recommended these warranted further exploration using nuclear genes
342 and morphological analyses. Four of the 7 clades of *C. innoxius*, and the 2 Yunnan clades of *C. liui* and
343 2 Chinese *C. insignipennis* all exhibited interclade *cox1* distances of 0.06 or greater and all were
344 supported by *28S rRNA* data, excepting the latter 3 species as no *28S rRNA* data is available for the
345 publicly available BINs of these species. Unfortunately we do not have sufficient numbers of specimens
346 of most of these clades to perform meaningful morphological analysis to support the status of these
347 potential cryptic species. Three of the 7 BINs referable to *C. innoxius* and one of our BINs of *C. liui* had
348 *cox1* genetic distances between 0.03 and 0.06 from conspecific BINs so the evidence supporting their
349 status is not as strong however these populations could still represent distinct species should
350 morphological differences be found to support this proposal.

351 The inter- and intraspecific distances for *cox1* were significantly greater than those for *28S rRNA*
352 suggesting that *28S rRNA* is more highly conserved than *cox1*. Similar differences were also observed
353 between *cox1* and *28S rRNA* data for African species (Bakhoum et al., 2018) and between *cox1* and
354 CAD nuclear sequence data for Australasian and Oriental species (Gopurenko et al., 2015). These
355 results agree with previous suggestions that *rRNA* contains many conserved regions (Doris et al., 2015;
356 Gillespie et al., 2006; Michot et al., 1984; Ward et al., 1990). The functions of *28S rRNA* were based o
357 proper loop-stem structures which were formed by brush-fire base complementarity by itself. Hence,

28S rRNA has many highly conserved nucleotide elements (CNEs) to keep the shapes of the functional secondary structures (Doris et al., 2015; Gillespie et al., 2006). Due to this difference, *28S rRNA* was found to be useful in classifying *Culicoides* to species level but offered less resolution than *cox1*. The recovery rate of *28S rRNA* was also lower than that of *cox1*, possibly due to the presence of multiple copies of the gene arising from maternal and paternal sources. This lower recovery rate diminishes the usefulness of *28S rRNA* in species identification. The conservative nature of *28S rRNA* sequences, however, did appear to be more closely aligned with morphological placement of species into subgenera than was *cox1*.

Fig. 1 Collection sites of *Culicoides* in Yunnan Province, China. Filled circles indicate sites where species belonging to *C. subg. Hoffmania* were collected. FM Fumin, GM Gengma, JC Jiangcheng, JH Jinghong, LF Lufeng, ML Menglian, MLa Mengla, MS Mangshi, SZ Shizong, YL Yiliang, YY Yuanyang, ZY Zhanyi.

Fig. 2 Wing photographs of representative female specimens of BINs referable to *C. subg. Hoffmania* collected in Yunnan Province, China.

Fig. 3 Neighbor-Joining (NJ) tree of 185 *cox1* sequences from species of *C. subg. Hoffmania* collected from Yunnan province, China and a further 25 sequences mined from BOLD and GenBank. The species names, vouchers, and BIN numbers are provided. New BINs are highlighted by five-pointed star and blue color.

Fig. 4 Maximum Likelihood (ML) tree of concatenated *cox1 + 28S rRNA*. Phylogenetic tree was concatenated by ML algorithm using concatenated *cox1 + 28S rRNA* sequences obtained from *Culicoides* species belonging to six subgeneric groupings. Subgeneric affiliations are indicated by different colors. The tree was separated to two parts; dotted lines A and B were connected directly in the intact tree.

Fig. S1 ML tree of *28S rRNA* sequences from species of *C. subg. Hoffmania* collected from Yunnan province, China. Novel BINs are highlighted by five-pointed star and blue color.

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Disclosure

The authors declare no conflict of interest.

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516

Figure 1

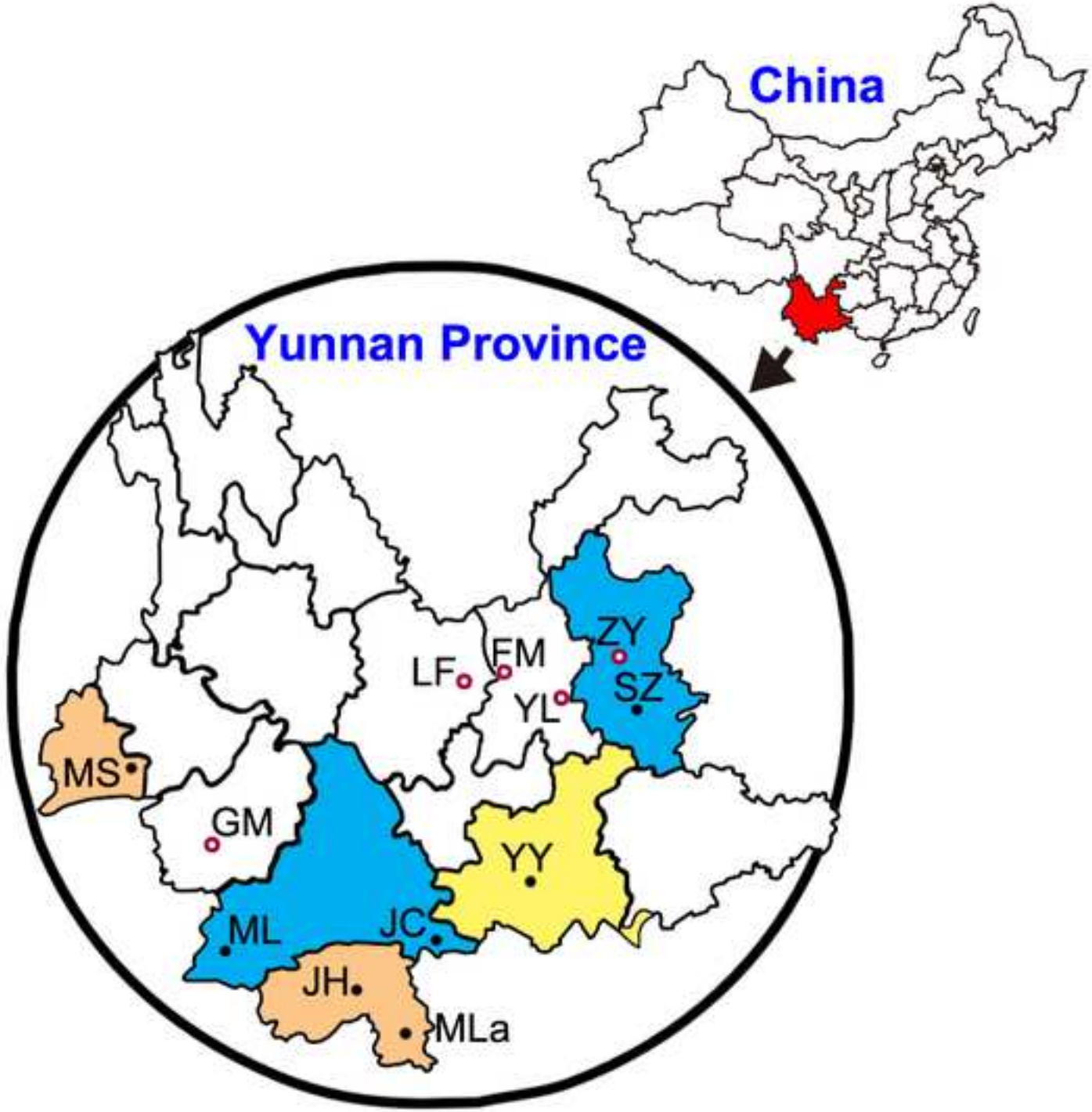

Figure 2

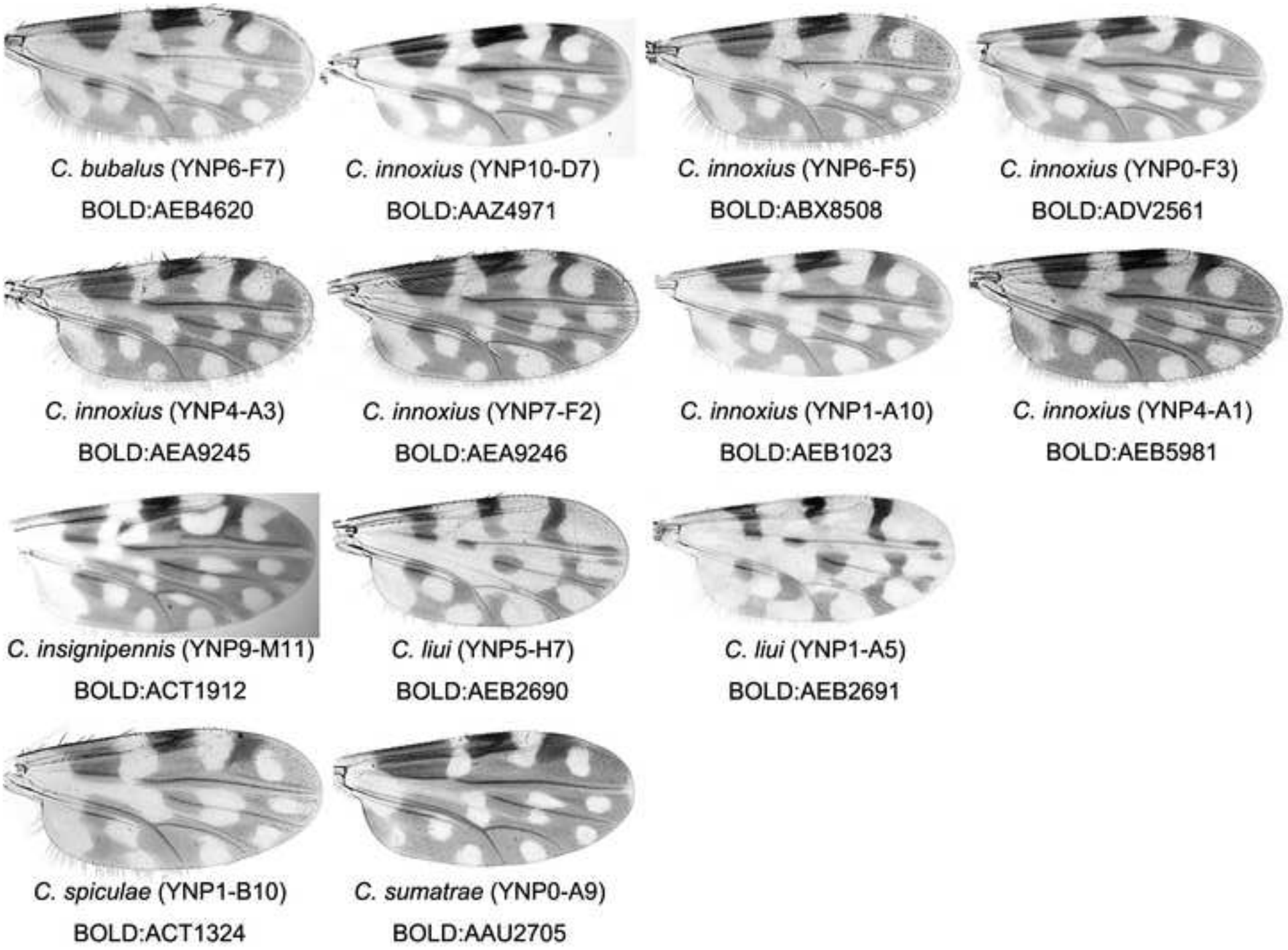

Figure 3

Click here to access/download;Figure;Fig 3=NJ of COI x210 (APE).eps

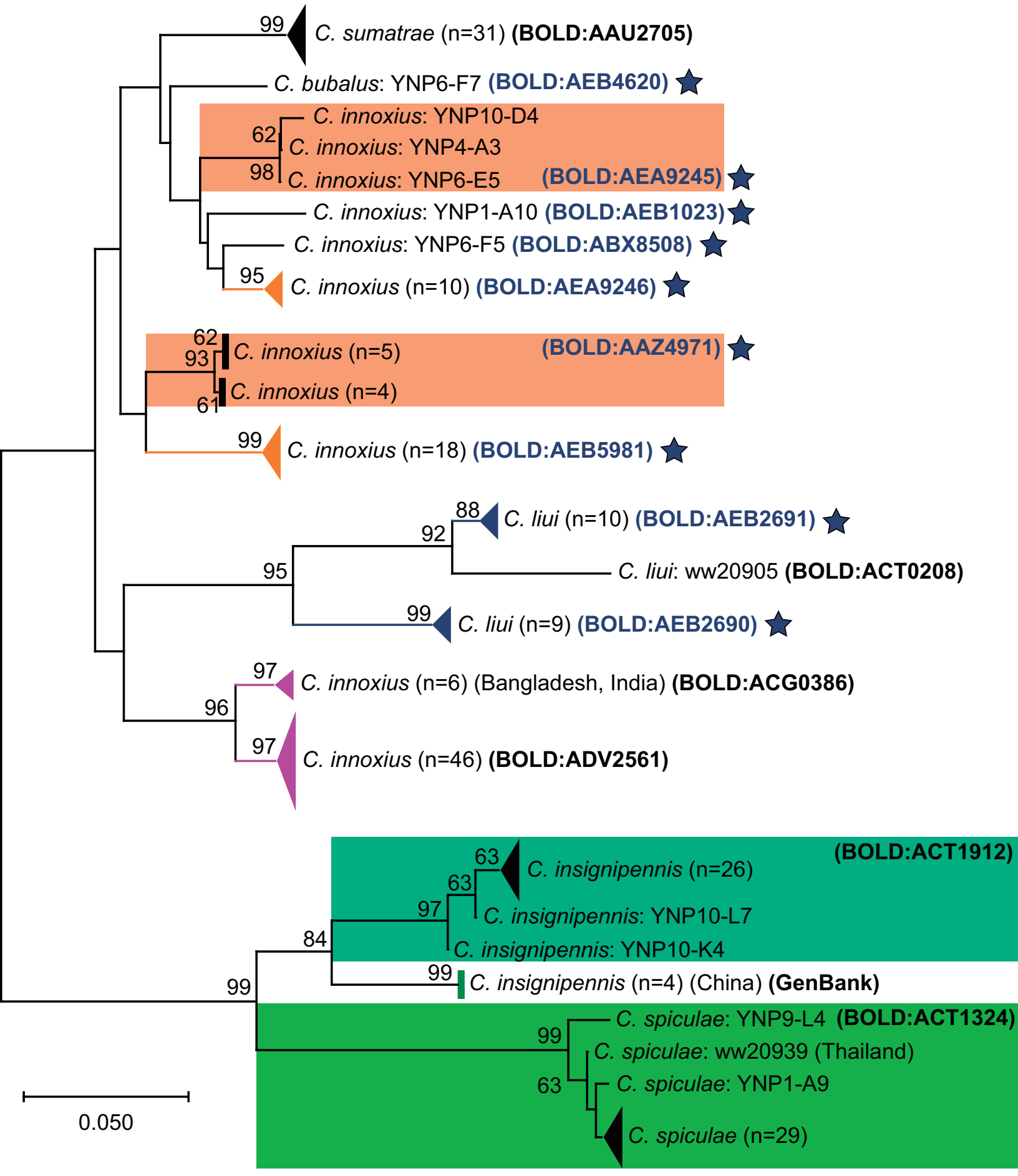

Figure 4

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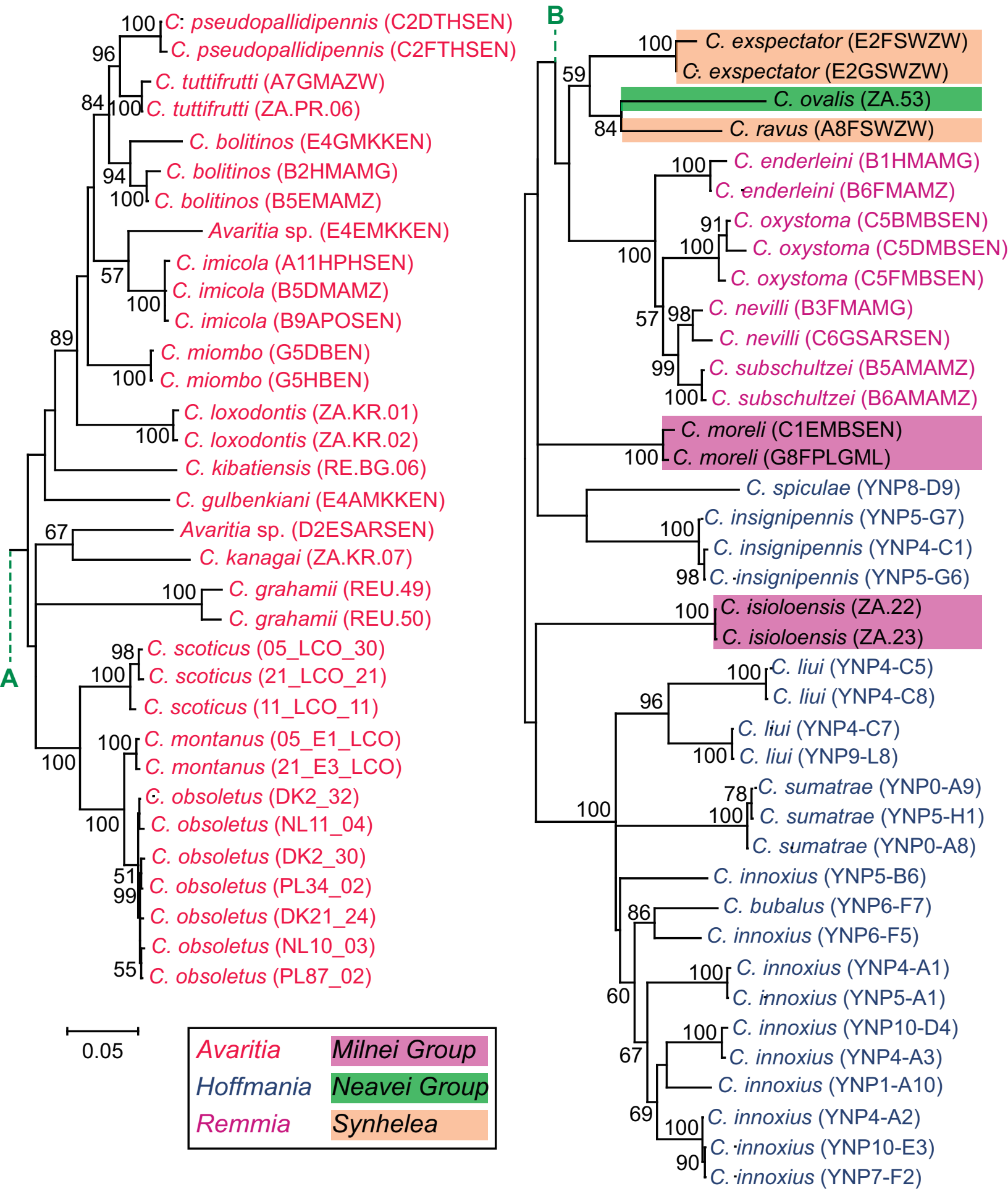

Figure S1

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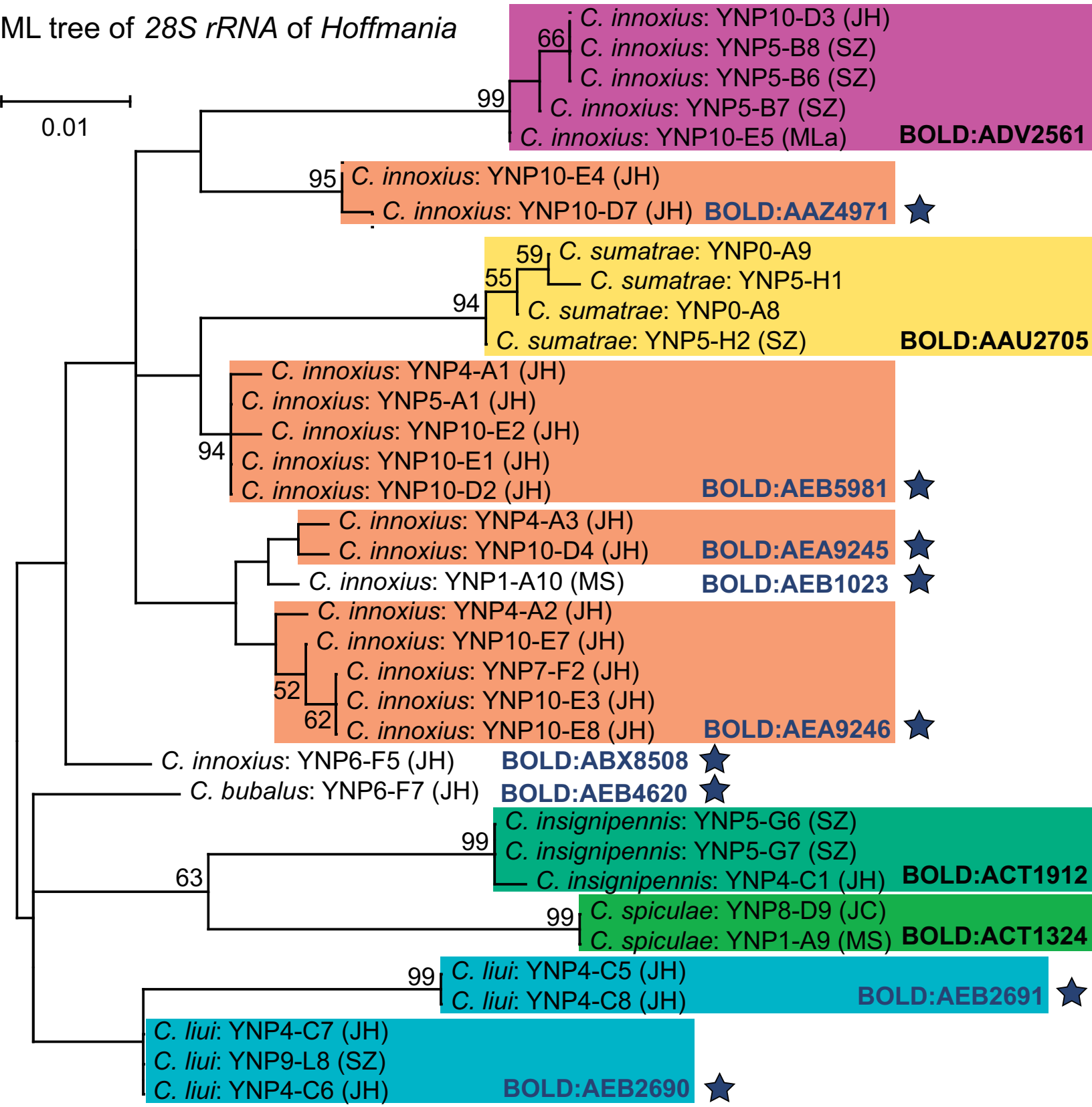

Table 1 Collection sites for *Culicoides* in Yunnan Province, China

City/County	Exact sites	Hosts	Collection date	Latitude (°N)	Longitude (°E)	Elevation (m a.s.l.)
Fumin*	NA	Cattle/goats	16 Aug 2019	25.20	102.26	1780
Gengma*	NA	Cattle	18 Jun 2019	23.54	99.41	1102
Jiangcheng	NA	Cattle	5 Jul 2019	22.60	101.85	1091
Jinghong	Elephant Valley	Elephants	14-15 Nov 2019	22.18	100.86	737
			16 Jun 2021			
	Forest site 1#	nil	15 Nov 2019	22.17	100.87	783
	Forest site 2#	nil	15 Nov 2019	22.17	100.88	766
	Gasa	Buffalo	9 Jul 2019 &	22.00	100.64	726
			15 Nov 2019			
	Mengyang	Buffalo/Chicken	14 Nov 2019	22.09	100.85	739
	Near Gasa	Chicken	15 Nov 2019	21.99	100.71	585
		Goats#	15 Nov 2019	NA	NA	NA
Lufeng*	NA	Cattle/goats	27 Aug 2019	25.80	102.30	1560
Mangshi	NA	Buffalo	7 Aug 2019	24.23	98.28	843
Mengla	NA	Cattle	17 Jun 2021	21.56	101.61	643
Menglian	NA	Cattle	1 Aug 2019	22.15	99.35	971
		Cattle/buffalo	3 Aug 2019			
Shizong	Wulong	Cattle/goats	22 May 2019	24.64	104.29	975
			1 Apr 2020			
			6 Sep 2020			
Yiliang*	NA	Goats	23 Aug 2019	24.92	103.14	1524
Yuanyang	NA	Cattle/goats	6 Aug 2018	23.24	102.79	183
Zhanyi*	NA	Cattle/goats	20-21 Aug 2019	25.41	103.45	2078

* No specimens belonging to *C.* subg. *Hoffmania* were collected at these sites.

Almost no *Culicoides* collected at these sites.

Table 2 Diversity of BINs and number of sequences obtained from species belonging to *C.* subg. *Hoffmania* collected from Yunnan Province, China

Species and BIN	Number of sequences		Geographic Distribution
	COI	28S	
1) *C. bubalus*			
BOLD:AEB4620	1	1	Jinghong
2) *C. innoxius*			
BOLD:AAZ4971	9	2	Jinghong
BOLD:ABX8508	1	1	Jinghong
BOLD:ADV2561	46	5	Jinghong, Mangshi, Mengla, Shizong, Yuanyang
BOLD:AEA9245	3	2	Jinghong
BOLD:AEA9246	10	5	Jiangcheng, Jinghong
BOLD:AEB1023	1	1	Mangshi
BOLD:AEB5981	18	5	Jinghong, Mengla
3) *C. insignipennis*			
BOLD:ACT1912	27	3	Jiangcheng, Jinghong, Mengla, Sizhong
4) *C. liui*			
BOLD:AEB2690	9	3	Jinghong, Mengla, Sizhong
BOLD:AEB2691	10	2	Jinghong, Mangshi
5) *C. spiculae*			
BOLD:ACT1324	24	2	Jiangcheng, Menglian, Mangshi
6) *C. sumatrae*			
BOLD:AAU2705	26	4	Jiangcheng, Jinghong, Sizhong, Yuanyang
Total	185	36	

Disclosure:

All the authors declare no conflict of interest.

Table S1

Table S2

Table S3

Table S4

Table S5

Table S6