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Taxonomy and phylogeny of rodents parasitic fleas in southeastern China with description of a new subspecies of *Ctenophthalmus breviprojiciens*

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Accurate differentiation and identification of flea species are essential for both basic and applied research on fleas, as well as for the diagnosis of flea-borne diseases. However, distinguishing between flea species can be challenging, especially among those with minimal morphological differences. Therefore, some scholars have suggested the necessity of comprehensive revisions to the classification of fleas, incorporating morphological, molecular, and phylogenetic data. In this study, we focused on classifying the rodents' parasitic fleas in southeastern China and provided molecular and phylogenetic data. We also described a new subspecies *Ctenophthalmus breviprojiciens fujiansis* n. ssp. A total of 392 fleas were collected from 8 species of rodents in 10 counties. Morphologically, they belonged to 10 species, 9 genera and 5 families. Barcode identification based on *COI* gene and phylogenetic analysis based on five genetic markers (*18S rDNA*, *28S rDNA*, *EF-1a*, *COI*, *COII*) revealed that the molecular and morphological identification of *Xenopsylla cheopis*, *Aviostivalius klossi bispiniformis*, *Leptopsylla segnis*, *Monopsyllus anisus* and *Ctenocephalides felis felis* were consistent. The taxonomic status of *Neopsylla specialis minpiensis* and *Peromyscopsylla himalaica sinica* as subspecies is questionable due to significant intraspecific genetic distance, and further morphological and molecular data are required to determine if they should be elevated to species level. The molecular identification of *C. breviprojiciens* n. ssp., *N. dispar fukienensis*, and *Nosopsyllus nicanus* could not be completed at this time due to a lack of sequences for related species in existing GenBank databases. Additionally, phylogenetic relationships of 31 species from 9 genera and 5 families of Siphonaptera were inferred based on five molecular markers (*18S rDNA*, *28S rDNA*, *EF-1a*, *COI* and *COII*) using Maximum Likelihood analyses. The analyses revealed that various taxa of Siphonaptera are monophyletic at the subspecies, species, and genus levels. However, at the family level, Leptopsyllidae, Ceratophyllidae, Pulicidae, and Pygiopsyllidae are all monophyletic, while Ctenophthalmidae is paraphyletic. we support the view of some authors that revising the catchall group Ctenophthalmidae and elevating each of its constituent subfamilies to family status.

Keywords Taxonomy, Phylogenetic analyses, Fleas, New subspecies, China

Siphonaptera (Insecta: Mecoptera) is a relatively small order of blood-feeding insects ectoparasitic on mammals and birds, the majority of their species are adapted to rodents[1]. Fleas are of tremendous medical and economic importance as vectors of several diseases important to human health including bubonic plague, murine typhus, and tularaemia[1–4]. Worldwide, more than 2500 flea species belonging to 16 families and 238 genera have been described[3,5–7]. In China alone, as of 2007, 649 species and subspecies of fleas belonging to 10 families and 74 genera had been recorded[3]. Since then, an additional 12 species and 2 subspecies have been reported[8–17].

In the 20th century, Phylogenetic studies of fleas were primarily based on comparative morphology, occurrence and association of hosts, ecology and zoogeography of fleas[3]. In 1948, Jordan first proposed a classification system of Siphonaptera, which included 2 Superfamilies, 18 families and 31 subfamilies[18]. Building

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upon this classification system, Holland[19]devised a general phylogenetic diagram of fleas of worldwide that encompassed three Superfamilies and 15 families. In 1982, Smit further divided the large Ceratophylloidea into Vermipsylloidea and Hystrichopsylloidea[20]. Wang et al.[21] elevated the subfamily Liuopsyllinae[22] to the family Liuopsyllidae, and suggested a system consisting of five Superfamilies and 16 families. This system is currently accepted by most scholars[2,3,23,24]; however, there are still some who hold differing views. Medvedev[25,26] recognized 18 families and placed them under 4 infraorders, and this classification system was cited by Whiting[27]. Therefore, the classification system of fleas remains incomplete and requires further exploration.

Accurate differentiation and identification of flea species are crucial for diagnosing diseases and conducting fundamental and applied research on these important ectoparasites[6,28]. The morphological identification of fleas is primarily based on the shape and structure of their complex genitalia and the distribution of setae, spines and ctenidia, ect[29–31]. However, it can be challenging to distinguish among species with small morphological differences, especially among females, based solely on the minute variations in spermatheca and sternum VII. This difficulty arises from the fact that fleas exhibit a high degree of morphological specialization associated with ectoparasitism, resulting in a lack of sufficient morphological characteristics for taxonomic research[27,32–34]. Therefore, classifying fleas based solely on morphology often leads to controversy in academic circles[3,18,20,27,28,31,33]. Some scholars have suggested the need for comprehensive revisions of flea classification, integrating morphological, molecular, and phylogenetic data[27,31,33,34]. There are various types of molecular marker genes widely used in insect phylogenetic studies. These include cytochrome coxidase subunit I (*COI*), cytochrome coxidase subunit II (*COII*), 12S ribosomal DNA (*12S rDNA*) and 16S ribosomal DNA (*16S rDNA*) from mitochondrial DNA (*mtDNA*), as well as 18S ribosomal DNA (*18S rDNA*), 28S ribosomal DNA (*28S rDNA*) and internal transcribed spacer (*ITS*) from ribosomal DNA (*rDNA*), and elongation factor 1-alpha (*EF-1a*) from protein coding gene[35]. It has been found that phylogenetic analysis based on a single gene is insufficient to resolve the phylogeny of Siphonaptera due to the lack of adequate information. However, when using multiple genes for phylogenetic analysis, more accurate and reliable results can be obtained[36,37]. In 2002 and 2008, Whiting et al.[27,36] established polygenic phylogenetic trees based on *18S rDNA*, *28S rDNA*, *EF-1a*, and *COII* of fleas. They analyzed the phylogenetic relationships among tribe level and above units of Siphonaptera. Based on Whiting et al.'s study, Zhu et al.[1] expanded the flea species and added five marker genes (*12S rDNA*, *16S rDNA*, *COI*, cytochrome b (*CytB*) and histone H3 (*H3*)) to reconstruct an updated phylogenetic tree, estimate divergence times of Siphonaptera for the first time, and reconstruct ancestral host affiliation and biogeographic history. Zurita et al.[31] analyzed the phylogeny of related fleas based on nuclear markers (*18S rDNA*, *ITS1*, *ITS2*) and two mitochondrial markers (*COI* and *cytb*). The results did not support the classification of *Ctenophthalmus baeticus boisseauorum and C. apertus allani*as two distinct species. In China, Shu et al.[7] also provided molecular and phylogenetic results based on four genetic markers (*18S rDNA*, *28S rDNA*, *COI*, and *COII*) for 19 species of fleas on wild mammals in northern region of Xinjiang.

Between 2019 and 2023, we collected flea specimens from the body surface of rodents in southeastern China. The specimens were identified by morphology and DNA barcoding based on the *COI* gene. Molecular data of *18S rDNA*, *28S rDNA*, *EF-1a*, *COI*, and *COII* of some specimens were provided. In addition, phylogenetic relationships of fleas at the family, genus, and species levels were analyzed to further improve the classification of fleas in southeastern China and provide a reference for future taxonomic studies of fleas.

In this study, we collected a new subspecies of *Ctenophthalmus*, named *C. breviprojiciens fujiansis* n. ssp., and then we provided a detailed description of its morphological features, with complete photographs and type drawings.

Materials and methods

Ethical aspects

All applicable international, national, and institutional guidelines for the care and use of animals were followed. Ectoparasitic fleas of rodents were collected with permission of the Ethics Committee of Fujian Center for Disease Control and Prevention (No: FJCDCNT1811-2015). All methods were performed in accordance with the relevant guidelines and regulations. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Sample collection

The rodents were captured, the fleas were collected between 2019 and 2023 in ten Counties in southeastern China. Rodents were captured in live-capture traps baited with corn. Live traps were placed every night at each surveillance point for three consecutive nights at locations where rodent activities were detected, and retrieved the following morning.

The traps containing a live rodent were packed into cloth bags respectively and brought back to the lab, where the rodents were euthanized with carbon dioxide and fleas were collected from the body surface of the rodents and from the cloth bags. The collected fleas were preserved in Eppendorf tubes with 75% ethanol for subsequent identification and DNA extraction. All the rodents were identified according to morphological characteristics, including body shape, tail structure, coat color, and other relevant traits by experienced zoologists who have long been engaged in the prevention and control of mouse-borne diseases and the classification of rodents. Some rodent species were further molecularly identified by *COI*[38].

Morphological identification

Most of the flea samples were identified based on the morphological characteristics (the shape and structure of their complex genitalia, distribution of setae, spines and ctenidia, etc.) utilizing a stereomicroscope (Olympus SZX9, Japan). Some samples underwent conventional procedures[3] for clearing and mounting on glass slides using Canadian balsam, followed by observation using a biological microscope (Leica DM3000, Switzerland)

and identified according to Wu et al.[3]. Additionally, the patterns of the new subspecies were photographed and depicted using a Leica microsystems (Leica DMC5400 BZ01, Switzerland). We used a Leica microsystems (Leica DMC5400 BZ01, Switzerland) to photograph and draw patterns of the new subspecies.

Molecular identification

After morphological identification, two samples of each flea species were selected for DNA extraction and amplification. Genomic DNA of a single flea was extracted using the DNeasy Blood and Tissue Kit of Qiagen following the manufacturer's instructions (no grinding, complete specimens were digested at 56 °C for approximately one hour). The extracted DNA was either immediately utilized or stored at -20 °C until molecular analysis. Voucher exoskeletons of DNA-extracted fleas were preserved in 75% ethanol solution, or curated as slide-mounted specimens.

The molecular markers (*18S rDNA*, *28S rDNA*, *EF-1a*, *COI*, *COII*) were subjected to polymerase chain reaction (PCR) amplification using a thermal cycler (Applied biosystems ProFlex Base, Singapore). The complete list of PCR conditions and primers can be accessed in Supplementary Table S1. These primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). Each reaction mixture contained 25 µL with 12.5 µl Premix Taq (Takara Taq Version 2.0 plus dye), 0.5 µl each of forward and reverse primers at a concentration of 10 µM, 3 µl of DNA template, and 8.5 µl double-distilled water. Subsequently, The PCR products (3 µl) were separated by electrophoresis on a 1.5% agarose gel, followed by visualization using the Analytikjena Gel Imaging System (UVP GelSolo, USA). Positive products were subsequently sequenced bidirectionally by Sangon Biotech Co., Ltd (Shanghai, China).

The bidirectional sequences were assembled into contigs and manually edited using the SeqMan software. Subsequently, these sequences were subjected to BLAST comparison by NCBI (www.ncbi.nlm.nih.gov) to assess their reliability. In cases where contigs exhibited numerous ambiguities, the corresponding sequences were reamplified and resequenced. Molecular identification of fleas involved selecting reference species from GenBank with the highest similarity to reliable *COI*sequences through BLAST comparisons. Consistency between morphological and DNA barcoding identification outcomes was assessed based on a criterion requiring less than 2% intraspecific genetic distance[39].

The reliable sequences of all five molecular markers obtained in this study have been submitted to GenBank.

Phylogenetic analysis

The sequences of fleas and outgroups were obtained from GenBank (Supplementary Table S2). All sequences for each gene region, acquired in this study as well as downloaded, were aligned, manually edited, and trimmed at both ends separately using the software BioEdit[40]. Subsequently, the resulting aligned sequences were concatenated into a single multiple alignment following the order of *18S rDNA*, *28S rDNA*, *EF-1a*, *COII*, and *COI*.

Maximum likelihood (ML) phylogenetic analyses of each unpartitioned gene alignment were performed using MEGA 7.0[41]. The most appropriate likelihood model for the analysis was determined based on the Bayesian information criterion[42]. Initial trees for the heuristic search were automatically generated using the Maximum Parsimony method. The resulting tree was drawn to scale with branch lengths measured in substitutions per site. Nodal support was assessed for the combined dataset with 1000 bootstrap replicates.

Results

Morphological identification result

By morphological identification, a total of 392 fleas collected from 8 species of rodents in 10 Counties, belonged to 10 species, 9 genus, and 5 families. The information of fleas including number, host and sampling sites are shown in Table 1.

Taxonomy of the new subspecies (Figs. 1, 2 and 3)

Siphonaptera Latreille, 1825.

Ctenophthalmidae Rothschild, 1915.

Ctenophthalminae Rothschild, 1915.

Ctenophthalmus Kolenati 1856.

Ctenophthalmus (Sinoctenophthalmus) breviprojiciens Li & Huang, 1980.

Ctenophthalmus (Sinoctenophthalmus) breviprojiciens fujiansis Zhou & Liu, n. ssp.

Material examined

Holotype; 1 male, Zhongxin Town (27°55′ N, 118°32′ E; elevation 350 m; reed bushes beside farmland), Pucheng County, Fujian Province, China, 12-V-2020, on *Microtus fortis*, S. H. Zhou. Allotype; 1 female, Siqiao Town (27°9′ N, 119°13′ E; elevation 890 m; reed bushes beside farmland), Zhouning County, Fujian Province, China, 22-X-2019, on *Microtus fortis*, S. H. Zhou. Paratypes; 3 males, same data as holotype.

Deposition of types

The holotype, allotype, and 3 paratypes were deposited in Fujian Provincial Key Laboratory of Zoonosis Research, Fujian Center for Disease Control and Prevention, Fuzhou, Fujian, China.

Etymology

The subspecies name is derived from the name of the Fujian Province of China, where the type specimens were collected.

Family	Genus	Species	Host species	No. captured	Sampling sites
Pulicidae	*Xenopsylla*	*X. cheopis*	*Rattus losea*	♂1	Luoyuan
			Rattus norvegicus	♀54♂44	Changtai, Datian
			Rattus tanezumi	♀42♂35	Changtai, Datian, Yanping, Yongtai
	Ctenocephalides	*C. felis felis*	*Rattus norvegicus*	♂1	Changtai
			Rattus tanezumi	♀1♂1	Yanping
Pygiopsyllidae	*Aviostivalius*	*A. klossi bispiniformis*	*Niviventer fulvescens*	♀5♂6	Yong'an
			Rattus bowersi	♀a	Yong'an, Zhouning
			Rattus losea	♀1	Zhouning
Ctenophthalmidae	*Ctenophthalmus*	*C. breviprojiciens fujiansis* n.ssp.	*Microtus fortis**	♀1♂9	Zhouning, Pucheng
	Neopsylla	*N. dispar fukienensis*	*Apodemus agrarius*	♀2♂3	Zhouning
			Rattus bowersi	♀31♂66	Zhouning, Yong'an
			*Niviventer andersoni**	♀5♂12	Jianyang, Youxi
		N. specialis minpiensis	*Apodemus agrarius*	♀1♂1	Zhouning, Pucheng
Leptopsyllidae	*Peromyscopsylla*	*P. himalaica sinica*	*Apodemus agrarius,*	♀4♂1	Zhouning
			Niviventer andersoni	♀4♂1	Zhouning
			Rattus losea	♀1	Zhouning
	Leptopsylla	*L. segnis*	*Rattus norvegicus*	♀2♂4	Changtai, Datian, Yongtai
			Rattus tanezumi	♀21♂10	Datian, Yanping, Yongtai
Ceratophyllidae	*Monopsyllus*	*M. anisus*	*Niviventer fulvescens*	♀2♂2	Youxi
			Rattus norvegicus,	♂1	Yanping
			Rattus tanezumi	♂1	Yanping
	Nosopsyllus	*N. nicanus*	*Rattus losea*	♀4♂10	Luoyuan

Table 1. The information of fleas on rodent surfaces including number, host and sampling sites. The asterisk (*) indicate that these host species were further molecularly identified by *COI*.

Diagnosis

Two subspecies of *C. breviprojiciens*have been recorded[3]: *C. breviprojiciens breviprojiciens* Li & Huang, 1981 and *C. breviprojiciens yongjiaensis* Lu, Zhang & Li, 1999. We can easily distinguish *C. breviprojiciens fujiansis* n. ssp. from these two subspecies through the characteristics in Table 2.

Head

Frontal tubercles conspicuous in both sexes. Frontal (preantennal) region with two rows of large setae, first row consisting of five or six, second row consisting of three large setae (longer than those of first row); about 10 intercalary minute setae located between first row and genal ctenidium. Genal ctenidium with three sharp pointed spines; the first (lowermost) slightly shorter than the second (middle), the third (uppermost) longest, about 1.4 times as long as first, second spine slightly broad from other spines. Eyes significantly reduced. Occipital (postantennal) region with three rows of setae, arranged 2-3-5 in male and arranged 2-3-6 in female; third row with 4~5 intercalary minute setae in both sexes. Labial palps and stylets extending almost to the apex of fore coxae.

Thorax

Pronotum bearing a single row of six long setae with intercalary setae. Pronotal comb with total 18 spines in both sexes. Mesonotum with two rows of setae; main row of 5~6 large setae and intercalary setae; second row of seven setae in male, five setae in female; second rows preceded by many scattered minute setae; mesonotal collar with 3~4 pseudosetaes (two above the midpoint, 1~2 below) in male, and two pseudosetaes above the midpoint in female. Metanotum with two rows of setae; main row of five setae with intercalary setae, second row of six setae in male, seven setae in female; metanotal collar without setae, pseudosetae or spinelet. Pleural rod prominent. Mesepisternum, mesepimeron, metepisternum, metasternum, and metepimeron with 2, 5~6, 1~2, 1 and 5~6 setae, respectively.

Legs

Outer surface of fore coxa with 35~40 setae, dorsal and ventral margin of fore coxa both with 3~5 setae. Trochanters with two setae. Length of hind tarsomere II about equal to sum of tarsomere III and IV, its longest apical seta extending to 1/4~1/2 of tarsomere IV. Tarsomere V with 5-5-4 pairs of lateral plantar setae and one pair apical plantar setae in fore-, middle and hind legs, respectively; first pairs of lateral plantar setae displaced mesad, and lying between the members of the second pairs in all legs.

Unmodified abdominal segments

Terga I-VI with two rows of setae (these two rows preceded by one or two setae on some terga); main rows consisting of 4~6 setae with intercalary setae; secondary rows consisting of 4~7 setae; the lowermost seta of the

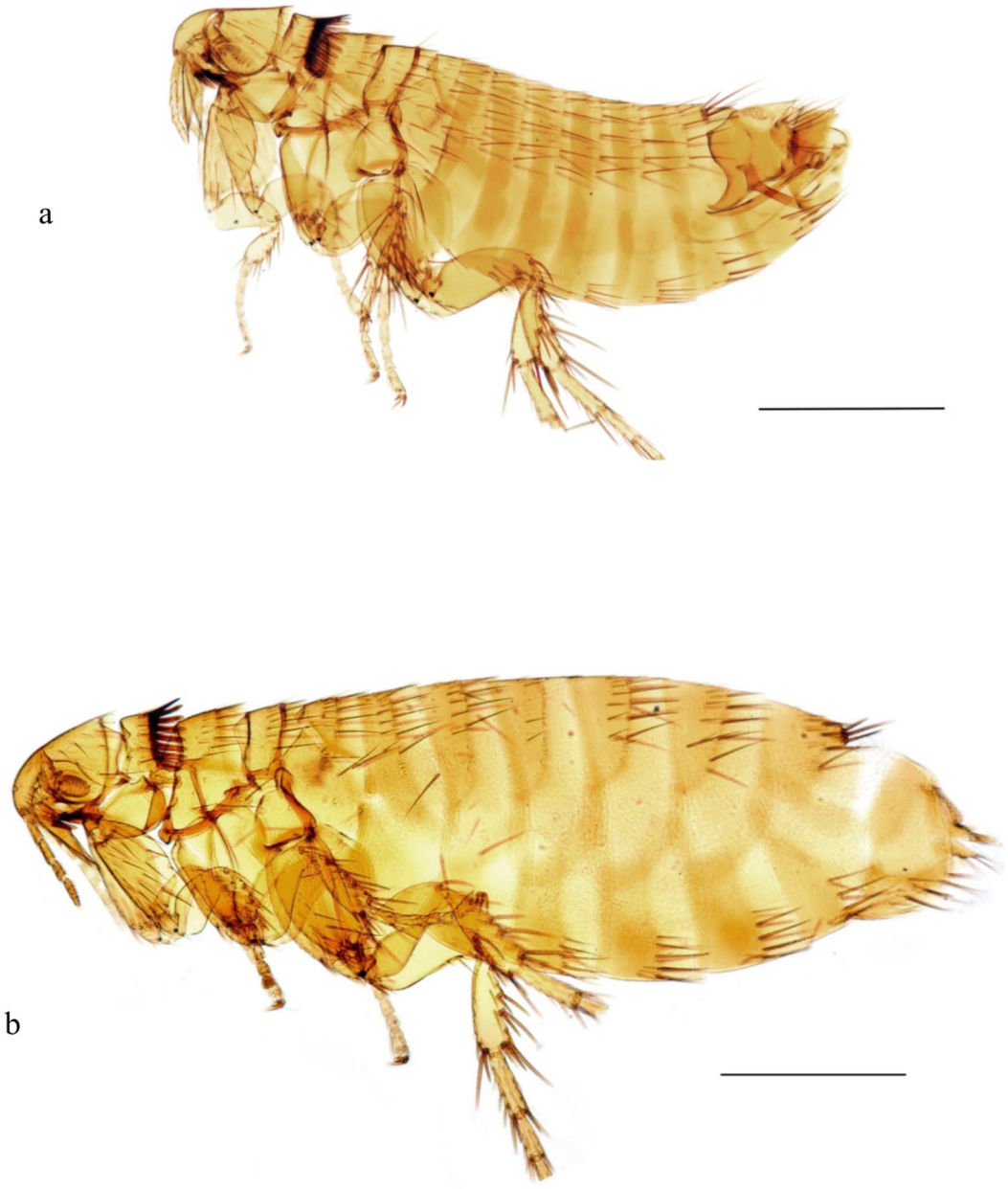

Fig. 1. *Ctenophthalmus breviprojiciens fujiansis* n. ssp. (**a**) Holotype (Male), (**b**) Allotype (female) (scale bar = 500 μm).

main row of terga II-VI located below the spiracular fossa; terga I-IV usually with one or two apical spinelets per side, arranged 1(or 2)-1(or 2)-1-1 in males and arranged 1-1-1-0 in female. Sternum II with single ventral seta. Sterna III-VI with two or three rows total of 4~5 setae in males and 6~9 setae in female.

Modified abdominal segments of male

Tergum VII with two rows of setae, main row with five setae and intercalary setae, the lowermost seta located below the spiracular fossa; second row with three (or four) setae. Three antesensilial setae with approximate ratio starting from the most dorsal: 0.35/1/0.55. Sternum VIII (Fig. 2-a) with two (or three) large and two mid-size stout setae; caudal margin rounded. Basimere (Fig. 2-b) divided by a very shallow sinus into two lobes; processus basimeris dorsalis forming rounded hump and bearing two long stout and four medium-size setae at apex; processus basimeris ventralis Short and forming circular triangular projection; basimere additionally bearing a long stout seta near apex of sinus; single acetabular bristle long. Telomere (Fig. 2-b) wide and large, nearly twice as long as width (at apex); apex and posterior margin almost straight; the antero-dorsal angle broadly rounded;

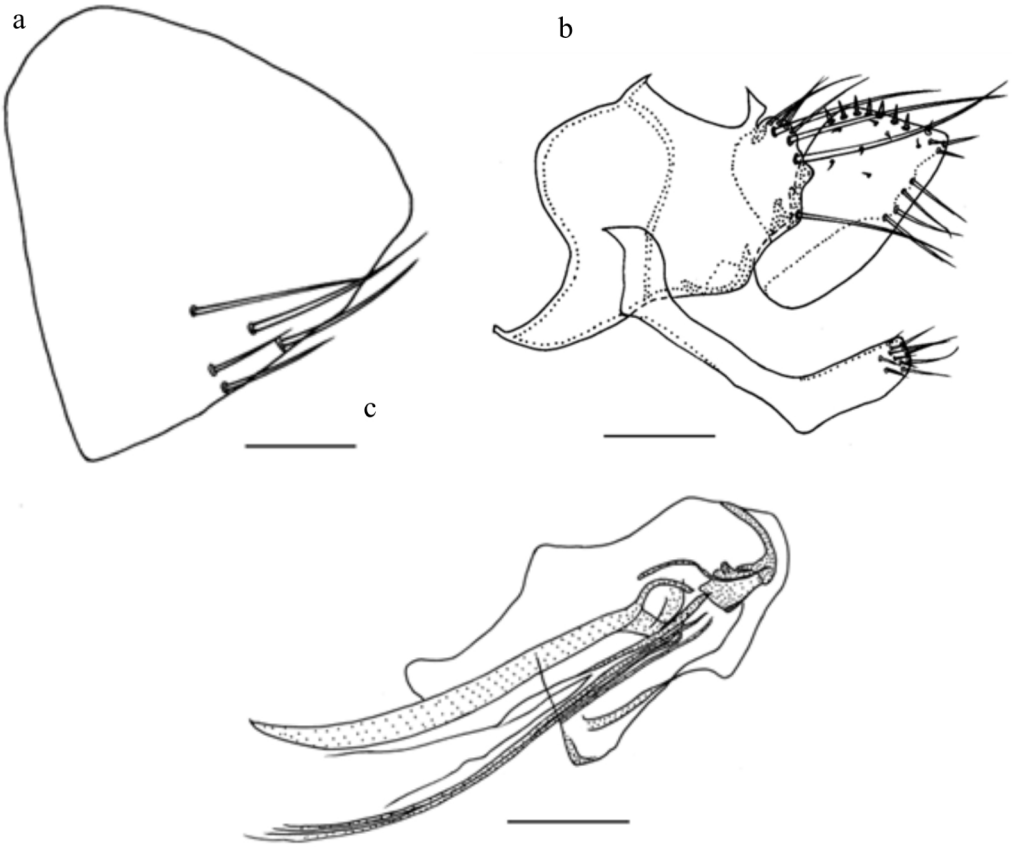

Fig. 2. *Ctenophthalmus breviprojiciens fujiansis* n. ssp. Holotype (Male). (**a**) Sternum VIII, (**b**) Segment IX, (**c**) Phallosome. (scale bar = 100 μm).

Identification point	*C. breviprojiciens breviprojiciens*	*C. breviprojiciens yongjiaensis*	*C. breviprojiciens fujiansisn.* ssp.
Telomere of male	Apex margin slightly concave, postero-dorsal angle slightly pointed	Apex margin almost straight, postero-dorsal angle blunt	Apex margin almost straight, postero-dorsal angle blunt
Distal arm of the sternum IX of male	Relatively short, about 1/2 the length of proximal arm, no obvious narrowing at the base	Relatively long, about 2/3 the length of proximal arm, narrowed at the base	Relatively short, about 1/2 the length of proximal arm, no obvious narrowing at the base
Lateral lobes of aedeagal	Blunt and unhooked at the projection of ventral margin	Hook-shaped at the projection of ventral margin	Blunt and unhooked at the projection of ventral margin
Sternum VII of female	Dorsal lobe about as large as middle lobe, and middle lobe slightly more projecting posteriorly than dorsal lobe	Middle lobe larger and posterior process than dorsal lobe	Dorsal and middle lobe about equal in size, with Caudal margin more rounded than those of the other two subspecies, and dorsal lobe slightly more projecting posteriorly than middle lobe
Tergum VIII of female	With hook-shaped sclerotization at base	With S-shaped sclerotization at base	No sclerotization at base

Table 2. The distinguishing characteristics of the three subspecies of *Ctenophthalmus breviprojiciens.*

apex bearing seven sensilla. Distal arm of the sternum IX (Fig. 2-b) bearing 13–14 fine setae at apex, and apex margin straight; proximal arm twice as long as distal arm. Phallosome as illustrated (Fig. 2-c), blunt at ventral margin projection of lateral lobes of aedeagal.

Modified abdominal segments of female

Tergum VII with three rows of setae, main row of four setae and intercalary setae; the main row preceded by two rows of four and one setae; the lowermost seta of main row located below the spiracular fossa. Three antesensilial setae with approximate ratio starting from the most dorsal: 0.35/1/0.60. Sternum VII (Fig. 3-a) with five setae; caudal margin of dorsal and median lobes rounded, with the dorsal lobe projecting slightly posteriorly than the middle lobe. Tergum VIII with rounded caudal margin, bearing 11 setae of varying sizes, and without sclerotization at base. Sternum VIII (Fig. 3-a) finely rod-like, with two very minute setae at apex. Ductus bursae

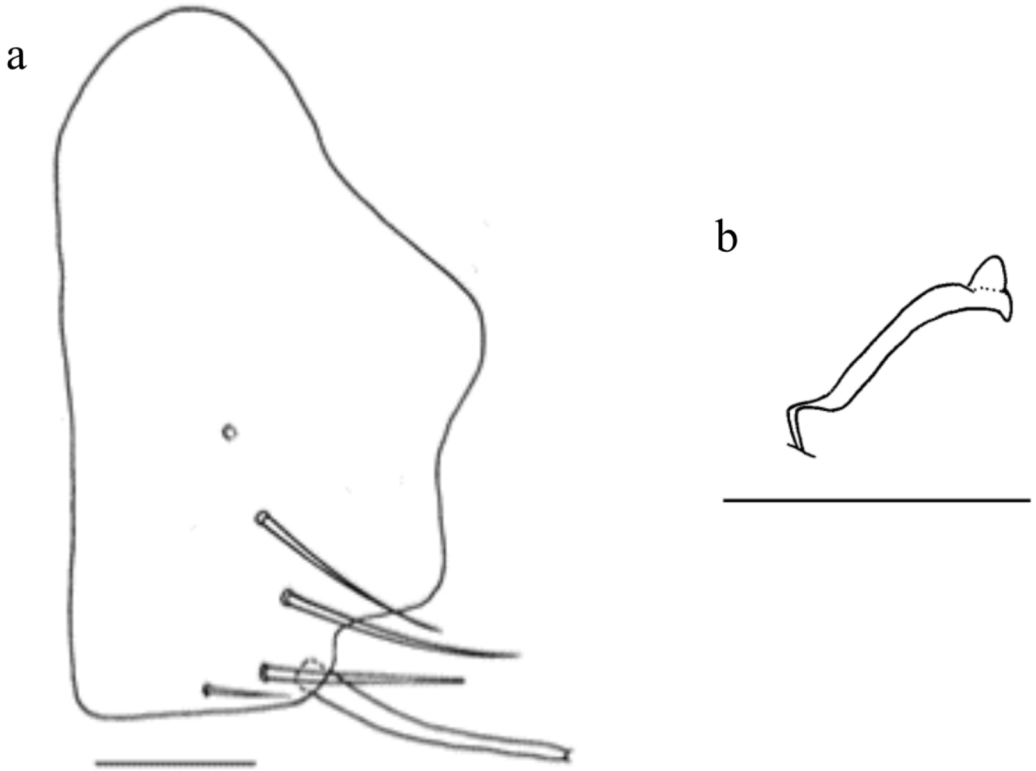

Fig. 3. *Ctenophthalmus breviprojiciens fujiansis* n. ssp. Allotype (female). (**a**) Sterna VII–VIII. (**b**) Bursa copulatrix. (scale bar = 100 μm).

(Fig. 3-b) (approximately 180 μm) slightly shorter than dorsal spine of pronotal ctenidium (approximately 200 μm); spermatheca absence.

Dimensions (slide-mounted insects)
Holotype: 1.9 mm; allotype: 2.4 mm.

Molecular identification

The sequences of 20 *18S rDNA*, 18 *28S rDNA*, 20 *EF-1a*, 11 *COI*, and 17 *COII* were successfully obtained and deposited in GenBank. The information and accession numbers of fleas are shown in Supplementary Table S3. Through BLAST comparison of five marker genes from 10 species of fleas in this study, it is evident that at the subspecies level, five marker genes of *Aviostivalius kossi bispiniformis*, *Neopsylla specialis minpiensis* and *Peromyscopsylla himalaica sinica* were preferentially submitted to GenBank; at the species level, five marker genes of *C. breviprojiciens*, *N. dispar and Nosopsyllus nicanus*; *28S rDNA* and *EF-1a* of *Monopsyllus anisus*, *18 S rDNA* and *EF-1a* of *N. specialis*, *EF-1a* of *Xenopsylla cheopis* and *Leptopsylla segnis*, *COI* genes of *P. himalaica* were also preferentially submitted.

Through BLAST comparison of the 17 *COI* sequences obtained, we identified the species from GenBank that showed the highest identity (minimum genetic distance) with these sequences as the reference species (Table 3). The results indicate that *X. cheopis*, *A. klossi*, *L. segnis* and *M. anisus* are consistent with the species names of the reference species, all showing genetic distances of less than 2% therefore, the morphological and molecular identification results of these four species were consistent. *N. specialis* matches the name of its reference species; however, its genetic distances exceed 2% (5.56% and 4.54%), leading us to question the taxonomic status of subspecies *N. specialis minpiensis*. *C. breviprojiciens*, *N. dispar*, *P. himalaica* and *N.nicanus* do not align with their respective reference species names as their genetic distances all exceed 2%, which is attributed to a lack of *COI* sequences for these four species in GenBank.

Phylogenetic analyses

The concatenated Muscle alignment resulted in 3727 aligned positions of 53 nucleotide sequences. The estimated nucleotide frequencies are: A = 0.263, C = 0.224, G = 0.237, and T = 0.276. ModelTest selected the GTR [General Time Reversible[41]] + G + I (Gamma distributed with Invariant sites) model for likelihood

Name	Sample No.	Reference species	GenBank No.	Sequence identity (%)	Genetic distances (%)
Xenopsylla cheopis	XC01	*Xenopsylla cheopis*	MW310242	99.85	0.15
Xenopsylla cheopis	XC02	*Xenopsylla cheopis*	MW310242	99.71	0.29
Aviostivalius klossi bispiniformis	AK02	*Aviostivalius klossi klossi*	KM890499	98.65	1.35
Ctenophthalmus breviprojiciens n. ssp.	CB01	*Ctenophthalmus yunnanus*	NC_085277	96.19	3.81
Ctenophthalmus breviprojiciens n. ssp.	CB02	*Ctenophthalmus yunnanus*	NC_085277	96.05	3.95
Neopsylla dispar fukienensis	NF01	*Neopsylla paranoma*	KJ470954	86.73	13.27
Neopsylla dispar fukienensis	NF02	*Neopsylla paranoma*	KJ470954	86.73	13.27
Neopsylla specialis minpiensis	NS01	*Neopsylla specialis*	NC_073019	94.44	5.56
Neopsylla specialis minpiensis	NS02	*Neopsylla specialis*	NC_073019	95.46	4.54
Peromyscopsylla himalaica sinica	PH01	*Amphalius clarus*	MG138161	87.14	12.86
Peromyscopsylla himalaica sinica	PH02	*Amphalius clarus*	MG138161	87.14	12.86
Leptopsylla segnis	LS01	*Leptopsylla segnis*	MG138247	99.66	0.34
Leptopsylla segnis	LS02	*Leptopsylla segnis*	MG138247	99.66	0.34
Monopsyllus anisus	MA01	*Monopsyllus anisus*	MG138206	99.84	0.16
Monopsyllus anisus	MA02	*Monopsyllus anisus*	MG138206	99.85	0.16
Nosopsyllus nicanus	NN01	*Nosopsyllus laeviceps*	MG138196	94.68	5.32
Nosopsyllus nicanus	NN02	*Nosopsyllus laeviceps*	MG138196	94.83	5.17

Table 3. The genetic distances between the 17 fleas individuals in this study and the corresponding reference species from the GenBank. The reference species was obtained through BLAST comparison in GenBank, and it showed the highest identity with the corresponding *COI* sequences.

analysis of this alignment. The evolutionary history was inferred based on the GTR model, with the parameter for the discrete gamma distribution estimated at 0.3591 and the proportion of invariable sites ratio at 0.3467. ML analysis produced a single best tree with a likelihood of -lnL = 26706.15.

The phylogenetic tree (Fig. 4) shows that this analysis included 31 species from 9 genera and 5 families of Siphonaptera. At the subspecies level, the ML analyses provide support for the monophyly of all four subspecies with multiple samples: *P. himalaica sinica* [bootstrap value (BS) > 95], *C. felis felis* (BS = 45), *A. klossi bispiniformis* (BS > 95), *A. klossi klossi* (BS = 56). At the species level, 12 species with more than one sample (clade C1-C12) form monophyly respectively, each with high support (BS > 95) except for *C. felis* (clade C8, BS = 58). Additionally, they exhibit small intraspecific genetic distance (< 0.02), except for *P. himalaica*. The intraspecific genetic distance between *P. himalaica sinica* and *P. himalaica* (F222) exceeds 0.02 significantly, leading to doubts about the taxonomic status of the subspecies *P. himalaica sinica*. At the genus level, the analyses provide support for the monophyly of all nine genera (clade C3, C9, C13-C19). At the family level, Leptopsyllidae (clade C20, BS > 95), comprising sister groups *Peromyscopsylla* and *Leptopsylla*, Ceratophyllidae (clade C21, BS = 84) consisting of sister groups *Monopsyllus* and *Nosopsyllus*, Pulicidae (clade C22, BS > 95), comprising sister groups *Xenopsylla* and *Ctenocephalides*, and Pygiopsyllidae (clade C9, BS > 95) are all supported as monophyletic with high support values. However, it is worth noting that Ctenophthalmidae is paraphyletic, with *Neopsylla* occurring in clade C24 as the sister group of Pygiopsyllidae, and *Ctenophthalmus* located on clade C19. At the Superfamily level, Ceratophyllidea (clade C23, BS > 95) consisting of sister groups Leptopsyllidae and Ceratophyllidae, and Pulicidea (clade C22) are both monophyletic with high support values; obviously, Hystrichopsylloidea consisting of Pygiopsyllidae and Ctenophthalmidae is paraphyletic.

Discussion

The *COI* gene is easy to amplify, and mitochondrial gene content is strongly conserved, with very few duplications, no introns, and very short intergenic regions[43]. Therefore, for most animals, the *COI* gene has become the standard barcode region[44]. In this study, a total of 10 species of fleas from rodents were identified based on morphology. Barcode identification utilizing the *COI* gene and phylogenetic analysis based on five genetic markers of *18S rDNA*, *28S rDNA*, *EF-1α*, *COI*, *COII* revealed that the molecular and morphological identification of *X. cheopis*, *A. klossi*, *L. segnis*, *M. anisus* and *C. felis felis* were consistent. The taxonomic status of *N. specialis minpiensis and P. himalaica sinica* as subspecies is questionable due to significant intraspecific genetic distance, and additional morphological and molecular data are required to confirm whether they should be elevated to species status. The molecular identification of *C. breviprojiciens* n. ssp., *N. dispar fukienensis*, and *N. nicanus* could not be completed at this time due to the absence of sequences of related species in the existing GenBank. In addition, We have deposited the successfully obtained sequences of *18S rDNA*, *28S rDNA*, *EF-1a*, *COII* and *COI* into GenBank. Relevant gene sequences of some species and subspecies were uploaded preferentially, effectively filling gaps existing in the GenBank database and enhancing its resources. As a result, this study will facilitate future molecular research on fleas.

Phylogenetic analyses suggest that the classification of fleas at the subspecies, species, and genus levels largely reflects phylogeny based on ML analysis using five genetic markers (*18S rDNA*, *28S rDNA*, *EF-1a*, *COI*, and *COII*). However, some bootstrap values are not high enough, which we attribute to the lack of complete sequences for some samples. At the family level, Ctenophthalmidae does not accurately reflect phylogeny. This

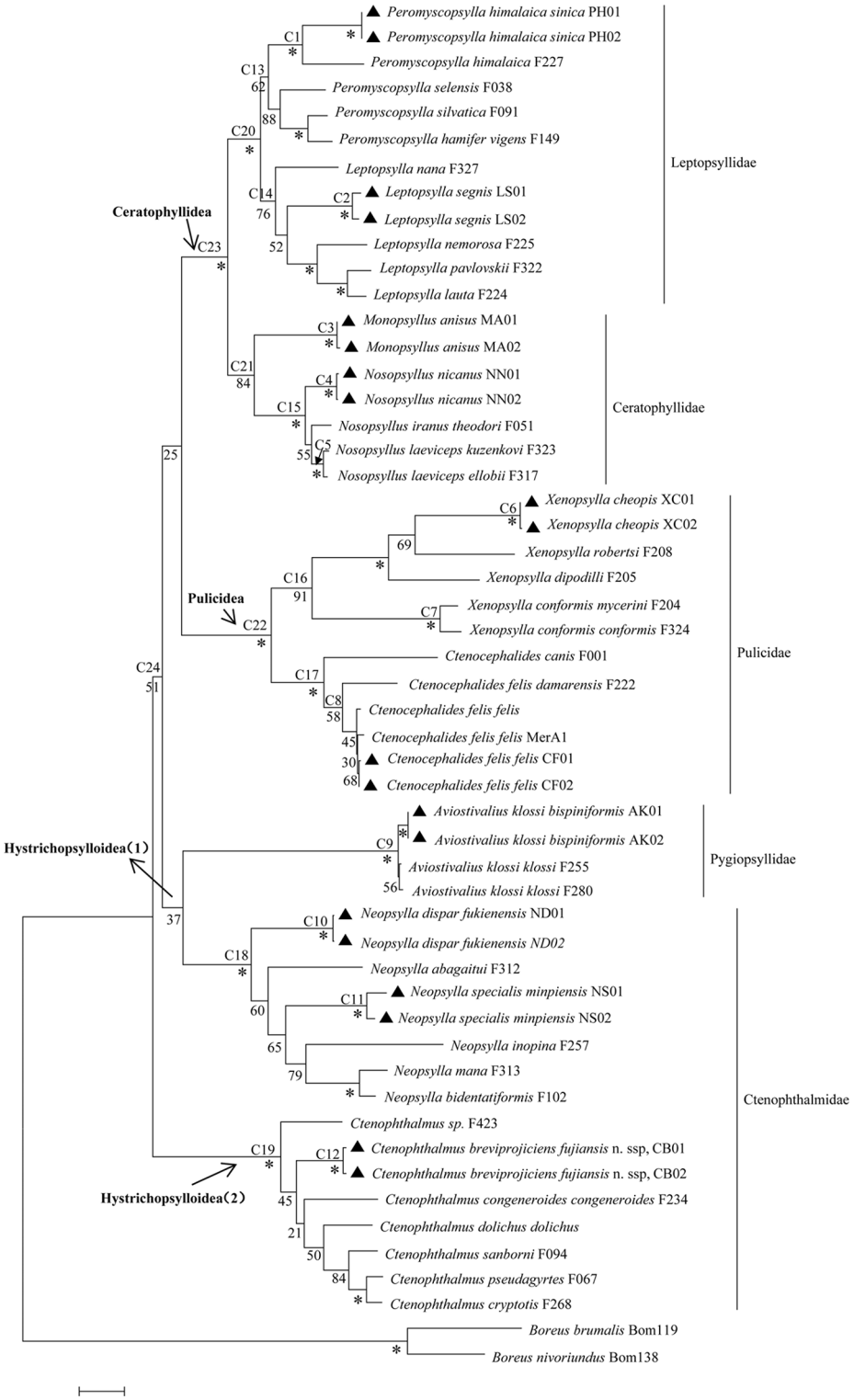

Fig. 4. Phylogenetic tree of the *18S–28S–EF-1a–COII-COI* concatenated sequences of fleas. The tree was constructed based on maximum-likelihood (ML; 1000 bootstrap replicates) analyses using MEGA7. The self-tested sequences in this study are marked with black triangles (▲).The marks in the upper left of the nodes represent the partial branch number, those in the lower left of the nodes represent the bootstrap value (BS), and the asterisks (*) indicate bootstrap values greater than 95. scale bar = 0.02.

is consistent with Whiting et al.'s[27] phylogenetic analysis of fleas based on *18S rDNA*, 28S rDNA, *COII* and *EF-1a*. The Ctenophthalmidae[45] consists of nine subfamilies and 17 tribes, with 42 genera and 664 species[31,46]. It is the most diverse family in Siphonaptera, approximately one quarter of flea species are placed within this group. Some scholars[27,31,46] have suggested that Ctenophthalmidae has been traditionally used as a 'catchall' family for fleas -- an unnatural grouping that includes a wide range of divergent taxa and is almost certainly paraphyletic assemblages. Therefore, elevating each of its constituent subfamilies to family level would better reflect phylogeny. In conclusion, we support this view and look forward to the re-revision of the family level.

Morphologically, male fleas are easily distinguishable based on the size, shape, and chaetotaxy of their genitalia. In contrast, the identification and classification of female fleas rely heavily on the characteristics of sternum VII and spermatheca. However, in some cases, the morphological differences among female fleas are so small that specific identification can be more challenging, especially when isolated without males for comparison[2,31]. There has never been a collection record of the genus *Ctenophthalmus* in Fujian Province, southern China, and *C. breviprojiciens fujiansis* n. ssp. collected in this study is a new record for the genus *Ctenophthalmus* in this area. In this study, a total of 10 *C. breviprojiciens fujiansis* n. ssp. specimens were obtained, of which 9 males were collected from the surface of 4 *M. fortis* in Zhongxin Town, Pucheng County in May 2020, and one female was collected from a *M. fortis* in Siqiao Town, Zhouning County in October 2019. Therefore, the single female specimen was collected at different times and places from the other nine male specimens. Additionally, it had lost the spermatheca, which is important for classification. As a result, the female specimen can only be distinguished from *C. breviprojiciens breviprojiciens* and *C. breviprojiciens yongjiaensis* by slight differences in the lobes of Sternum VII and the base of Tergum VIII, greatly increasing the difficulty of its classification. We determined that the female specimen is the same species as the nine male specimens based on the fact that they were all collected from the same ecological environment (reed bushes beside farmland) and hosts (*M. fortis*), which has been well verified by the molecular data we provided (Fig. 4). Unfortunately, we could not obtain the molecular data of *C. breviprojiciens breviprojiciens* and *C. breviprojiciens yongjiaensis*.

Data availability

The experimentally obtained sequences of *18S rDNA*, *28S rDNA*, EF-1α, *COII* and *COI* have been uploaded to Genbank (https://www.ncbi.nlm.nih.gov/), and accession numbers can be found in Supplementary Table S3.

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Author contributions

F.X. is the Corresponding Author, and designed and supervised the study. S.Z. was the main performer of this study, responsible for collecting and identifying fleas, conducting experiments and data analysis, preparing Figs. 1, 2, 3 and 4, and drafting the manuscript. W.L. and Z.Z. participated in rodent trapping, experiments, and data analysis. J.W., T.H., G.X., and L.H. all participated in rodent trapping. F.X. and S.W. provided financial support. All authors reviewed and approved the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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