Whipworms of south-east Asian rodents are distinct from *Trichuris muris*

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Abstract

The whipworm *Trichuris muris* is known to be associated with various rodent species in the northern hemisphere, but the species identity of whipworm infecting rodents in the Oriental region remains largely unknown. We collected *Trichuris* of Muridae rodents in mainland and insular Southeast Asia between 2008 and 2015 and used molecular and morphological approaches to identify the systematic position of new specimens. We discovered two new species that were clearly distinct from *T. muris*, both in terms of molecular phylogenetic clustering and morphological features, with one species found in Thailand and another one in Borneo. We named the new species from Thailand as *Trichuris cossoni* and the species from Borneo as *Trichuris arrizabalagai.* Molecular phylogeny using internal transcribed spacer region (ITS1-5.8S-ITS2) showed a divergence between *T. arrizabalagai* n. sp., *T. cossoni* n. sp. and *T. muris*. Our findings of phylogeographically distinct *Trichuris* species despite some globally distributed host species requires further research into the distribution of different species, previously assumed to belong to *T. muris*, which has particular relevance for using these species as laboratory model organisms.

Keywords: *Trichuris*, Borneo, Thailand, Ribosomal DNA, helminth diversity, phylogeography

1. Introduction

The whipworm *Trichuris muris* was originally described by Schrank [1] as *Trichocephalus muris* from an unspecified mouse species in Germany, termed as "Maus" (which is a general term for all mice species in German, but most likely referring to the house mouse (*Mus musculus*) as the most common species in anthropogenic habitats rather than forest-dwelling mice (*Apodemus sylvaticus* and *Apodemus flavicollis*); all three rodent species have been reported to harbour this parasite; see below). Later, the priority of the name *Trichuris muris* (Schrank, 1788) Hall, 1916 was established [2]. A redescription of *T. muris* by Roman [3] in 1951 based on multiple specimens collected from both the wood mouse (*A. sylvaticus*) and the house mouse (*Mus musculus domesticus*) from France provided morphometric attributes that provide a basis to distinguish *T. muris* from potentially novel species from other rodent species or geographic regions. In the last decade, various studies have established molecular evidence that representative host species of Murinae (*Apodemus*, *Mus* and *Rattus*) sampled in several localities across Europe are associated with *T. muris* as a unique whipworm species [4,5,6]. This molecular evidence is of particular importance as morphometrical traits are not conclusive to distinguish *Trichuris* species from rodents [7] and other mammals (e.g.: [8]). This difficulty in a proper identification at species level can be illustrated in case of the studies *Trichuris* from rodents in Belgium by Bernard [9] suspected for the presence of a different entity than *T. muris* in European Microtidae, that could not be confirmed with aid of molecular methods since 40 years later by Feliu et al. [10]. During the last decades, the geographic distribution of *T. muris* has been expanded outside Europe by studies reporting this parasite species from Africa (reviewed by Ribas et al. [11]), Australia (e.g.: [12]), and also Southeast Asia (SEA) (e.g.: [13]) based on morphological species identification alone. However, taking the limited accuracy of morphological

traits for distinguishing *Trichuris* species into account and the lack of molecular information in these studies, it remains unclear if *T. muris* has indeed such broad geographic distribution or is replaced by other species.

Given the global distribution of invasive host species from the genus *Mus* and *Rattus*, that share parasites with a large range of wildlife in different areas [14], as well as the origin of these host species in Asia, one may expect *T. muris* to be present in SEA, as broadly assumed. However, geographic or environmental barriers may also disrupt host-parasite interactions and can result in distinct parasite species across geographic space [15,16]. Hence, it is also possible that *T. muris* is replaced by other whipworm species in SEA. This expectation is supported by the record of other whipworms from rodents elsewhere in the Oriental Region [17,18].

Despite this taxonomic uncertainty, whipworms from various geographic areas believed to belong to *T. muris* have been used extensively as a laboratory model. Such studies address aspects as diverse as host infection [19,20], pathology [21,22], and immune responses [23], the interaction of helminths with microbiota and bacteria [24,25,26], whipworm metabolism [27], and the efficiency of chemotherapeutic agents [28]. Since such studies are likely to use specimens of what is assumed to be *T. muris* from different host species or even geographic areas, reaffirming the phylogenetic uniqueness of these specimens is crucial to make such studies fully repeatable.

The genus *Trichuris* has been rarely recorded from rodents from SEA. An undetermined species was reported by Betterton [29] in Malaysian forest-dwelling rodents (*Leopoldamys sabanus*, *Maxomys surifer* and *Maxomys whiteheadi*). Another single individual was reported from *Rattus rattus diardii* after examining 1,117 rats in Singapore and Kuala Lampur (Schacher and Cheong [30]. Additionally, Leong et al. [31] reported *Trichuris* sp. from a single rat (*R. rattus diardii*) in Malaysia of examining 151 host in-

dividuals. Reports of *T. muris* from Thailand are confined to some of our previous studies [32]. Following the general assumption of no distinct species and because of strong morphological and metrical resemblance of specimens with *T. muris*, we assigned all species to *T. muris* in this previous work. Additional records of *T. muris* have been reviewed from New Guinea Island and Sulawesi Island by Hasegawa and Dewi [18] and Smales [17]. With the recent increase of our helminth collection from SEA rodents, including a growing number of specimens preserved for molecular studies, we here combine a genetic approach in combination with morphometrics to refine the systematics of whipworm specimens from SEA. The use of internal transcribed spacer (ITS) have been proven useful as a tool to resolve relationships of helminths such as *Trichuris* at the species level, including whipworms studies from Murinae (e.g.: [5,11] and more generally in Rodentia collected elsewhere [33]). The present study aimed to shed light on the taxonomy of whipworms from rodents in SEA and to test whether specimens can be confirmed as "*T. muris"* as widely assumed. In addition our individuals were compared with the three previously described *Trichuris* species from SEA rodents: *T. germani* recorded in New Guinea Island from *Pogonomys loriae* and *Pogonomys championi* (Murinae) [17]; *T. mallomyos* from *Mallomys rothschildi* (Murinae) in New Guinea Island [18] and *T. musseri* recorded in Sulawesi from *Echiothrix centrosa* (Murinae) [18].

2. Materials and methods

We studied specimens of *Trichuris* collected during surveys of Muridae rodents in mainland SEA, namely Cambodia, Lao PDR, Thailand and Vietnam, and one insular location, namely Borneo. All specimens were collected between 2008 and 2015. For the trappings of mainland rodents, we used locally made and baited cage-traps or Sherman traps. Rodents were identified by morphology or using species primer specific and/or barcoding assignments (consultable on the 'Barcoding Tool/RodentSEA' section of the

Community Ecology of Rodents and their Pathogens in South-East Asia (CERoPath) project web site [www.ceropath.org]).

In Borneo, we conducted small mammal trapping in the urban and suburban landscapes of the coastal city of Kota Kinabalu and the surrounding landscape in northern Borneo (lat. 6.0°N long. 116.1°E, ca. 600,000 inhabitants) from March 2012 – May 2013. Small mammals were captured with wire-mesh cage traps baited with mixtures of raw banana, fried banana and dried fish and placed on the ground. As the focus of our study was on nocturnal species, traps were opened at 7 – 9 p.m. and checked the next morning. Sampling locations covering an area of ca. 15 * 10 km in extent. We randomly placed 10 – 40 traps per night in various areas and habitats, usually with distances of 10 – 500 meters lying between adjacent traps. A detailed report of the occurrence of species in different habitat type is provided in [34]. Captured animals were transferred to a field laboratory and sacrificed by an overdose via inhalation of narcotics and then cervical dislocation (according to guidelines by the American Veterinary Medical Association, https://www.avma.org; see also [35]. Species identification was based on morphological characters according to various sources [35,36]. However, as individuals of Asian black rats (*Rattus rattus* complex) cannot be distinguished based on morphometry alone and as several species are likely to co-occur elsewhere in SEA [37,38], we noted all individuals as "*Rattus rattus* sp. complex" while emphasizing that this species complex might contain unknown genetic structure at the species level.

From all rodents including mainland and insular, the viscera were removed and preserved in 70% ethanol and dissected in the laboratory or freshly killed rodents dissected in our field labs when possible. Isolated worms belonging to genus *Trichuris* after [39] were preserved in 95% ethanol until DNA extraction was performed.

For the morphometrical study, specimens from the same host specimens from which molecular samples were obtained, were cleared in Amman lactophenol, examined under a compound microscope and measured with a microscope-mounted camera. All measurements are given in micrometres; listed as holotype or allotype, followed by the range of the paratypes in parentheses. A subset of samples was examined by scanning electron microscopy (SEM) examination, dehydrated in an ethanol series and critical point dried with carbon dioxide in a Polaron CPD 7501. Finally, specimens were mounted on stubs with adhesive tape and colloidal silver, sputter-coated with gold in a Fisons Instrument SC 510, and examined using a JEOL JSM6440LV at 10 kV**.**

The total genomic DNA of the individual adult worm was extracted using E.Z.NA® Tissue DNA kit (Omega bio-tek, USA) following the manufacture's protocol. The Internal transcribed spacer (ITS1-5.8S-ITS2) region was amplified using NC2 and NC5 primers [40]. The thermal cycling condition is as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min and a final extension at 72°C for 5 min. The PCR products were separated on 1% agarose gel and cut for gel-purification.

All PCR products were gel-purified using E.Z.NA® Gel Extraction kit (Omega bio-tek, USA). The purified PCR products were cycle-sequenced using ABI BigDye v3.1 (Warrington, UK) and run on an ABI Prism 377 automated sequencer (Perkin-Elmer Corp., Foster City, CA, USA). In case of unreadable chromatograms observed due to multiple potentially divergent copies present in a particular specimen, the purified PCR product was cloned into a pGEM-T easy vector (Promega, Madison, WI), according to manufacturer's instructions. The recombinant plasmid was introduced and propagated in *Escherichia coli* JM109, and four to six white colonies were randomly selected for plasmid DNA extraction using the FastGene® Plasmid Mini kit (Nippon

Genetics Co., Ltd., Japan). Plasmid DNA was used as template for cycle sequencing in both directions.

Phylogenetic analyses were conducted on the sequences obtained during the present study including *T. muris* from Europe from Genbank (FM955260.1: *T. muris* from *A. sylvaticus* in Spain) and *T. muris* analysed from *M. domesticus* from Catalonia (Spain) from first author's collection); also, other four sequences of *Trichuris* species of rodents from GenBank, namely X683525.1: *T. mastomysi* from *Mastomys natalensis* from Tanzania; KX669085.1: *Trichuris* sp. from *M. natalensis* in Benin, JX683524.1: *T. carlieri* s.l. from *Gerbilliscus vicinus* in Tanzania; KX669087.1: FN543185.1: *T. arvicolae* from *Myodes glareolus* Spain were included. Whereas *T. duplantieri* from *Gerbillus gerbillus* in Mauritania was used as outgroup. Obtained sequences in this study were deposited in the Genbank with accession numbers MN428339- MN428406. Phylogenetic tree analyses were performed based on Kimura-2-parameter model [41] for constructing Maximum Likelihood (ML), and Neighbour-Joining (NJ) by using MEGA7 program [41,42] with nodal support estimated using 1,000 bootstrap re-sampling. A consensus tree was selected to represent topology with a supporting node with ML/NJ in each branch of the tree.

3. Results

3. 1. Samples

A total of 3,665 rodents were examined, with whipworm specimens obtained in a proportion of samples as follows: Cambodia (2/571) in *M. surifer* and *Rattus* R3 (sensus [36]; Lao PDR (1/310) in *Rattus tanezumi*; Thailand (18/2158): *Bandicota indica* (12), *Bandicota savilei* (1), *Niviventer fulvescens* (1), *Rattus exulans* (1), *Rattus sakaretensis* (2) and *R. tanezumi* (1); and Vietnam (0/136) and Borneo (35/490) (details on Bornean samples are presented in Table 1).

3. 2. Description

3.2.1. Trichuris cossoni n. sp.

Males (n = 7): Anterior portion of the body 9,589 (range: 7,381 – 11,322); posterior portion 5,374 (3,286 – 7,619); esophago - intestinal junction width 203 (159 – 260); maximum posterior width 347 (296 – 463); ratio of the posterior-anterior body lengths 1:1.84 (1:1.52 – 1:2.24) ; spicule length 1,079 (689 – 1,443) with distal tip rounded; width of the spicule at end 38 (22 – 69); width of the spicule sheath (distal) end 38 (28 – 59); distal cloacal tube 523 (320 – 703); proximal cloacal tube 621 (296 – 1,296); distance from the posterior testes to the tail end 1,270 (792 – 2,000) (n = 6) (Fig 1C).

Gravid females (n = 5): Anterior portion of the body 14,712 (11,000 – 16,333); posterior portion 8,257 (6,857 – 10,619); esophago - intestinal junction width 223 (182 – 270); maximum posterior width 434 (396 – 490); ratio of posterior-anterior body lengths 1:1.8 (1:1.43 – 1:2.16); distance between oesophago - intestinal junction and vulva 90 (33 – 140); vagina length 886 (792 – 981); protruding vulvar appendage (Fig.1B); distance from the ovary loop to the tail end 359 (182 – 509) (n = 4); the egg size was 68 (65 – 72) × 30 (28 – 30) (n = 30).

3.2.1.1 Taxonomic summary

***Type host species*:** *Bandicota indica*

***Type locality*:** Huay Muang village, San Tong subdistrict, Tha Wang Pha district, Thailand (19°08'21.3396"N, 100°43'09.2964"E).

Data of collection: 23 February of 2015

***Site of infection*:** Caecum.

***Type specimens*:** Deposited at “Museu de Ciències Naturals de Barcelona” (Natural Science Museum of Barcelona), including a holotype (MZB 2017-1238), allotype (MZB 2017-1239) and paratypes (MZB 2020-0219).

***Etymology*:** The specific name refers to our colleague Jean François Cosson for our long-standing scientific collaboration, including the study of SEA rodents and its parasites.

3.2.1.2 Remarks

T. cossoni n. sp. resembles *T. muris* and *T. germani* in having a round tip of the spicule, but clearly differs from *T. musseri* and *T. mallomyos*, both possessing a sharply pointed distal tip of the spicule [18]. *T. cossoni* n. sp. is also distinguished from *T. germani*, which has a much longer spicule (1,700 – 3,300 μm long) [17]. The spicule length of *T cossoni* n. sp. overlaps partially with that of *T. muris* (600 - 800 μm [3] or 580 - 990 μm [10]). However the new species is distinguishable from *T. muris* by having a protruding vulvar appendage [10].

3.2.2. Trichuris arrizabalagai n. sp.

Males (n = 14): Anterior portion of the body 12,451 (range: 10,581 – 14,333); posterior portion 6,813 (4,857 – 8,809); esophago - intestinal junction width 204 (171 – 234); maximum posterior width 411 (333 – 453); ratio of the posterior-anterior body lengths 1:1.87 (1:1.3 – 1:2.58); spicule length 1,960 (1,648 – 2,215) with distal tip rounded; width of the spicule end (proximal) 51 (31 – 87); width of the spicule sheath (distal) end 34 (18 – 38) (n = 13); distal cloacal tube 786 (574 – 981); proximal cloacal tube 935 (537 – 1,185); distance from the posterior testes to the tail end 1,581 (1,207 – 2,025) (Fig. 1D).

Gravid females (n = 17): Anterior portion of the body 14,730 (9,714 – 19,190); posterior portion 8,736 (4,857 – 12,000 μm); esophago - intestinal junction width 213 (164 – 276); maximum posterior width 409 (358 – 490); ratio of posterior-anterior body lengths 1:1.72 (1: 1.22 – 1: 2.27); distance between oesophago -intestinal junction and vulva 144 (37 – 342); vagina length 1,357 (830 – 2,428); highly protruding vulvar ap-

pendage covered with spines visible on optical microscope (Fig. 1A, 2); distance from the ovary loop to the tail end 295 (117 – 641) (n=13); the egg size was 71 (65 – 74) × 30 (28 – 30) (n =30).

3.2.2.1 Taxonomic summary

Type host species**:** *Sundamys muelleri.*

Type locality**:** Kota Kinabalu, Likas subdistrict, Malaysia, Borneo (0°39'49"N, 066°08'21"E).

Data of collection: 20 of July of 2012

Site of infection**:** Caecum.

Type specimens: Deposited at "Museu de Ciències Naturals de Barcelona", including a holotype (MZB 2017-1240) and allotype (MZB 2017-1241). Paratypes are deposited at the Sabah Parks Museum in Sabah, Malaysia (SP32315 and SP32316).

Etymology**:** The specific name refers to Antoni Arrizabalaga, director of Museu de Granollers-Natural Sciences, Barcelona, Spain who was a crucial mentor to the first author early in his career.

3.2.2.2 Remarks

T. arrizabalagai n. sp. resembles *T. cossoni* n. sp, *T. muris* and *T. germani* in having a round tip of the spicule, but clearly differs from *T. musseri* and *T. mallomyos*, both possessing a sharply pointed distal tip of the spicule [18]. *T. arrizabalagai* n. sp. is also distinguished by having much longer spicule than *T. cossoni* n. sp (689 – 1,443 μm) and *T. muris* (600 - 800 μm [3] or 580 - 990 μm [10]). The new species is distinguishable from *T. germani* by having a protruding vulvar appendage [17].

3. 3. Molecular analyses

A total of 68 sequences of 37 whipworm individuals from SEA including Thailand and Boneo Island, and one from *M. domesticus* from Spain were successfully amplified

(Fig. 3; Table 1 for details). After trimmed all sequences found that the nucleotides length 803 – 816 bp including 4 polymorphic and 13 InDel sites, 963 – 990 bp including 38 polymorphic and 27 InDel sites, and 932 – 936 bp including 7 polymorphic and 4 InDel sites were observed in whipworms from Thailand, Borneo Island and Spain, respectively (data not shown). Comparison between the whipworms from Thailand and Borneo found 90 positions (11.95%) were differences. Whereas comparison between *T. muris* from Spain with Thai and Bornean whipworms found 271 positions (32.65%) and 253 positions (31.70%) differences, respectively. The nucleotide variation and differentiation sites were found only in ITS1 and ITS2 regions, whereas 5.8S region was monomorphic. A consensus tree from ML and NJ analyses revealed that the SEA whipworms were separated into two distinct clades, namely Thai clade and Bornean clade, whereas a clade of *T. muris* from Spain was closely clustered with another species of *Trichuris* retrieved from GenBank database, but distinct from the SEA whipworms with high supporting bootstrap values (Fig. 4).

4. Discussion

Both described new species are clearly distinguished by molecular criteria from *T. muris*, revealing also clear biogeographic structure and between Thai and Bornean individuals. The whipworm samples from SEA clustered in two closely related groups, suggesting a potential common ancestor. No molecular information exists on previously reported specimens from SEA and Oriental *Trichuris* from rodents, but the clear morphological and metrical differences provide the first evidence of distinct species. As more species of *Trichuris* are described, additional molecular studies are necessary to avoid the shortfall of morphometrical characters to clearly distinguish different species [7, 11].

Despite the geographic structure of specimens found in our study with one species associated to the island of Borneo (Sundaic bioregion) and another to peninsular SEA (Indochinese bioregion), geographic distance alone is unlikely the sole driver of speciation. This is because repeated changes in sea level, resulting in the periodic connection of the landmasses across the shelf [43], several rodents are shared between Borneo and Malay Peninsula (e.g. *L. sabanus*, *Maxomys rajah*, *M. surifer* and *M. whiteheadi*) [44]. A second factor is the recent translocations of rodents mediated by humans [45]. Therefore, future research into the biogeographic distribution and range limits of the different whipworm species is necessary to unravel whether the two newly described species *T. cossoni* n. sp. and *T. arrizabalagai* n. sp. exhibit overlapping or discrete geographic ranges and which drivers determine their distribution.

Despite successfully obtaining *Trichuris* samples from extensive trapping efforts on Muridae hosts (subfamily Murinae), we currently lack information on potential additional rodent host species such as Sciuridae (terrestrial and arboreal squirrels exhibit a high species diversity in SEA) that should be considered in future research. The original description of *Trichuris megaloon* from a single female worm in Prevost's squirrel (*Callosciurus prevostii*) from Sumatra Island, for example, gave eggs measurements 88 – 96 μm length by 44 – 48 μm wide (according to the original description [46]. These eggs sizes were larger than our measurements for *T. megaloon*: (65 – 72 μm) × (28 – 30 μm) in *T. cossoni* n. sp. and (65 – 74 μm) × (28 – 30 μm) in *T. arrizabalagai* n. sp.. However, no morphometrical data is available for *T. megaloon* despite additional records made elsewhere (e. g., in Vietnam by Phan [47]). The original description provides limited information to conclude on the taxonomic relationships of whipworms from squirrels and those found in Murinae in SEA. Future research is necessary to perform a redescription of *T. megaloon* and other species to better

understand whipworm diversification, patterns of host sharing and geographic distribution.

The study of *T. muris* in the house mouse in Europe by Wasimuddin et al. [6] reported a pan-European clade, that included strains carried by the house mouse or rat in recent times, but our novel species descriptions lead to the question if the distribution and dispersal of this whipworm species by far-ranging invasive host species such as *Rattus norvegicus*/*rattus* is reduced, as we report distinct *Trichuris* species from Murinae in SEA. Nevertheless, important details to conclude of the true presence/absence of *T. muris* in Asia (including our study region) is currently lacking, including missing molecular information from Chinese *Trichuris* from rodents, as the location of origin of the host species *R. norvegicus* before its worldwide expansion including Europe [48], together with lack of molecular information in the far west to corroborate this possible origin [6]. Our results suggest that for species such as the two cosmopolitan invasive rats *R. norvegicus* and *R. rattus* (present in SEA), which previously have been assumed to harbour *T. muris* across all its geographic range [14,44], associations with *T. muris* and conspecific parasites need to be reconsidered for its taxonomic validity.

We report the newly described species from a range of different host species such that *T. cossoni* n. sp. is currently known to be shared by *B. indica* and *Mus pahari* and *T. arrizabalagai* n. sp. by *R. rattus* complex, *R. norvegicus* and *Sundamys muelleri*. Given the large geographic ranges of some of the host species (and likely additional host species unknown to date because of no exhaustive surveillance), we believe that both *Trichuris* likely have much larger geographic distributions as reported here. The host species *B. indica*, for example, is found throughout most of the Indian subcontinent [49], so we can expect a wide distribution of *T. cossoni* n. sp.. Among *Trichuris*

specimens of interest for determining its taxonomic status are also those from peninsular Malaysia [29], as one of the geographic locations in between our two study locations.

According to our results, all previous reports of *T. muris* in SEA, as well as other nearby areas (e. g.: China and India) should be reconsidered [50,51,52]. Our results urges for caution when using specimens of *Trichuris* of different geographic origin for laboratory, given that established evidence from our study question the unique species identity. As an example, laboratory studies which are using whipworms from India [53], where the rodent fauna overlap with those of Thailand, may risk to use *T. cossoni* n. sp. instead of *T. muris* or perhaps even other yet to be described species.

5. Conclusions

Our molecular data with the support of additional morphometric attributes show specimens of *Trichuris* from SEA rodents to be distinct from *T. muris* as described elsewhere. We propose two new species from Thailand and Borneo Island: *T. cossoni* n. sp. and *T. arrizabalagai* n. sp. as *T. muris* is used extensively as a laboratory model, the taxonomic identity should be taken into consideration for the repeatability of experiments.

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559 **Figure 1.** Microphotographs showing vulvar region in *T. arrizabalagai* n. sp. (A) and *T.*
560 *cossoni* n. sp. (B) and posterior region of male of *T. arrizabalagai* n. sp. (C) *and T.*
561 *cossoni* n. sp. (D).

562 **Figure 2.** Scanning electronic microscope of the vulvar region of *T. arrizabalagai* n.
563 sp..

564 **Figure 3.** Geographical distribution of the whipworms studied in the present study: *T.*
565 *cossoni* n. sp. (black squares) and *T. arrizabalagai* n. sp. (black star).

566 **Figure 4.** Strict consensus tree of ML- NJ analyses using sequences of ITS1-5.8S-ITS2
567 of whipworm isolates of Southeast Asia and Spain. The sequences of other isolates as
568 well as related species available in GenBank were also included. Nodal supports are of
569 bootstrap values obtained by ML and NJ analyses, respectively. The scale-bar indicates
570 the expected number of substitutions per site. A sequence of *T. duplantieri* was used as
571 out-group.

572 **Table 1.** Prevalence of *T. arrizabalagai* n. sp. in Borneo rodents.