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VERSITA

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New data on *Malayometastrongylus diardinematus* (Gibbons and Krishnasamy, 1986) (Metastrongyloidea: Angiostrongylidae) a lungworm occurring in *Rattus tanezumi* (Temminck, 1844) from South-East Asia

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Summary

A survey of rodents from Luang Prabang Province (Lao PDR) in February 2010 and May 2012 allowed examining lungs of 95 rodents belonging to 7 different genera (*Bandicota*, *Berylmys*, *Cannomys*, *Leopoldamys*, *Maxomys*, *Mus*, *Rattus*). The helminth *Malayometastrongylus diardinematus* (Nematoda: Metastrongyloidea) was identified in *Rattus tanezumi* living in close contact with humans, being the first report after its original description in *R. tanezumi* from Malaysia. A molecular study using mitochondrial gene cytochrome *c* oxidase subunit I (COI) of *M. diardinematus* placed the genus *Malayometastrongylus* in a separate clade in comparison with *Angiostrongylus cantonensis* (subgenus *Parastrongylus*) and other representatives of the genus, both present in rodents of South East Asia.

Keywords: Metastrongyloidea; genes; Rodentia; Laos

Introduction

Malayometastrongylus diardinematus (Gibbons & Krishnasamy, 1986) was described as a new genus of nematode from lungs of the Asian house rat *Rattus tanezumi* Temminck, 1844 (described originally as *Rattus rattus diardii*) in Malaysia. The original description of *M. diardinematus* lacks of information concerning locality, number of hosts studied and if other rodent species were examined for the presence of this lungworm. This nematode has never been reported afterwards despite the Asian house rat shows a wide distribution (Aplin *et al*., 2003).

Other four angiostrongylids have been reported in rodents from South-East Asia (SEA): *Angiostrongylus cantonensis* (subgenus *Parastrongylus*) (Chen, 1935), *Angiostrongylus malaysiensis* (subgenus *Parastrongylus*) (Bhaibulaya & Cross, 1971), *Angiostrongylus siamensis* (subgenus *Parastrongylus*) (Ohbayashi *et al*., 1979) and *Thaistrongylus harinasutai* (Ohbayashi *et al*., 1979).

Angiostrongylus cantonensis (subgenus *Parastrongylus*) is recognized as a zoonotic helminth that has caused hundreds of human angiostrongyliasis worldwide (Wang *et al*., 2012). This nematode has been reported in central Thailand (Bangkok-Thonburi near the mouth of the Chao Phraya River); North-East (Nakornrajsrima, Ubonratchatani and Udornratchatani); North (Chiang Mai, Sukothai and Nakhon Sawan); West (Kanchanaburi and small villages up the two forks of the Khwae River); South-East (Trat, Chantaburi and Rayong) and South Peninsula (Narathiwat, Yala, Songkhla, Trang, Nakhon Si Thamarat, Krabi, Ranong, Chumpon and Prachuap Khiri Khan) (Crook *et al*., 1968) from rodents as *Bandicota bengalensis* Gray, 1835, *Bandicota indica* (Bechstein, 1800), *Rattus exulans* (Peale, 1848), *Rattus norvegicus* (Berkenhout, 1769), *Rattus rattus* (Linnaeus, 1758) and *Rattus* sp. It was also reported from North-East Thailand in *R. norvegicus* and *B. indica* (Pipitgool *et al*., 1997) and North Thailand (Chiang Mai) in *R. norvegicus* and *R. rattus* (Namue & Wongsawad, 1997). In other countries as Philippines in *R. tanezumi* (Antolin *et al*., 2006) and *Rattus rattus mindanesis* (syn. *Rattus rattus*) (Fedorko, 1999); in Malaysia in *Rattus argentiventer* (Robinson & Kloss, 1916), *Rattus tiomanicus* (Miller, 1900) (syn. *Rattus jalorensis*) (Singh & Cheong, 1971) and additionally in *Leopoldamys sabanus* (Thomas, 1887) (syn. *Rattus sabanus*) (Liat *et al*., 1975), also in *Rattus exulans concolor* (syn. *Rattus exulans*) and *Rattus rattus diardii* (syn. *Rattus tanezumi*) (Schacher & Cheong, 1960); and in Indonesia (Java Island) from rodents as *R. tanezumi* (syn. *R. r. diardii*) and *Maxomys bartelsii* (Jentink, 1910) (syn. *Rattus bartelsii*) (Wiroreno, 1978). The presence of *A. cantonensis* has not been reported in rodents of other countries of SEA.

Angiostrongylus malaysiensis (subgenus *Parastrongylus*) has been reported in Malaysia from *R. tanezumi* (syn. *R. r. diardii*) (Gibbons & Krishnasamy, 1986; Leong *et al*.,

1979), *R. norvegicus* and *R. rattus* (Mohd Zain *et al*., 2012), *Rattus jalorensis* (syn. *Rattus tiomanicus*) (Bhaibulaya & Cross, 1971), *R. tiomanicus* (Krishnasamy *et al*., 1980), and in Rattus spp. (*R. tiomanicus*, *R. r. diardii*, *R. argentiventer*, *R. norvegicus*) (Nur Syazana *et al*., 2013) in central and western regions of Thailand in *R. tiomanicus* (syn. *R. jalorensis*) (Bhaibulaya & Techasoponmani, 1972) and in Indonesia in *B. indica*, *Niviventer lepturus* (Jentink, 1879) (syn. *R. lepturus*), *R. tanezumi* (syn. *R. r. diardii*), *R. exulans*, *R. norvegicus* and in *R. tiomanicus* (syn. *R. jalorensis*) (Krishnasamy *et al*., 1980).

Angiostrongylus siamensis (subgenus *Parastrongylus*) was reported in Thailand from *L. sabanus* (Ohbayashi *et al*., 1979), *Berylmys berdmorei* (Blyth, 1851), *R. rattus* and *Maxomys surifer* (Miller, 1900) (syn. *Rattus surifer*) (Kamiya *et al*., 1980) and in *Bandicota savilei* Thomas, 1916 and *B. indica* (Ohbayashi *et al*., 1983).

Finally, *T. harinasutai* was recovered from *B. berdmorei* in Thailand and identified by comparison with the original materials of description (Gibbons & Krishnasamy, 1986), and additional morphological characters were provided. In conclusion, previous studies on angiostrongylids of rodents from SEA, except for *A. cantonensis* are limited to few studies on Indonesia, Malaysia and Thailand.

The phylogenetic position of some angiostrongylids were studied using cytochrome *c* oxidase subunit I (COI) (*A. cantonensis* and *A. malaysiensis*) (Eamsobhana *et al*., 2010) and with the complete internal transcribed spacer 2 (ITS-2) and a fragment of the small subunit ribosomal RNA(SSU rRNA) nucleotide sequences (*A. cantonensis*) (Foronda *et al*., 2010). Excluding *A. cantonensis* and *A. malaysiensis*, genetic information is lacking on the other species of angiostrongylids occurring on rodents of SEA.

In the framework of CERoPath and BiodivHealthSEA projects, we report and provide new data: metrical, molecular and biogeographical on the angiostrongylid *M. diardinematus* found in *R. tanezumi* associated to human habitats in Lao PDR.

Material and methods

Helminth collection

A total of 95 rodents: *B. indica* (n = 13); *B. berdmorei* (n = 1); *Cannomys badius* (Hodgson, 1841) (n = 7); *Leopoldamys edwardsi* (Thomas, 1882) (n = 4); *Leopoldamys neilli* Marshall, 1976 (n = 2); *M. surifer* (n = 2); *Mus cokii* Ryley, 1914 (n = 3) and *R. tanezumi* (n = 63) captured from Luang Prabang Province (Lao PDR) in Xiang Gneun district (19°36'44''N; 102°2'41''E) in 2010 and from the village of Lak Sip (19°50'55''N; 102°10'4''E) in 2012, were dissected in order to examine its lungs for helminths. Rodents were euthanized and dissected following protocols that maximize animal care, health and safety of field parasitologists and generation of quality data (Herbreteau *et al*., 2011) (see http://www.ceropath.org/research/protocols). All rodents were identified by morphology following the nomenclature and classification in (Aplin *et al*., 2003; Marshall, 1988; Pagès *et al*., 2010). For the problematic species, a molecular identification by using species specific primers and/or barcoding assignments were applied (Galan *et al*., 2012), details available in *Barcoding Tool/RodentSEA* section of the CERoPath web site: http://www.ceropath.org.

Nematodes were isolated from lung tissues, transferred to 70 % alcohol and examined in the Laboratory of Parasitology of the Faculty of Pharmacy (University of Barcelona, Spain). For morphological characterization, helminths were mounted in Amman lactophenol. Measurements are in millimetres and compared to measurements of original description by Gibbons & Krishnasamy (1986). To complete the molecular study we included *Parastrongylus dujardini* (Drozdz & Doby, 1970) from the rodents *Apodemus* sp. and *Myodes glareolus* (Schreber, 1780) captured in Pyrenees Mountains (France) (42°29′47.76″N;2°21′4.68″E) and *A. cantonensis* (subgenus *Parastrongylus*) from rodents captured in Thailand: *R. tanezumi* (captured in Chiangrai (19º50'38.11"N;99º57' 55.14"E) and Ranong (10º28'32.88"N;98º48'33.96"E)) and *B. indica* (captured in Chiangrai (19º50'38.11"N;99º57' 55.14"E)).

DNA extraction

Genomic DNA of each worm (*P. dujardini*, *A. cantonensis* (subgenus *Parastrongylus*) and *M. diardinematus*) was extracted using tissue Genomic DNA Mini Kits (Favor-Prep^{TM}, Taiwan), according to the manufacturer's protocol.

PCR amplification and DNA sequencing

The DNA amplification by Polymerase Chain Reaction (PCR) was conducted with primers designed from COI sequences of *A. cantonensis* (subgenus *Parastrongylus*) (GenBank accession number GQ398121), as Ac-COI Forward 5'GTTCTAATCATAAGGATATTG3' and Ac-COI Reverse 5'CAAACAAACAAACTCATATG3'. PCR amplicons were approximately 500 bp in length. 50 µL of each PCR reaction was composed of the 1xKAPA TaqReadyMix DNA Polymerase (1U KAPA Taq Polymerase, 1.5 mM MgCl2 and KAPA Taq Polymerase Buffer), 0.25 µM of each primer, and 1 µL of DNA template. Amplification conditions were initially heated at 94 °C for 3 min, followed by 34 amplification cycles, consisting of denaturation at 95 °C for 30 sec, annealing at 53 °C for 30 sec and elongation 72 °C for 1 min and final extension at 72 °C for 5 min. PCR products were run into 1.0 % agarose gel and visualized on UV-transilluminator. Each PCR amplicons was sequenced using the 2 amplification primers on an ABI's 3730XL DNA Analyzers and BigDye Terminator at AITBIOTECH Pte Ltd (Singapore).

COI sequences from GenBank

COI sequences from *Angiostrongylus vasorum* (Baillet, 1866), *A. cantonensis* (subgenus *Parastrongylus*), *Parelaphostrongylus tenuis* Dougherty, 1945, *Metastrongylus pudendotectus* (Wostokow, 1905), *Metastrongylus salmi* (Gedoelst, 1823) and *Schistosoma mekongi* Voge *et al*., 1978 (used as an outgroup) were obtained from the

Table 1. Uncorrected 'p' distance (%) between angiostrongylids

	A. dujardinii, France, *Apodemus* sp.	*A. dujardinii*, France, *M. glareolus*	*A. cantonensis*, GQ398121, China	*A. cantonensis*, NC013065, China	*A. cantonensis*, Thailand, *B. indica*	*M. pudendotectus*, NC013813, Estonia, *Sus* sp.	*A. vasorum*, GQ982876, Germany, *V. vulpes*	*A. vasorum*, GQ982785, United Kingdom, *V. vulpes*	*A. vasorum*, GQ982734, Germany, *V. vulpes*	*A. vasorum*, GQ982777, United Kingdom, *V. vulpes*	*A. vasorum*, GQ982875, Portugal, *V. vulpes*	*A. vasorum*, GQ982835, Denmark, *V. vulpes*	*A. vasorum*, GQ982858, Denmark, *C. familiaris*	*A. cantonensis*, Thailand, *R. tanezumi*	*A. cantonensis*, Thailand, *R. tanezumi*	*M. salmi*, NC013815, Estonia, *Sus* sp.	*P. tenuis*, EF173723, USA, *O. virginiamus*	*M. diardinematus*, Laos, *R. tanezumi*	*S. mekongki*, AF217449, Laos
A. dujardinii, France, *Apodemus* sp.		0.000	0.013	0.013	0.013	0.015	0.013	0.013	0.013	0.012	0.012	0.012	0.012	0.013	0.014	0.016	0.016	0.016	0.022
A. dujardinii, France, *M. glareolus*	0.000		0.013	0.013	0.013	0.015	0.013	0.013	0.013	0.012	0.012	0.012	0.012	0.013	0.014	0.016	0.016	0.016	0.022
A. cantonensis, GQ398121, China	0.100	0.100		0.000	0.010	0.016	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.012	0.012	0.016	0.016	0.016	0.023
A. cantonensis, NC013065, China	0.100	0.100	0.000		0.010	0.016	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.012	0.012	0.016	0.016	0.016	0.023
A. cantonensis, Thailand, *B. indica*	0.109	0.109	0.049	0.049		0.016	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.013	0.013	0.016	0.015	0.016	0.022
M. pudendotectus, NC013813, Estonia, *Sus* sp.	0.169	0.169	0.183	0.183	0.171		0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.017	0.017	0.014	0.016	0.016	0.023
A. vasorum, GQ982876, Germany, *V. vulpes*	0.095	0.095	0.120	0.120	0.132	0.171		0.003	0.000	0.003	0.003	0.003	0.003	0.016	0.016	0.016	0.015	0.016	0.022
A. vasorum, GQ982785, United Kingdom, *V. vulpes*	0.095	0.095	0.120	0.120	0.132	0.171	0.005		0.003	0.003	0.003	0.003	0.003	0.015	0.015	0.016	0.015	0.016	0.022
A. vasorum, GQ982734, Germany, *V. vulpes*	0.095	0.095	0.120	0.120	0.132	0.171	0.000	0.005		0.003	0.003	0.003	0.003	0.016	0.016	0.016	0.015	0.016	0.022
A. vasorum, GQ982777, United Kingdom, *V. vulpes*	0.093	0.093	0.116	0.116	0.127	0.169	0.005	0.005	0.005		0.000	0.000	0.000	0.015	0.015	0.016	0.015	0.015	0.022
A. vasorum, GQ982875, Portugal, *V. vulpes*	0.093	0.093	0.116	0.116	0.127	0.169	0.005	0.005	0.005	0.000		0.000	0.000	0.015	0.015	0.016	0.015	0.015	0.022
A .vasorum, GQ982835, Denmark, *V. vulpes*	0.093	0.093	0.116	0.116	0.127	0.169	0.005	0.005	0.005	0.000	0.000		0.000	0.015	0.015	0.016	0.015	0.015	0.022
A. vasorum, GQ982858, Denmark, *C. familiaris*	0.093	0.093	0.116	0.116	0.127	0.169	0.005	0.005	0.005	0.000	0.000	0.000		0.015	0.015	0.016	0.015	0.015	0.022
A. cantonensis, Thailand, *R. tanezumi*	0.111	0.111	0.081	0.081	0.093	0.192	0.150	0.148	0.150	0.146	0.146	0.146	0.146		0.002	0.016	0.015	0.015	0.023
A. cantonensis, Thailand, *R. tanezumi*	0.113	0.113	0.081	0.081	0.093	0.194	0.150	0.148	0.150	0.146	0.146	0.146	0.146	0.002		0.016	0.016	0.015	0.023
M. salmi, NC013815, Estonia, *Sus* sp.	0.171	0.171	0.164	0.164	0.153	0.132	0.164	0.164	0.164	0.160	0.160	0.160	0.160	0.171	0.171		0.017	0.017	0.023
P. tenuis, EF173723, USA, *O. virginiamus*	0.144	0.144	0.150	0.150	0.150	0.169	0.137	0.134	0.137	0.137	0.137	0.137	0.137	0.155	0.157	0.178		0.016	0.023
M. diardinematus, Laos, *R. tanezumi*	0.157	0.157	0.162	0.162	0.157	0.150	0.148	0.148	0.148	0.144	0.144	0.144	0.144	0.155	0.155	0.162	0.162		0.022
S. mekongki, AF217449, Laos	0.412	0.412	0.424	0.424	0.428	0.426	0.421	0.419	0.421	0.417	0.417	0.417	0.417	0.410	0.412	0.419	0.417	0.389	

GenBank as follows: *A. vasorum* GQ982777, GQ982875, GQ982835, GQ982858, GQ982785, GQ982876, GQ982734; *A. cantonensis* (subgenus *Parastrongylus*) GQ398121, NC013065; *P. tenuis* EF173723; *M. pudendotectus* NC013 813; *M. salmi* NC013815; and *S. mekongi* AF217449.

Sequence alignment and phylogenetic analysis

The electropherogram of each sequence was examined for sequence accuracy by BioEdit program version 7.0 (Hall, 1999). The DNA sequences of COI were aligned by CLUSTAL X (Thompson, 1997). All gap sites in the sequences were removed from the alignment. Phylogram was constructed from COI sequences by neighbour-joining (NJ) method by estimating 1000 replications with *p*-distance in MEGA 5 (Tamura *et al*., 2011).

Results and discussion

Measurements of worms: oesophagus length 0.277 – 0.307 (0.288), n = 3; right spicule 0.195 – 0.198 (0.196), n = 2; left spicule 0.195 – 0.204 (0.200), n = 2; distance from anus to tip of tail 0.064 – 0.072 (0.068), n = 2; distance from vulva to tip of tail 0.193 – 0.250 (0.214), n = 3; eggs (length x width) 0.050 – 0.059 (0.056) x 0.034 – 0.040 (0.037), n = 52; width at the base of oesophagus 0.035 – 0.039 (0.037), n = 2:, and oesophagus maximum width 0.060 – 0.069 (0.063), n = 3.

Helminths were found in lungs of 9 *R. tanezumi* captured in February 2010 from Xiang Gneun district, being all other captured rodents (86) negative for this worm. The worm fragility and as are imbricate in lungs do not allow counting the number of individuals in each host, additionally the presence of male nematodes was scarce.

Measures of worms are in accordance with original description (Gibbons & Krishnasamy, 1986) of *M. diardinematus*: oesophagus length 0.266 – 0.353, n = 8; spicules 0.171 – 0.240, n = 13; 0.163 – 0.225, n = 13; distance from anus to tip of tail 0.042 – 0.089, n = 4; distance from vulva to tip of tail 0.228 – 0.318, n = 4; eggs (length x width) 0.057 – 0.068 x 0.034 – 0.046, n = unknown.

This is the first report of *M. diardinematus* after the original description in *R. tanezumi* from Malaysia, so consequently this nematode has a wider distribution than previously expected as has been found in two points separated more than 2000 km (Malaysia and Lao PDR). *M. diardinematus* has a prevalence of 23.08 % in 2010 and was absent in 2012 samples. We suggest the lack of reports in SEA is related to the small size and fragility of these worms if we compare with *A. cantonensis* (subgenus *Parastrongylus*) and *A. malaysiensis* (subgenus *Parastrongylus*), being its zoonotic potential known (Wang *et al*., 2012).

A molecular study using mitochondrial gene cytochrome *c* oxidase subunit I (COI) of *M. diardinematus* confirms the validity of the genus *Malayometastrongylus* differing from

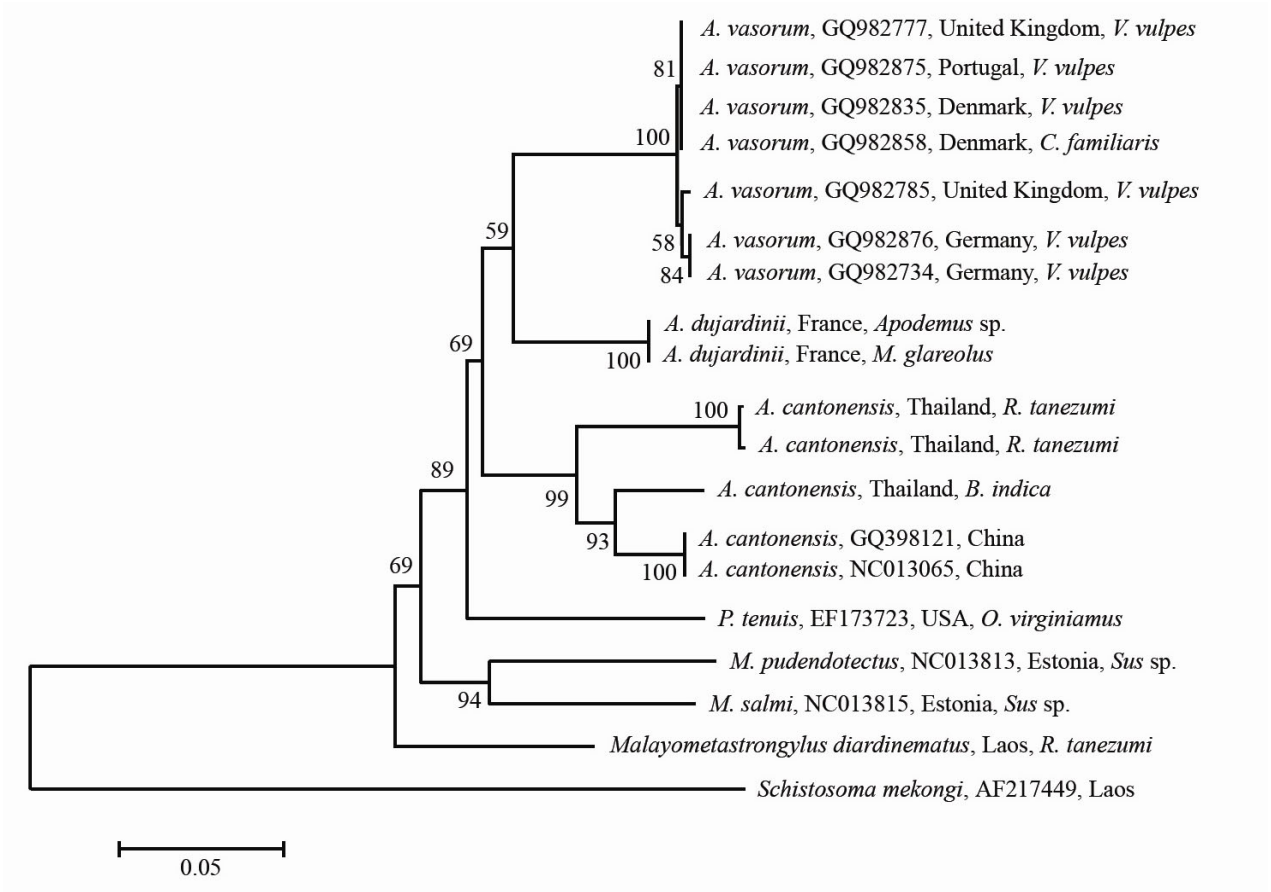

Fig. 1. Phylogeny tree using partial COI gene sequences by neighbour-joining (NJ) method

subgenus *Angiostrongylus* and *Parastrongylus* (Fig. 1). Our molecular results show that the phylogenetic analysis does not support the differentiation of the subgenus *Angiostrongylus* and *Parastrongylus* based in the mitochondrial gene COI as has also been concluded in previous studies (Eamsobhana *et al*., 2010). Molecular analysis shows that *Malayometastrongylus* is placed in a separate clade distant of *Angiostrongylus* and *Parastrongylus* (Figure 1; Table 1). Future research should be addressed in solve the phylogenetic position of the other four angiostrongylid reported in SEA rodents to a better understanding of this group of nematodes. Additionally, the limited number of available sequences of COI gene in angiostrongylids despite its high diversity of genera reveals further to research for better understanding of phylogenetic relationships.

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