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Angiostrongylus mackerrasae and *A. cantonensis* (Nematoda: Metastrongyloidea) belong to same genetic lineage: evidence from mitochondrial protein-coding genes

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Abstract

Angiostrongylus mackerrasae is a parasitic nematode of rats found in Australia. When first reported, it was referred to as *A. cantonensis*. Recent molecular studies, including the mitochondrial genome, indicate that it is highly similar to *A. cantonensis*. These studies did not include *A. malaysiensis*, another member of the *A. cantonensis* species complex, for comparison. The present study examined the genetic distance and phylogenetic relationship between the component taxa (*A. cantonensis*, *A. mackerrasae* and *A. malaysiensis*) of the *A. cantonensis* species complex, based on the 12 protein-coding genes (PCGs) of their mitochondrial genome. Both the nucleotide and amino acid sequences were analysed. *Angiostrongylus mackerrasae* and *A. cantonensis* are members of the same genetic lineage and both are genetically distinct from *A. malaysiensis*. The genetic distance based on concatenated nucleotide sequences of 12 mt-PCGs between *A. mackerrasae* and *A. cantonensis* from Thailand is p = 1.73%, while that between the Thai and Chinese taxa of *A. cantonensis* is p = 3.52%; the genetic distance between *A. mackerrasae* and *A. cantonensis* from China is p = 3.70%. The results indicate that *A. mackerrasae* and *A. cantonensis* belong to the same genetic lineage, and that *A. mackerrasae* may be conspecific with *A. cantonensis*. It remains to be resolved whether *A. mackerrasae* is conspecific with *A. cantonensis* or undergoing incipient speciation.

Introduction

The genus *Angiostrongylus* is represented by 21 valid named species and an unnamed species (Spratt, 2015). Rodents serve as the definitive host for 15 species of this genus of nematode parasites (Eamsobhana, 2014). Two species, *A. cantonensis* (Chen, 1935) and *A. costaricensis* (Morera & Céspedes, 1971) are zoonotic parasites of public health importance (Cross & Chen 2007; Eamsobhana, 2014; Spratt, 2015). Two other species of the *A. cantonensis* species complex, *A. mackerrasae* (Bhaibulaya, 1968) and *A. malaysiensis* (Bhaibulaya & Cross, 1971), have not been unequivocally shown to be involved in human infections, but their potential needs to be investigated as they share a similar life cycle with *A. cantonensis*.

When first documented in Malaysia, *A. malaysiensis* was referred to as *A. cantonensis* (Schacher & Cheong, 1960; Lim *et al.*, 1965). Both mitochondrial and nuclear genes

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have been used to differentiate *A. cantonensis* and *A. malaysiensis*. The mitochondrial genes cytochrome *c* oxidase subunit I (*cox1*) (Eamsobhana *et al*., 2010b, 2017a, b) and cytochrome *b* (*cob*) (Yong *et al*., 2015a) as well as the complete mitochondrial genome (Yong *et al*., 2016) unequivocally separated *A. cantonensis* and *A. malaysiensis*. Likewise, the nuclear genes, 66-kDa protein gene (Eamsobhana *et al*., 2010a) and ribosomal RNA small subunit (18S rRNA) gene (Eamsobhana *et al*., 2015) clearly separated *A. cantonensis* and *A. malaysiensis*.

As for *A. malaysiensis*, *A. mackerrasae*, when first documented in Australia, was referred to as *A. cantonensis* (Mackerras & Sandars, 1954, 1955). Recent studies based on molecular markers indicate that it is highly similar to *A. cantonensis* (Aghazadeh, 2015; Aghazadeh *et al*., 2015; Chan *et al*., 2015). These studies did not include *A. malaysiensis*, another member of the *A. cantonensis* species complex, for comparison. Furthermore, only a single mitogenome of *A. cantonensis* from China (GQ398121) was included for comparison based on amino acid sequence (Aghazadeh *et al*., 2015).

In the present study, we examined the genetic distance and phylogenetic relationships between the component taxa (*A. cantonensis*, *A. mackerrasae* and *A. malaysiensis*) within the *A. cantonensis* species complex, based on the 12 protein-coding genes from their mitochondrial genomes. Both the nucleotide and amino acid sequences were analysed. The results indicate that *A. mackerrasae* and *A. cantonensis* are members of the same genetic lineage and both are genetically distinct from *A. malaysiensis*.

Materials and methods

The complete mitochondrial genome of *A cantonensis* (KT947978 from Thailand; NC_013065 from China) and *A. malaysiensis* (NC_030332 from Malaysia) were downloaded from GenBank (National Center for Biotechnology Information, NCBI). The nucleotide sequences of the 12 protein-coding genes (PCGs) of *A. mackerrasae* from Australia were from Aghazadeh (2015). Other mitogenomes of *Angiostrongylus* available in NCBI's GenBank were included for comparison – *A. costaricensis* Costa Rica (KR827449), *A. costaricensis* Brazil (NC_013067) and *A. vasorum* (NC_018602). The mitogenomes of *Metastrongylus pudendotectus* (NC_013813) and *M. salmi* (NC_013815) were included as outgroup taxa.

Only the 12 PCGs were used for phylogenetic analysis as sequences for rRNA and tRNA genes were not available for *A. mackerrasae*. Phylogenetic analysis based on nucleotide sequences was performed as described in Yong *et al*. (2016). Evolutionary analyses based on amino acid sequences were conducted in MEGA7 (Kumar *et al*., 2016). The median joining (MJ) network (Bandelt *et al*., 1999) was used to estimate the genealogical relationships of the haplotypes. The MJ network was calculated using NETWORK v5.0.0.1 (http://www.fluxus-engineering.com).

Results and discussion

Figure 1 depicts the molecular phylogeny of *A. mackerrasae* in relation to other members (*A. cantonensis* and *A. malaysiensis*) of the *A. cantonensis* species complex and other taxa of the Angiostrongylidae, as well as *Metastrongylus* taxa, based on 12 mt-PCGs. *Angiostrongylus mackerrasae* formed a sister lineage with *A. cantonensis* and both were distinctly different from *A. malaysiensis*. The *A. cantonensis* species complex formed a distinct clade from that comprising *A. costaricensis* and *A. vasorum*.

The percentage of uncorrected 'p' genetic distances between different pairs of *Angiostrongylus* taxa and *Metastrongylus* taxa, based on nucleotide sequence and amino acid sequence of 12 PCGs, are summarized in table 1. Based on the nucleotide sequence, the genetic distance between *A. mackerrasae* and *A. cantonensis* from Thailand was p = 1.73%, while that between *A. mackerrasae* and *A. cantonensis* from China was p = 3.70%; the genetic distance between *A. cantonensis* from Thailand and from China was p = 3.52%. The genetic distance between *A. mackerrasae* and *A. malaysiensis* was p = 12.07% while that between *A. malaysiensis* and *A. cantonensis* was 11.89% (from Thailand) and 12.15% (from China). The genetic distance between other congeners was p > 15%.

The magnitude of genetic difference was similar based on amino acid sequence (table 1). The genetic distance

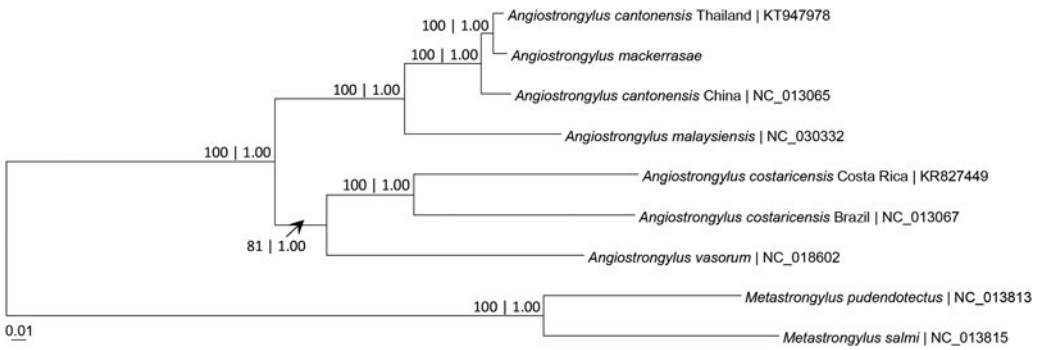

Fig. 1. Maximum likelihood and Bayesian inference tree based on 12 protein-coding genes of the whole mitogenomes of *Angiostrongylus* and *Metastrongylus* taxa. The total nucleotide sequence was 10,429 bp, AIC model = GTR + Gamma and BIC model = SYM + Gamma. Numeric values at the nodes are ML bootstrap/Bayesian posterior probabilities.The *A. cantonensis* species complex formed a distinct clade, with *A. mackerrasae* and *A. cantonensis* forming a sister lineage.

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Table 1. Percentage of uncorrected 'p' genetic distance between different pairs of *Angiostrongylus* taxa and *Metastrongylus* taxa based on the nucleotide sequence (below diagonal) and amino acid sequence (above diagonal) of 12 protein-coding genes (PCGs) of the mitochondrial genome.

Taxon	1	2	3	4	5	6	7	8	9
1 *Angiostrongylus mackerrasae*, Australia	–	2.6	5.8	20.2	29.9	29.6	28.0	36.5	37.5
2 *Angiostrongylus cantonensis*, Thailand KT947978	1.73	–	5.6	19.6	29.5	29.1	27.7	36.4	37.5
3 *Angiostrongylus cantonensis*, China NC_013065	3.70	3.52	–	20.7	29.1	29.3	28.0	37.1	38.0
4 *Angiostrongylus malaysiensis*, Malaysia NC_030332	12.07	11.89	12.15	–	29.6	29.8	29.7	36.6	38.7
5 *Angiostrongylus costaricensis*, Costa Rica KR827449	18.31	18.14	18.14	18.10	–	26.1	29.8	37.1	38.7
6 *Angiostrongylus costaricensis*, Brazil NC_013067	18.21	18.03	17.95	18.29	16.17	–	28.1	37.8	39.1
7 *Angiostrongylus vasorum*, NC_018602	16.62	16.41	16.70	18.10	18.10	17.47	–	38.2	39.4
8 *Metastrongylus pudendotectus*, NC_013813	22.95	22.78	22.98	22.72	23.14	23.31	23.84	–	25.0
9 *Metastrongylus salmi*, NC_013815	22.54	22.69	22.92	23.31	23.32	23.82	23.20	15.29	–

between *A. mackerrasae* and *A. cantonensis* was p = 2.6% (for the Thai taxon) and p = 5.8% (for the Chinese taxon), while that between the Thai and Chinese taxa of *A. cantonensis* was p = 5.6%. Compared to *A. malaysiensis*, the genetic distance was p = 20.2% for *A. mackerrasae*, p = 19.6% for *A. cantonensis* from Thailand, and p = 20.7% for *A. cantonensis* from China.

Based on the nucleotide sequence of cytochrome *c* oxidase subunit I (*cox1*) gene, the genetic distance between *A. mackerrasae* and *A. cantonensis* from Thailand was p = 1.90%, while that between the Thai and Chinese taxa of *A. cantonensis* was p = 3.80% (table 2). The genetic distance between *A. mackerrasae* and *A. malaysiensis* was p = 9.00%, and that between *A. cantonensis* and *A. malaysiensis* was p = 8.56–9.13%. Other congeners had genetic distances of p > 11%.

Haplotype network analysis based on individual mt-PCG revealed close genealogical relationships of *A. mackerrasae* and *A. cantonensis* compared to *A. malaysiensis* (table 3; fig. 2). *Angiostrongylus mackerrasae* and *A. cantonensis* from Thailand had the smallest difference in the number of base pairs in 11 of the 12 mt-PCGs (ATP6, COI, COB, COII, COIII, ND2, ND3, ND4, ND4L, ND5 and ND6), while *A. cantonensis* from Thailand and China had a larger difference (table 3). For the ND1 gene, *A. mackerrasae* differed from *A. cantonensis* from Thailand by 37 bp (4.35%) and from *A. cantonensis* from China by 50 bp (5.88%), while the difference between *A. cantonensis* from Thailand and China was 29 bp (3.41%).

Until recently, molecular studies were lacking for *A. mackerrasae* compared to the other members (*A. cantonensis* and *A. malaysiensis*) of the *A. cantonensis* species complex. A recent study indicates that *A. mackerrasae* and *A. cantonensis* are almost identical at two genetic loci (the nuclear internal transcribed spacer (ITS)-1 and 18S rRNA genes), indicating that these two taxa are conspecific (Chan *et al*., 2015). Another study, based on restriction enzymes for the *cox3* region, failed to distinguish *A. mackerrasae* from *A. cantonensis* (Aghazadeh, 2015). Based on the amino acid sequence of the 12 mitochondrial PCGs, the genetic distance between *A. mackerrasae* and *A. cantonensis* is p = 2.4% (Aghazadeh *et al*., 2015), indicating that these two taxa are genetically very similar.

Compared to *A. mackerrasae*, molecular markers (both mitochondrial and nuclear genes) could distinguish *A. malaysiensis* from *A. cantonensis* unambigiously (Eamsobhana *et al*., 2010a, b, 2015, 2017a, b; Yong *et al*., 2015a, 2016). The genetic distance bwteen *A. cantonensis* and *A. malaysiensis* is p = 11.9% based on 12 PCGs, p = 9.5% based on 2 rRNA genes, and p = 11.6% based on 14 mt-genes (Yong *et al*., 2016).

In the present study, the genetic distance based on concatenated nucleotide sequences of 12 mt-PCGs indicates that *A. mackerrasae* falls within the intraspecific range for *A. cantonensis* – p = 1.73% between *A. mackerrasae* and *A. cantonensis* from Thailand compared to p = 3.52% between the Thai and Chinese taxa of *A. cantonensis*. Both *A. mackerrasae* and *A. cantonensis* are genetically distinct from *A. malaysiensis* (p > 11%). A large genetic difference between sibling species is also found in the Costa Rican and Brazilian taxa of *A. costaricensis* (p = 16.17%, table 1; Yong *et al*., 2015b). The magnitude of the genetic

Table 2. Percentage of uncorrected 'p' genetic distance between different pairs of *Angiostrongylus* taxa and *Metastrongylus* taxa based on the nucleotide sequence of the complete cytochrome *c* oxidase subunit I (*cox1*) gene.

Taxon	1	2	3	4	5	6	7	8
1 *Angiostrongylus mackerrasae*, Australia	–							
2 *Angiostrongylus cantonensis*, Thailand KT947978	1.90	–						
3 *Angiostrongylus cantonensis*, China NC_013065	4.06	3.80	–					
4 *Angiostrongylus malaysiensis*, Malaysia NC_030332	9.00	9.13	8.56	–				
5 *Angiostrongylus costaricensis*, Costa Rica KR827449	12.93	12.61	12.93	12.61	–			
6 *Angiostrongylus costaricensis*, Brazil NC_013067	14.07	13.94	14.01	13.69	11.72	–		
7 *Angiostrongylus vasorum*, NC_018602	11.83	11.63	12.14	13.87	13.09	13.15	–	
8 *Metastrongylus pudendotectus*, NC_013813	15.75	14.98	15.68	16.00	14.66	15.93	15.24	–
9 *Metastrongylus salmi*, NC_013815	13.84	14.09	14.54	15.62	13.40	15.11	14.79	11.87

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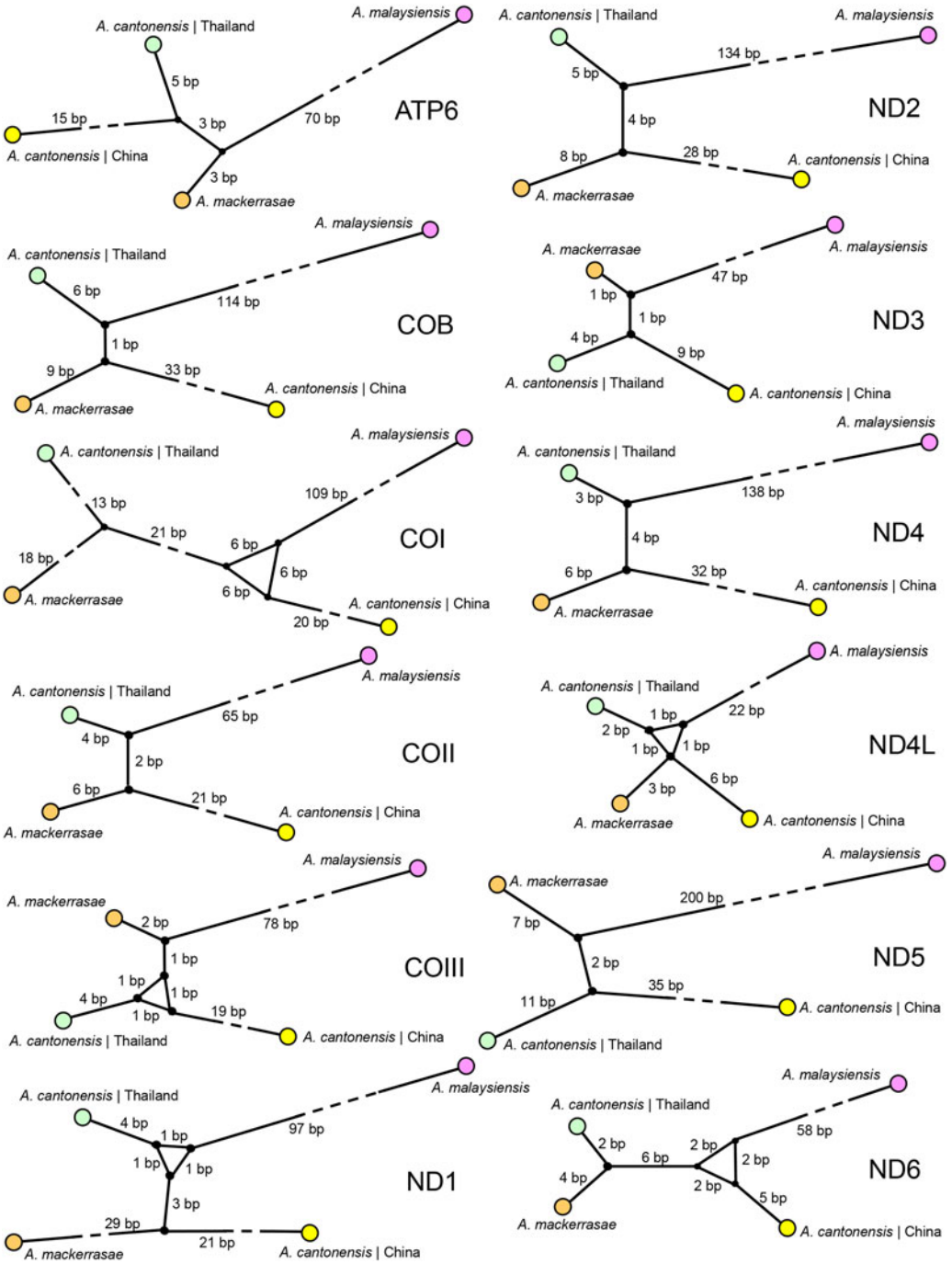

Fig. 2. Haplotype network of *Angiostrongylus cantonensis* species complex based on mitochondrial protein-coding gene sequences generated by NETWORK software. Abbreviations of gene names: ATP6, ATP synthase subunit 6; COB, cytochrome *b*; COI–III, cytochrome *c* oxidase subunit 1–3; ND1–6, nicotinamide-adenine-dinucleotide dehydrogenase subunit 1–6.

difference is similar for the amino acid sequence of the 12 PCGs (table 1) as well as the complete mitochondrial *cox1* nucleotide sequence (table 2).

Phylogenetic analysis based on 12 mt-PCGs indicates that *A. mackerrasae* is a member of the *A. cantonensis* genetic lineage (fig. 1). Based on haplotype network analysis, 11 of the 12 mt-PCGs also show a closer genetic relationship between *A. mackerrasae* and *A. cantonensis* from Thailand than between both of these and *A. cantonensis* from China (table 3; fig. 2). These results agree with the notion that *A. mackerrasae* may be conspecific with *A. cantonensis* (Chan *et al.*, 2015).

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Table 3. Differences in number of base pairs between component taxa of *Angiostrongylus cantonensis* species complex as determined by haplotype network analysis of 12 mitochondrial protein-coding genes. Amac, *A. mackerrasae*; AcanT, *A. cantonensis* Thailand; AcanC, *A. cantonensis* China; Amal, *A. malaysiensis*.

Gene (size, bp)	Amac vs. AcanT	Amac vs. AcanC	AcanT vs. AcanC	Amac vs. Amal	AcanT vs. Amal	AcanC vs. Amal
ATP6 (598)	11 (1.84%)	20 (3.34%)	21 (3.51%)	73 (12.21%)	78 (13.04%)	88 (14.72%)
COI (1579)	31 (1.96%)	65 (4.12%)	60 (3.80%)	154 (9.75%)	149 (9.44%)	135 (8.55%)
COB (1109)	16 (1.44%)	42 (3.79%)	41 (3.70%)	124 (11.18%)	120 (10.82%)	148 (13.35%)
COII (693)	12 (1.73%)	27 (3.90%)	27 (3.90%)	73 (10.53%)	69 (9.96%)	88 (12.70%)
COIII (766)	8 (1.04%)	23 (3.00%)	24 (3.13%)	80 (10.44%)	84 (10.97%)	99 (12.92%)
ND1 (850)	37 (4.35%)	50 (5.88%)	29 (3.41%)	130 (15.29%)	102 (12.00%)	122 (14.35%)
ND2 (850)	17 (2.00%)	36 (4.24%)	37 (4.35%)	146 (17.18%)	139 (16.35%)	164 (19.29%)
ND3 (334)	6 (1.80%)	11 (3.29%)	13 (3.89%)	48 (14.37%)	52 (15.57%)	57 (17.07%)
ND4 (1230)	13 (1.06%)	38 (3.09%)	39 (3.17%)	148 (12.03%)	141 (11.46%)	174 (14.15%)
ND4L (231)	6 (2.60%)	9 (3.90%)	9 (3.90%)	26 (11.26%)	25 (10.82%)	29 (12.55%)
ND5 (1543)	20 (1.30%)	44 (2.85%)	46 (2.98%)	207 (13.42%)	213 (13.80%)	237 (15.36%)
ND6 (426)	6 (1.41%)	17 (3.99%)	15 (3.52%)	70 (16.43%)	68 (15.96%)	65 (15.26%)

ATP6, ATP synthase subunit 6; COB, cytochrome *b*; COI–III, cytochrome *c* oxidase subunit 1–3; ND1–6, nicotinamide-adenine-dinucleotide dehydrogenase subunit 1–6.

In Australia, *A. cantonensis* occurs in introduced rat species (*Rattus rattus* and *Rattus norvegicus*) while *A. mackerrasae* is found mainly in native bush rats, *Rattus fuscipes* (Spratt, 2015). In mainland Asia, various species of murid rodents (both native and introduced) are the final or definitive hosts of *A. cantonensis* (Yong & Eamsobhana, 2013; Eamsobhana *et al.*, 2016). An extensive phylogeographical study of the parasites from mainland Asia, Indonesia, New Guinea, Australia and other Pacific islands will provide conclusive evidence for the taxonomic status and origin of these parasites.

In this study, we have demonstrated that *A. mackerrasae* is genetically very similar to *A. cantonensis* and that these taxa form a sister lineage. The small genetic distance between these taxa (p = 1.73–3.70%), compared to that with *A. malaysiensis* (p = 11.89–12.15%), indicates the need to re-examine the taxonomic status of *A. mackerrasae* (referred to as *A. cantonensis* when first documented in Australia). Further studies, particularly phylogeography, are needed to resolve whether it is conspecific with *A. cantonensis* or undergoing incipient speciation.

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Conflict of interest

None.

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