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First Report of *Thelazia Callipaeda* Infection in *Amiota Okadai* and Wildlife in Wildlife Home Range, China

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Short report

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Abstract

Background: *Thelazia callipaeda* is a nematode of the sucking nematode genus of the sucking family, with *Amiota okadai* as the intermediate host and vector in China. China has the largest number of cases of thelaziosis in humans in the world. It is generally believed that domestic animals (dogs and cats) are the most important reservoir host of *Thelazia callipaeda* and directly threaten humans. At present, there is not much research and attention on the role of wildlife in the transmission cycle of thelaziosis in wildlife home range.

Methods: During 2016-2019, we selected four wildlife national nature reserve across the country as monitoring points for *Amiota okadai* and wildlife. And we chose to use fly-trap method for monitoring *Amiota okadai* density. Morphological analysis of the parasites collected from the conjunctival sac of the wildlife was taken as the first step, and a specific PCR was used for exact confirmation.

Results: In 2019, the density of *Amiota okadai* in Foping National Nature Reserve, in China, increased sharply and infected *Amiota okadai* were newly found in wildlife home range. And it was newly found that wild giant pandas, wild boars, leopard cats, black bears were infected with *Thelazia callipaeda*. A total of 4 nematodes were collected. The morphologic characteristics of the nematode led to its identification as *Thelazia callipaeda*, which was molecularly confirmed by a specific PCR amplification.

Conclusions: This is the first time in China that *Amiota okadai* has been reported to be infected with *Thelazia callipaeda* in wildlife home range, while a variety of wildlife, including wild giant pandas, have been infected. This suggests that there has been a transmission cycle of thelaziosis among wildlife in wildlife home range. This has undoubtedly increased the risk of infection of *Thelazia callipaeda* in villagers around wildlife home range.

Background

From 1917 to 2019, 643 cases of thelaziosis in human have been recorded in China [1, 2]. China has the largest number of cases of thelaziosis in human in the world. It can be seen that the number of infection cases has increased significantly since 1970, and although the number of cases has decreased compared with that from 1970 to 1999 in the past 20 years, it still tends to increase [1, 2]. The increase in the number of *A. okadai*, coupled with the proliferation of domestic animals (dogs and cats), has led to increased vigilance against *T. callipaeda* infections.

And the infection rate of villagers is obviously higher than that of urban people [2]. This is because the villages environment is more conducive to the survival of *A. okadai*. From 2016 to 2019, We conducted ocular examination on domestic animals in the villages around wildlife home range and found that the prevalence rate, in 2019, was as high as 84.62% (88 of 104), higher than that in 2016 (38.05% ,43 of 113), 2017 (53.92%,55 of 102) and 2018 (56.25%, 63 of 112).

However, the wildlife home range in china have a better climate for *A.okadai*, and most of wildlife home range are surrounded by villagers. And the number of wildlife in the wildlife home range is far more than the number of domestic animals in the surrounding villages. If the wildlife spread the *T. callipaeda* on a large scale, it will be a threat to the villagers and domestic animals.

In order to understand the prevalence of *T. callipaeda* in the wildlife home range and surrounding villages, we selected four wildlife national nature reserve across the country as monitoring points for *A. okadai* and wildlife, during 2016–2019. The four monitoring points are located in the giant panda national park, one of the most complex topographical regions in the world with the most obvious vertical zoning of climate, and home to more than 8000 kinds of wildlife and plants. There are villages around these reserves, and some villagers raise domestic animals. These areas have overlaps between the activities of wildlife and domestic animals [3]. In addition, these reserves were also selected because they are close to densely populated cities.

Methods

Monitoring points for *A. okadai* and wildlife

During 2016-2019, we selected four wildlife national nature reserve across the country as monitoring points for *A. okadai* and wildlife. They are Foping National Nature Reserve, Shaanxi, China (FNNR, 33°38'43"N, 107°47'38"E), Tangjiahe National Nature Reserve, Sichuan, China (TNNR, 32°34'44"N, 104°45'43"E), Wolong National Nature Reserve, Sichuan, China (WNNR, 31°02'20"N, 103°11'52"E) and Fengtongzhai National Nature Reserve, Sichuan, China (FTZNNR, 30°22'05"N, 102°48'52"E) (Fig. 1).

Monitoring *A. okadai* density and morphological identification

According to the “Surveillance methods for vector density – Fly” promulgated by the National Health Commission of the People's Republic of China [4], we chose to use fly-trap method for monitoring *A. okadai* density. 200 traps were placed at each monitoring point, of which 100 were distributed in wildlife home range and the other 100 were distributed in surrounding villages. The prepared fruit mud was put in the traps. Monitoring was started from the beginning of spring in April to the end of winter in November. The fly traps were placed at 9 am in the middle of

each month, and was taken back at 9 am the next day. Because there are many kinds of flies in the traps, *A. okadai* can be identified according to the morphological characteristics and crawling speed of various flies. Then it was taken out for further morphological analysis under microscope. Then the density of *A. okadai* was calculated and the infection was identified by dissection. Density is determined by dividing the number of *A.okadai* by the number of fly traps and represents the number of *A. okadai* per cage for 24h [4].

Sample collection

In 2019, 4 cases of ocular worm infection in wild giant panda (Fig. 2), wild boar, leopard cat and black bear were found during ophthalmic examination with injection anesthesia in FNNR.

Parasite collection and treatment

The worm was removed from the conjunctival sac and placed in a sampling tube containing 70% ethanol. Morphological analysis of the parasites collected was taken as the first step, and a specific PCR was used for exact confirmation.

Morphological analysis

The parasites collected from the eyes of each wildlife were identified on the basis of morphologic keys under a light microscopy combined with a camera.

Sequence and Phylogenetic analysis

we extracted genomic DNA from the *T. callipaeda* in the conjunctival sac of wild giant panda, wild boar, leopard cat, black bear with the HiPure Tissue & Blood DNA Kit (MAGEN, China). And a partial sequence of the mitochondrial cytochrome c oxidase subunit 1 gene (cox1, 689 bp) was amplified by PCR. Amplicons were purified by using HiPure Gel Pure Micro Kit (MAGEN, China) and sequenced in an ABI3730XL by using the BigDyeTr v3.1 Cycle Seq Kit (Applied Biosystems, USA). Sequences were determined in both directions (GenBank accession nos. MN719908, MN719912, MN719913 and MN719914) and performed genetic analyses using available sequences of related nematodes from GenBank and the GISAID database (https://www.gisaid.org). The phylogenetic tree was constructed by using MEGA version 6 (https://www.megasoftware.net) by the neighbor-joining method with 1,000 bootstrap replicates.

Results

After morphological observation under microscope, *A. okadai* body length was measured to be 2.7 ~ 3.6 mm. The compound eye was surrounded by a white band, and there were many brown spots of different sizes and irregular shapes on the back of the chest. There were three black bands at the base, middle, and end of the foot, one of the characteristic signs [5, 6]. The dorsal side of the 3rd-5th segment of the abdomen each had a dark brown shape similar to "hill" shaped transverse belt (Fig. 3).

It can be seen from our monitoring data (Table 1- Table 4) that before 2019 (in 2016, 2017 and 2018), there were fewer *A. okadai* in wildlife home range than in surrounding villages, but no infected *A. okadai* were found in the wildlife home range. Only in surrounding villages, a large number of infected *A. okadai* were found. However, in 2019, the density of *A. okadai* in FNNR increased sharply and infected *A. okadai* were newly found in wildlife home range. July and August were the two months with the highest distribution density of *A. okadai*, and the two months with the highest number of *A. okadai* with the early stage of pre-infection larval of *T. callipaeda* (Fig. 4a).

Table 1
The density and infection situation of Amiota okadai in wildlife home range and surrounding villages, China, 2016

Monitoring points	Region	Months	No. Fly Traps		No. *A.okadai*		Fly density (fly/cage /24 h)		No. infected *A.okadai*	
			surrounding villages	wildlife home range	surrounding villages	wildlife home range	surrounding villages	wildlife home range	surrounding villages	wildlife home range
FNNR	Shaanxi	April	100	100	141	85	1.41	0.85	69	0
		May			203	96	2.03	0.96	118	0
		June			202	101	2.02	1.01	111	0
		July			206	123	2.06	1.23	112	0
		August			221	124	2.21	1.24	126	0
		September			218	119	2.18	1.19	102	0
		October			137	113	1.37	1.13	104	0
TNNR	Sichuan	April	100	100	93	67	0.93	0.67	43	0
		May			102	81	1.02	0.81	67	0
		June			109	66	1.09	0.66	75	0
		July			114	76	1.14	0.76	87	0
		August			123	79	1.23	0.79	101	0
		September			103	65	1.03	0.65	79	0
		October			96	54	0.96	0.54	33	0
WNNR	Sichuan	April	100	100	0	0	0	0	0	0
		May			0	0	0	0	0	0
		June			0	0	0	0	0	0
		July			0	0	0	0	0	0
		August			0	0	0	0	0	0
		September			0	0	0	0	0	0
		October			0	0	0	0	0	0
FTZNNR	Sichuan	April	100	100	86	45	0.86	0.45	32	0
		May			85	33	0.85	0.33	66	0
		June			98	75	0.98	0.75	34	0
		July			103	88	1.03	0.88	79	0
		August			106	86	1.06	0.86	98	0
		September			101	76	1.01	0.76	77	0
		October			91	23	0.91	0.23	34	0

Abbreviations: FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve; No. Fly Traps, mean the total number of traps placed at monitoring point; No. *A.okadai*, mean the total number of *Amiota okadai* collected in the 24 h of sampling; Fly density (fly/cage /24 h), mean the number of *Amiota okadai* per cage for 24 h; No. infected *A.okadai*, mean the number of contain early stage of pre-infection larval of *Thelazia callipaeda* in the worm hemocoel

Table 2
The density and infection situation of Amiota okadai in wildlife home range and surrounding villages, China, 2017

Monitoring points	Region	Months	No. Fly Traps		No. *A.okadai*		Fly density (fly/cage /24 h)		No. infected *A.okadai*	
			surrounding villages	wildlife home range	surrounding villages	wildlife home range	surrounding villages	wildlife home range	surrounding villages	wildlife home range
FNNR	Shaanxi	April	100	100	181	81	1.81	0.81	90	0
		May			221	88	2.21	0.88	121	0
		June			238	103	2.38	1.03	143	0
		July			241	113	2.41	1.13	213	0
		August			272	129	2.72	1.29	222	0
		September			203	109	2.03	1.09	107	0
		October			180	99	1.80	0.99	111	0
TNNR	Sichuan	April	100	100	103	78	1.03	0.78	52	0
		May			116	86	1.16	0.86	56	0
		June			105	76	1.05	0.76	79	0
		July			124	72	1.24	0.72	94	0
		August			113	89	1.13	0.89	79	0
		September			97	71	0.97	0.71	59	0
		October			92	62	0.92	0.62	31	0
WNNR	Sichuan	April	100	100	0	0	0	0	0	0
		May			0	0	0	0	0	0
		June			0	0	0	0	0	0
		July			0	0	0	0	0	0
		August			0	0	0	0	0	0
		September			0	0	0	0	0	0
		October			0	0	0	0	0	0
FTZNNR	Sichuan	April	100	100	90	56	0.90	0.56	42	0
		May			81	32	0.81	0.32	55	0
		June			93	79	0.93	0.79	32	0
		July			112	82	1.12	0.82	68	0
		August			121	96	1.21	0.96	93	0
		September			110	74	1.10	0.74	66	0
		October			81	33	0.81	0.33	39	0

Abbreviations: FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve; No. Fly Traps, mean the total number of traps placed at monitoring point; No. *A.okadai*, mean the total number of *Amiota okadai* collected in the 24 h of sampling; Fly density (fly/cage /24 h), mean the number of *Amiota okadai* per cage for 24 h; No. infected *A.okadai*, mean the number of contain early stage of pre-infection larval of *Thelazia callipaeda* in the worm hemocoel

Table 3
The density and infection situation of Amiota okadai in wildlife home range and surrounding villages, China, 2018

Monitoring points	Region	Months	No. Fly Traps		No. *A.okadai*		Fly density (fly/cage /24 h)		No. infected *A.okadai*	
			surrounding villages	wildlife home range	surrounding villages	wildlife home range	surrounding villages	wildlife home range	surrounding villages	wildlife home range
FNNR	Shaanxi	April	100	100	180	92	1.80	0.92	91	0
		May			219	79	2.19	0.79	106	0
		June			205	131	2.05	1.31	129	0
		July			212	126	2.12	1.26	209	0
		August			284	123	2.84	1.23	223	0
		September			232	116	2.32	1.16	116	0
		October			149	83	1.49	0.83	109	0
TNNR	Sichuan	April	100	100	108	61	1.08	0.61	64	0
		May			121	71	1.21	0.71	63	0
		June			112	82	1.12	0.82	73	0
		July			132	89	1.32	0.89	85	0
		August			129	92	1.29	0.92	84	0
		September			82	83	0.82	0.83	67	0
		October			99	78	0.99	0.78	43	0
WNNR	Sichuan	April	100	100	0	0	0	0	0	0
		May			0	0	0	0	0	0
		June			0	0	0	0	0	0
		July			0	0	0	0	0	0
		August			0	0	0	0	0	0
		September			0	0	0	0	0	0
		October			0	0	0	0	0	0
FTZNNR	Sichuan	April	100	100	53	31	0.53	0.31	26	0
		May			83	53	0.83	0.53	42	0
		June			62	56	0.62	0.56	33	0
		July			124	72	1.24	0.72	71	0
		August			119	89	1.19	0.89	88	0
		September			102	88	1.02	0.88	71	0
		October			92	66	0.92	0.66	40	0

Abbreviations: FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve; No. Fly Traps, mean the total number of traps placed at monitoring point; No. *A.okadai*, mean the total number of *Amiota okadai* collected in the 24 h of sampling; Fly density (fly/cage /24 h), mean the number of *Amiota okadai* per cage for 24 h; No. infected *A.okadai*, mean the number of contain early stage of pre-infection larval of *Thelazia callipaeda* in the worm hemocoel

Table 4
The density and infection situation of Amiota okadai in wildlife home range and surrounding villages, China, 2019

Monitoring points	Region	Months	No. Fly Traps		No. *A.okadai*		Fly density (fly/cage /24 h)		No. infected *A.okadai*	
			surrounding villages	wildlife home range	surrounding villages	wildlife home range	surrounding villages	wildlife home range	surrounding villages	wildlife home range
FNNR	Shaanxi	April	100	100	198	197	1.98	1.97	101	115
		May			201	276	2.01	2.76	166	185
		June			225	224	2.25	2.24	186	166
		July			278	256	2.78	2.56	212	206
		August			266	299	2.66	2.99	202	218
		September			223	245	2.23	2.45	167	191
		October			187	198	1.87	1.98	101	142
TNNR	Sichuan	April	100	100	123	44	1.23	0.44	67	0
		May			102	82	1.02	0.82	55	0
		June			101	99	1.01	0.99	72	0
		July			115	83	1.15	0.83	71	0
		August			113	91	1.13	0.91	78	0
		September			79	74	0.79	0.74	41	0
		October			87	76	0.87	0.76	59	0
WNNR	Sichuan	April	100	100	0	0	0	0	0	0
		May			0	0	0	0	0	0
		June			0	0	0	0	0	0
		July			0	0	0	0	0	0
		August			0	0	0	0	0	0
		September			0	0	0	0	0	0
		October			0	0	0	0	0	0
FTZNNR	Sichuan	April	100	100	61	49	0.61	0.49	39	0
		May			78	67	0.78	0.67	48	0
		June			59	48	0.59	0.48	46	0
		July			109	84	1.09	0.84	62	0
		August			106	83	1.06	0.83	64	0
		September			99	52	0.99	0.52	32	0
		October			88	51	0.88	0.51	27	0

Abbreviations: FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve; No. Fly Traps, mean the total number of traps placed at monitoring point; No. *A.okadai*, mean the total number of *Amiota okadai* collected in the 24 h of sampling; Fly density (fly/cage /24 h), mean the number of *Amiota okadai* per cage for 24 h; No. infected *A.okadai*, mean the number of contain early stage of pre-infection larval of *Thelazia callipaeda* in the worm hemocoel

Through morphological observation of the collected parasites, it could be seen that under stereoscope, the worm body was a cylindrical shape with thin center at both ends, milky white and slightly transparent. All of worms were female. The mean body length and width of the female were 13.7 mm and 0.36 mm respectively. Under the optical microscope, the buccal capsule, pharynx, and esophagus of the anterior segment of the worm body and its serrated, wrinkled surface and the caudal end of the worm were visible (Fig. 4b, Fig. 4c). In the midsection of the female's

uterus, there were larvae and oval eggs (Fig. 4d), with the mode of reproduction of oviparous. According to the morphological characteristics of the worm, it could be identified as *T. callipaeda*.

A partial sequence of the mitochondrial cytochrome c oxidase subunit 1 gene (cox1, 689 bp) of the specimens were sequenced [7, 8, 9, 10]. According to alignment and phylogenetic analysis, the neighbor-joining method confirmed that the *T. callipaeda* cox1 sequences in wild giant panda, wild boar, leopard cat (GenBank accession nos. MN719908, MN719912 and MN719913) obtained clustered with that of *T. callipaeda* in dogs, cats, and humans from China, Japan and Korea. The sequences from black bears (GenBank accession nos. MN719914) were closely related to those from European specimens and they were clustered together. All cox1 sequences of *T. callipaeda* were clustered into one large branch, while the cox1 sequences of *Onchocerca lupi* from USA was clustered into one branch (Fig. 5).

Discussion

It is generally believed that domestic animals are the most important reservoir host of *T. callipaeda* and directly threaten humans [11–16]. However, in 2019, many species of wildlife that have not been reported before have been found to suffer from the thelaziosis. And through the investigation of the intermediate host, in 2019, it was found that there were a large number of infected intermediate hosts in wildlife home range. We believe that thelaziosis has already spread into the wildlife home range. The emerge of infected *A. okadai* in wildlife home range of FNNR shows that this transmission cycle has been established.

Summer is the most active season for the intermediate host of *T. callipaeda* [17], and it is also the season for many wildlife to migrate to the wildlife home range [3, 18, 19, 20]. The infected wildlife will establish the transmission cycle of thelaziosis between wildlife and *A. okadai* in wildlife home range. The newborn larvae of *T. callipaeda* are directly produced in the conjunctival sac of wildlife. When the intermediate host, *A. okadai*, licks the wildlife's eye secretions, it sucks the newborn larvae into its body. After ecdysis of the larvae 2 times, the newborn larvae develop into infective larvae and then enter the head and proboscis of the *A. okadai*. When *A. okadai* lick the wildlife's eye again, the infective larvae of *T. callipaeda* escape from the *A. okadai 's* proboscis and invade the conjunctival sac of the wildlife, it usually takes 15 to 20 days to develop into an adult (21). When it's about 35 days, the female *T. callipaeda* can continue to produce larvae, which are then licked by the intermediate host, *A. okadai*, and the cycle begins again. The infected *A. okadai* continue to threaten domestic animals and villagers.

There are nearly 2000 kinds of wildlife in China, and there are about 265 kinds of wildlife in FNNR alone. Once thelaziosis is widely spread among wildlife, with the frequent villager activities around the wildlife home range, it will inevitably threaten the human beings in turn. And, the infected wildlife gradually forms a reservoir host bank among wildlife. When the winter is approaching, wildlife migrates to the villages, and enter the overlapping areas between wildlife and domestic animals [3, 18, 19, 20]. The interaction creates an opportunity for the transmission of potential *T. callipaeda* between wildlife and domestic animals by *A. okadai*.

A. okadai of FNNR has the highest density, which is related to its geographical environment and climate [17]. It is located in Foping county, located in the northeast of Hanzhong city, Shaanxi province, belonging to the north sub-tropical climate zone, Qinling mountain in the north and Daba mountain in the south are two barriers, with an altitude of 980–2904 meters. The humid air is not easy to travel to the north. The climate is mild and humid. The suitable climate environment provided a good habitat for the *A. okadai*, which is the media of *T. callipaeda*. No intermediate host *A. okadai* is found in the WNNR monitoring points. The reason is that the altitude of most local mountain peaks is over 4000 meters and the average annual temperature is 8.5 ± 0.5℃, which is not conducive to the survival of *A. okadai*.

Although no wildlife infection cases have been found in the other three reserves (TNNR, WNNR, FTZNNR) and the infected *A. okadai* was only found in wildlife home range of FNNR and surrounding villages, the monitoring of the intermediate host needs to be carried out all the time. At the same time, the domestic animals in villages should be given deworming medicine regularly. Such efforts could monitor the epidemic trend of thelaziosis and weaken the threat to the public.

Conclusions

To the best of our knowledge, this is the first time in China that *A. okadai* has been reported to be infected with *T. callipaeda* in wildlife home range, while a variety of wildlife, including wild giant pandas, have been infected. This study illustrates the importance of wildlife in vector-borne zoonosis. Further studies need to focus on the assessment of the risk of *T. callipaeda* infection in villagers around the wildlife home range.

Declarations

Acknowledgements

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Authors' contributions

YPJ, ZCL, DGL, and JHL conceived the study. YPJ and ZCL organized the sampling plan. ZCL, JQW, YFW, NJH and LBT were responsible for the collection of cases of wildlife thelaziosis used in this study. And ZCL obtained the sequence of *Thelazia callipaeda* and analysed the results. All of members participated in the capture of *Amiota okadai* in wildlife nature reserve. YPJ and ZCL drafted the manuscript and all authors critically contributed to its final version. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analysed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

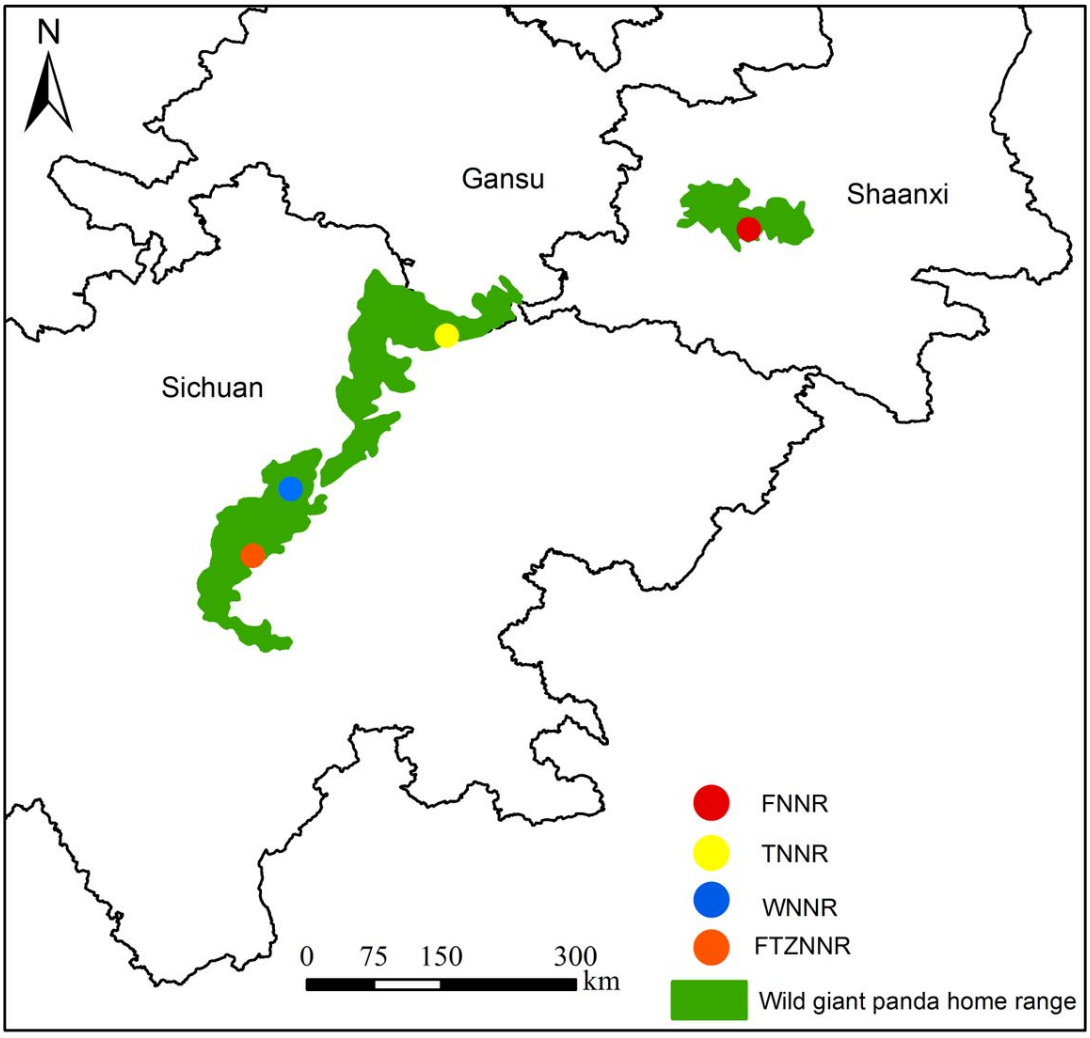

Figure 1

The wildlife home range in which the monitoring points of Amiota okadai and wildlife were set up, China, 2016-2019. FNNR, Foping National Nature Reserve; TNNR, Tangjiahe National Nature Reserve; WNNR, Wolong National Nature Reserve; FTZNNR, Fengtongzhai National Nature Reserve

Figure 2

Thelazia callipaeda in the eyes of wild giant panda

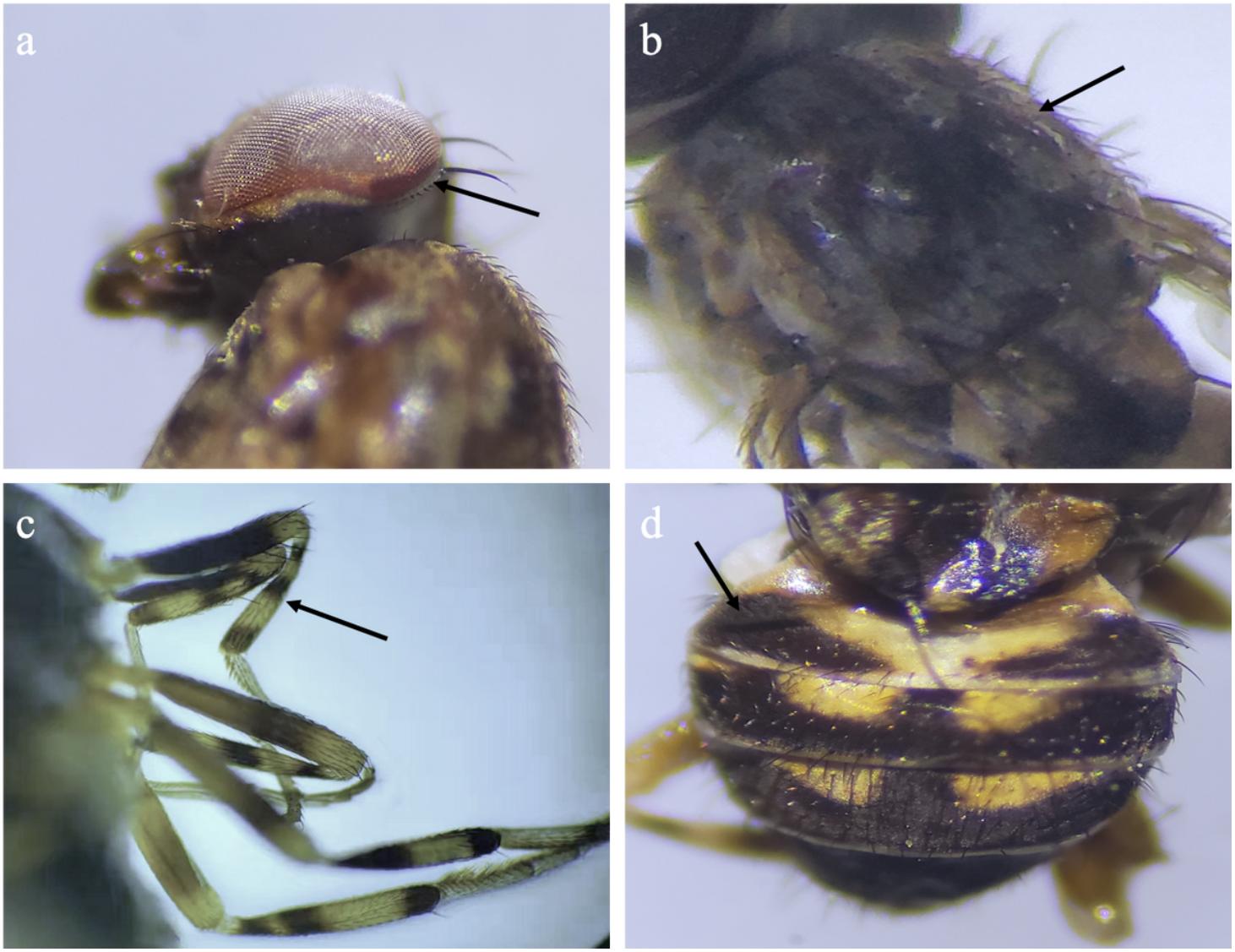

Figure 3

Light micrographs of Amiota okadai. a The white band around the compound eye (arrow); b Multiple dark brown spots on the back of the chest (arrow); c Three black bands on the shin of the foot (arrow); d The dorsal stripe of the abdomen similar to "hill" shaped transverse belt (arrow)

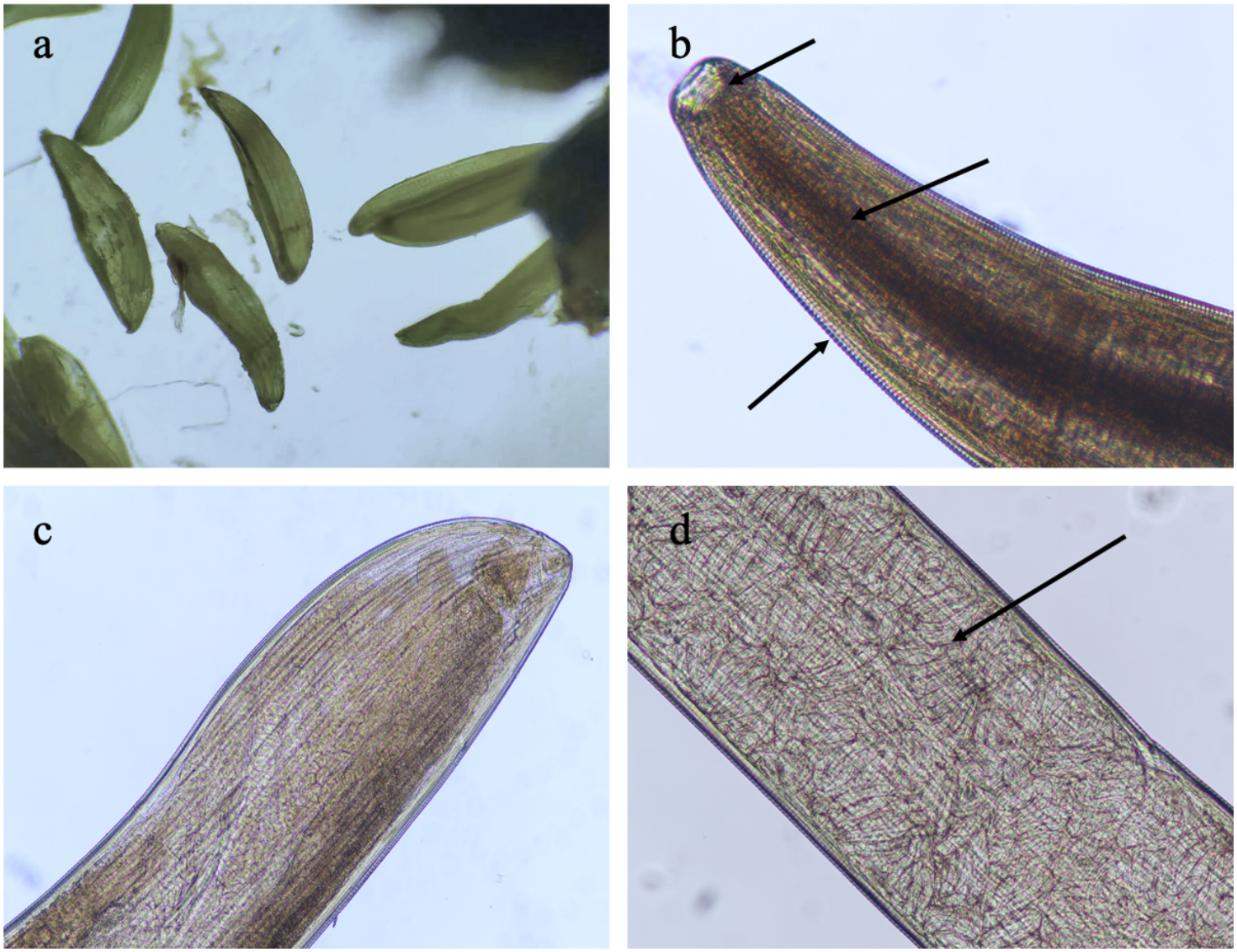

Figure 4

a The early stage of pre-infection larval of Thelazia callipaeda in surrounding tissues of hemocoel of Amiota okadai; b Thelazia callipaeda with the buccal capsule, pharynx, and esophagus of the anterior segment of the worm body and its serrated, wrinkled surface (arrow); c The caudal end of a worm; d Larvae in the female's uterus (arrow)

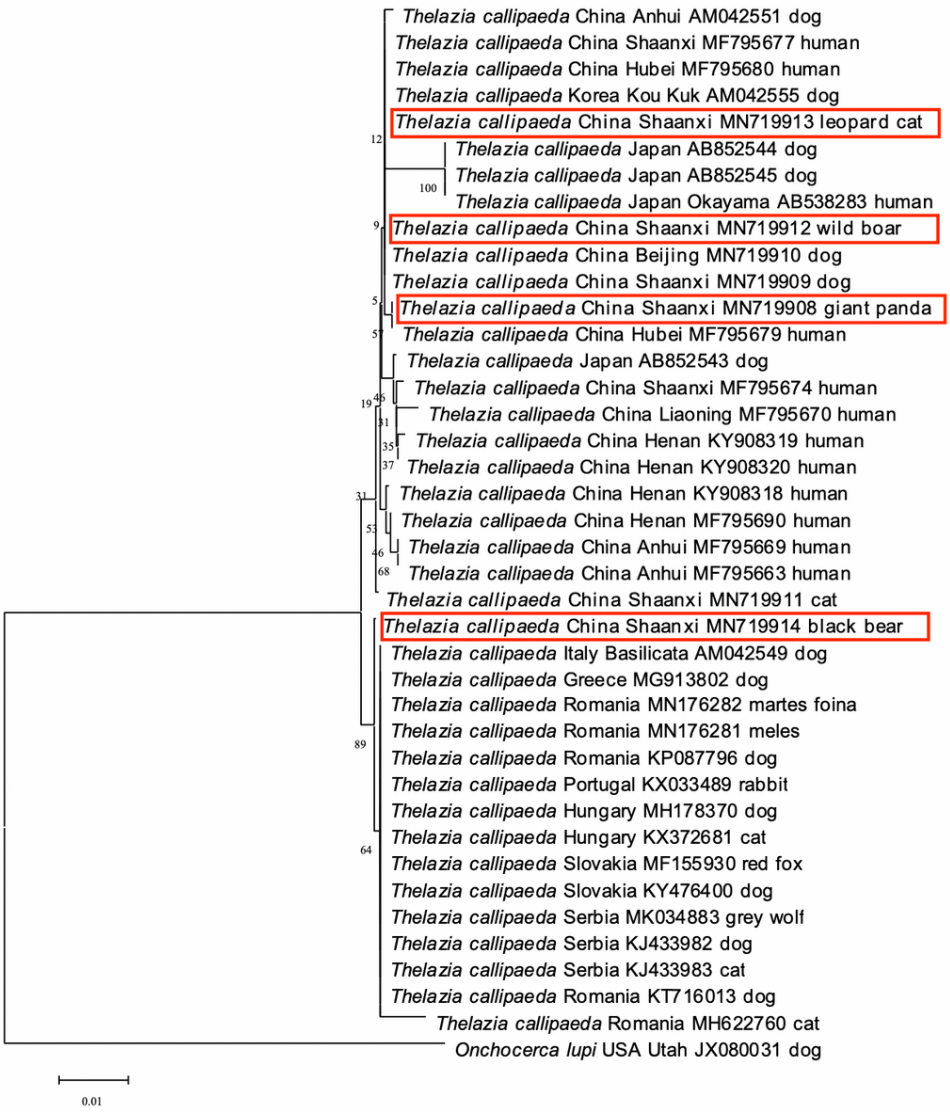

Figure 5

Phylogenetic tree of a partial sequence of the mitochondrial cytochrome c oxidase subunit 1 gene of Thelazia callipaeda. Bootstrap values are shown on the tree. The isolate sequenced in this study were labeled with a red rectangular box (GenBank accession nos. MN719908, MN719912, MN719913 and MN719914). The rest sequences of Thelazia callipaeda were downloaded from the National Center for Biotechnology information (NCBI). Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site