


RESEARCH

Open Access



Low diversity of *Angiostrongylus cantonensis* complete mitochondrial DNA sequences from Australia, Hawaii, French Polynesia and the Canary Islands revealed using whole genome next-generation sequencing

Barbora Červená^{1,2,3}, David Modrý^{2,4}, Barbora Fecková², Kristýna Hrazdilová⁵, Pilar Foronda^{6,7}, Aron Martin Alonso⁶, Rogan Lee⁸, John Walker⁹, Chris N. Niebuhr^{10,12}, Richard Malik¹¹ and Jan Šlapeta^{1*} 

Abstract

Background: Rats (*Rattus* spp.) invaded most of the world as stowaways including some that carried the rat lung-worm, *Angiostrongylus cantonensis*, the cause of eosinophilic meningoencephalitis in humans and other warm-blooded animals. A high genetic diversity of *A. cantonensis* based on short mitochondrial DNA regions is reported from Southeast Asia. However, the identity of invasive *A. cantonensis* is known for only a minority of countries. The affordability of next-generation sequencing for characterisation of *A. cantonensis* genomes should enable new insights into rat lung worm invasion and parasite identification in experimental studies.

Methods: Genomic DNA from morphologically verified *A. cantonensis* (two laboratory-maintained strains and two field isolates) was sequenced using low coverage whole genome sequencing. The complete mitochondrial genome was assembled and compared to published *A. cantonensis* and *Angiostrongylus malaysiensis* sequences. To determine if the commonly sequenced partial *cox1* can unequivocally identify *A. cantonensis* genetic lineages, the diversity of *cox1* was re-evaluated in the context of the publicly available *cox1* sequences and the entire mitochondrial genomes. Published experimental studies available in Web of Science were systematically reviewed to reveal published identities of *A. cantonensis* used in experimental studies.

Results: New *A. cantonensis* mitochondrial genomes from Sydney (Australia), Hawaii (USA), Canary Islands (Spain) and Fatu Hiva (French Polynesia), were assembled from next-generation sequencing data. Comparison of *A. cantonensis* mitochondrial genomes from outside of Southeast Asia showed low genetic diversity (0.02–1.03%) within a single lineage of *A. cantonensis*. Both *cox1* and *cox2* were considered the preferred markers for *A. cantonensis* haplotype identification. Systematic review revealed that unequivocal *A. cantonensis* identification of strains used in experimental studies is hindered by absence of their genetic and geographical identity.

Conclusions: Low coverage whole genome sequencing provides data enabling standardised identification of *A. cantonensis* laboratory strains and field isolates. The phenotype of invasive *A. cantonensis*, such as the capacity to establish in new territories, has a strong genetic component, as the *A. cantonensis* found outside of the original endemic area

*Correspondence: jan.slapeta@sydney.edu.au

¹ Sydney School of Veterinary Science, University of Sydney, Sydney, NSW 2006, Australia

Full list of author information is available at the end of the article



are genetically uniform. It is imperative that the genotype of *A. cantonensis* strains maintained in laboratories and used in experimental studies is unequivocally characterised.

Keywords: Rat lungworm, Mitochondrial genome, Genetic diversity, Invasive species, Next-generation sequencing, Rat lungworm, *cox1*, *Rattus*

Background

Biological invasions are a recognised outcome of global change [1, 2]. Historically, non-native species were introduced into new areas by the movement of people and goods, [3–6]. The rat (*Rattus* spp.) is recognised globally as an invasive species (GISD 2018: <http://www.iucngisd.org>) [7, 8]. Rats were accompanied by their pathogens, including viruses, bacteria, protozoans, helminths and ectoparasites [9]. The rat lungworm, *Angiostrongylus cantonensis* (Strongylida: Metastrongylidae), is a cause of eosinophilic meningoencephalitis in humans and animals [10]. The first documented outbreaks of human disease due to *A. cantonensis* occurred in Tahiti and Hawaii in the 1950s [11, 12]. The nematode *A. cantonensis* was described from rat lungs in the Guangzhou region in China and Taiwan more than 30 years earlier [13, 14], with the first human case reported during World War 2 (WW2) in a patient domiciled in Taiwan.

The life-cycle of *A. cantonensis* is sustained between rats, where the adult helminths are confined to the pulmonary arteries and right ventricle, and a range of molluscs, crustaceans, planarians and for example frogs serving as intermediate and transport hosts, respectively [15–17]. Humans, companion animals and wildlife are accidental dead-end hosts for *A. cantonensis*. Although such infections are unimportant to the sustainability of *A. cantonensis*, they may lead to severe, even fatal, infections in these accidental hosts [18–20]. One theory concerning the emergence of *A. cantonensis* outside Southeast Asia following WW2 states that invasion was facilitated by the introduction of the giant African snail, *Achatina fulica* that permitted the parasite to flourish. Alternatively, *A. cantonensis* might have been carried within the snail across the Pacific, where rats had already been established [21–23]. Reports of newly invaded territories keep occurring, e.g. Canary Islands (2010), Uganda (2012) and Oklahoma, USA (2015), although the exact timing and the route of the invasions remains largely uncharacterised [24–26].

Southeast Asia (including China, Taiwan, Vietnam, Thailand, Laos, Cambodia and Myanmar) is considered to be the original endemic region for *A. cantonensis* [21, 22, 27–35]. In Japan, Tokiwa et al. [33] suggested colonization of the area by multiple genetic lineages spreading from the south to the north of Japan. The majority of phylogeographic studies have local character and are

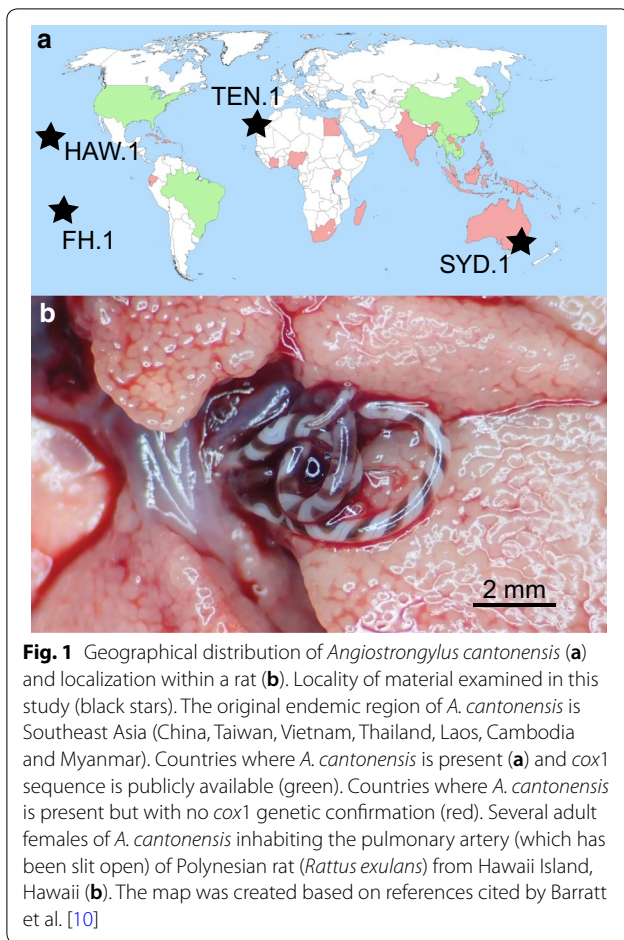
based on partial sequences [36]. Data on genetic and phenotypic diversity of *A. cantonensis* within invaded regions remain scarce [10, 37–40]. A recent study has shown that the pathogenicity of *A. cantonensis* for its laboratory host(s) varied between different *A. cantonensis* genetic lineages [41]. The range of *A. cantonensis* phenotypes in rats and molluscs from inside and outside of Southeast Asia may imply that only certain *A. cantonensis* genetic lineages are capable of invading other territories.

The aim of this study was to determine the extent of mitochondrial DNA (mtDNA) diversity of *A. cantonensis* outside Southeast Asia. We used whole genome low coverage next-generation sequencing to obtain complete mtDNA of *A. cantonensis* originating from four geographically distant regions where it represents an important public health concern: (i) Sydney (Mosman, NSW, Australia); (ii) Fatu Hiva Island (Marquesas, French Polynesia); (iii) Hawaii Island (Hawaii, USA); and (iv) Tenerife (Canary Islands, Spain) (Fig. 1). The low mtDNA diversity of invading *A. cantonensis* observed prompted us to review published experimental studies to determine the identity of *A. cantonensis* isolates used in experimental studies. The suitability of partial *cox1* to unequivocally identify genetic lineages for *A. cantonensis* was evaluated in the context of all available sequence data and the entire mtDNA genome.

Results

Angiostrongylus cantonensis from Pacific and Atlantic regions determined by morphology and *cox1* sequence

Adults of *A. cantonensis* from the Pacific region (Fatu Hiva, Marquesas, French Polynesia; Hawaii Island, Hawaii, USA; Sydney, Australia) and Atlantic region (Tenerife, Canary Islands, Spain) were morphologically consistent with the original description and re-description of *A. cantonensis sensu* Chen, 1935 and *sensu* Bhaibulaya, 1968, respectively [13, 42]. The spicules of the caudal end of *A. cantonensis* males examined exceeded 1 mm [Sydney specimen: 1.2–1.5 mm (SYD, $n=6$); Fatu Hiva specimen: 1.1–1.2 mm (FH, $n=3$); Hawaii specimen: 1.3 mm (HAW, $n=1$); Canary Islands: 1.2–1.3 mm (TEN, $n=5$), Fig. 2a]. Partial *cox1* sequences (primers LCO1490 and HCO2160) of selected vouchers from each series were >98% identical to the reference *A. cantonensis cox1* sequence (strain AC3; KT947978).



Low diversity of four invasive *Angiostrongylus cantonensis* mitochondrial genomes (mtDNA)

Low coverage whole genome next-generation sequencing of *A. cantonensis* DNA (quantity 36–57 ng) yielded 1.4–5.4 G bp with 89.49–92.99% high quality Q30 of the nucleotides (Table 1). Using MitoBIM, we assembled four (SYD.1, HAW.1, FH.1, TEN.1) complete mtDNA sequences, 13,500–13,508 bp (Fig. 2b). Three independent MitoBIM runs, each initially baited with *A. cantonensis* mtDNA (NC_013065), returned identical mtDNA sequences. With the aid of the MITOS Web Server and subsequent manual correction, 12 protein-coding genes representing 3426 amino acids (10,278 bp), 22 *trn* regions and two ribosomal subunits were annotated (Fig. 2b). The partial *cox1* sequence obtained by PCR (primers LCO1490-HCO2160, c.650 bp) from the same initial DNA following Sanger sequencing was 100% identical to the mtDNA assembled from the NGS data.

Nucleotide sequence alignment of four *A. cantonensis* complete mtDNA genomes obtained in this study with three published *A. cantonensis* mtDNA genomes originating from Southeast Asia [Taiwan

(AP017672), Thailand (Isolate AC3, KT947978), China (NC_013065)] was reconstructed (length 13,525 nt). Overall, the four new *A. cantonensis* mtDNA sequences (SYD.1, TEN.1, HAW.1, FH.1) differed in $\leq 0.02\%$ (3–139 residues) (Fig. 3a), the SYD.1 and TEN.1 differing in only 3 residues (synonymous mutations C/T and G/T located in *cox1* and *cox2*, respectively, and a single nucleotide change in *trnN*). Similarly, HAW.1 and FH.1 differed in 9 residues: seven indels in *rrnL*, one indel in a non-coding region between *trnP* and *trnA*, and one mutation C/T in *cob* (position 815) resulting in amino acid A in HAW.1 and V in FH.1, respectively. Compared to published Southeast Asian mtDNA sequences, the greatest similarity was between our isolates and the Taiwanese isolate AP017672, comprising two residue differences (one in *rrnL* and the other in a non-coding region) from TEN.1 to 138 residues for FH.1 (99 located in protein-coding genes). The highest difference was observed between the Chinese isolate NC_013065 and all the other isolates (463–475 residues/3.4–3.5%) (Fig. 3a).

The maximum likelihood phylogenetic trees reconstructed from complete mtDNA nucleotide (13,579 nt; Fig. 3b) and amino acid sequences (3,416 aa; Fig. 3c) showed clear separation of *A. cantonensis* and *A. malaysiensis* clades. The *A. cantonensis* clade was further divided in four sub-clades comprising (i) Chinese isolate (NC_013065); (ii) Thailand isolate (KT947978); (iii) isolates HAW.1, FH.1; and (iv) including Taiwanese isolate (AP017672), SYD.1 and TEN.1 isolates. All four sub-clades were supported by high bootstrap values (99–100 for nucleotide and 80–100 for amino acid tree; Fig. 3b, c).

The nucleotide pairwise sequence distance percentages (PSD_N) calculated for each of the protein-coding genes and rRNA gene subunits varied from 3.0% (*nad5*) to 4.4% (*nad2*) (Fig. 2b). The highest amino acid pairwise sequence distance percentage (PSD_{AA}) was observed in the shortest gene *nad4L* (77 aa). Interestingly, six of seven analysed sequences were identical in this gene, only the Chinese isolate (NC_013065) differed from the others in 5 amino acids. The lowest PSD_{AA} was observed in *cox2* (maximum difference 0.9%), representing two polymorphic sites (out of 230) where serine substituted for either asparagine or proline. The second lowest PSD_{AA} was detected in *cox1* (maximum difference 1.3%). Six of seven *cox1* sequences (525 aa), differed by a single amino acid (V/I in position 516). The sequence of the Chinese isolate (NC_013065) differed from the other six sequences in 6–7 amino acids. The interspecific PSD_{AA} of *cox1* sequences between *A. cantonensis* and *A. malaysiensis* (KT947979) was 12–14 residues (2.3–2.7%).

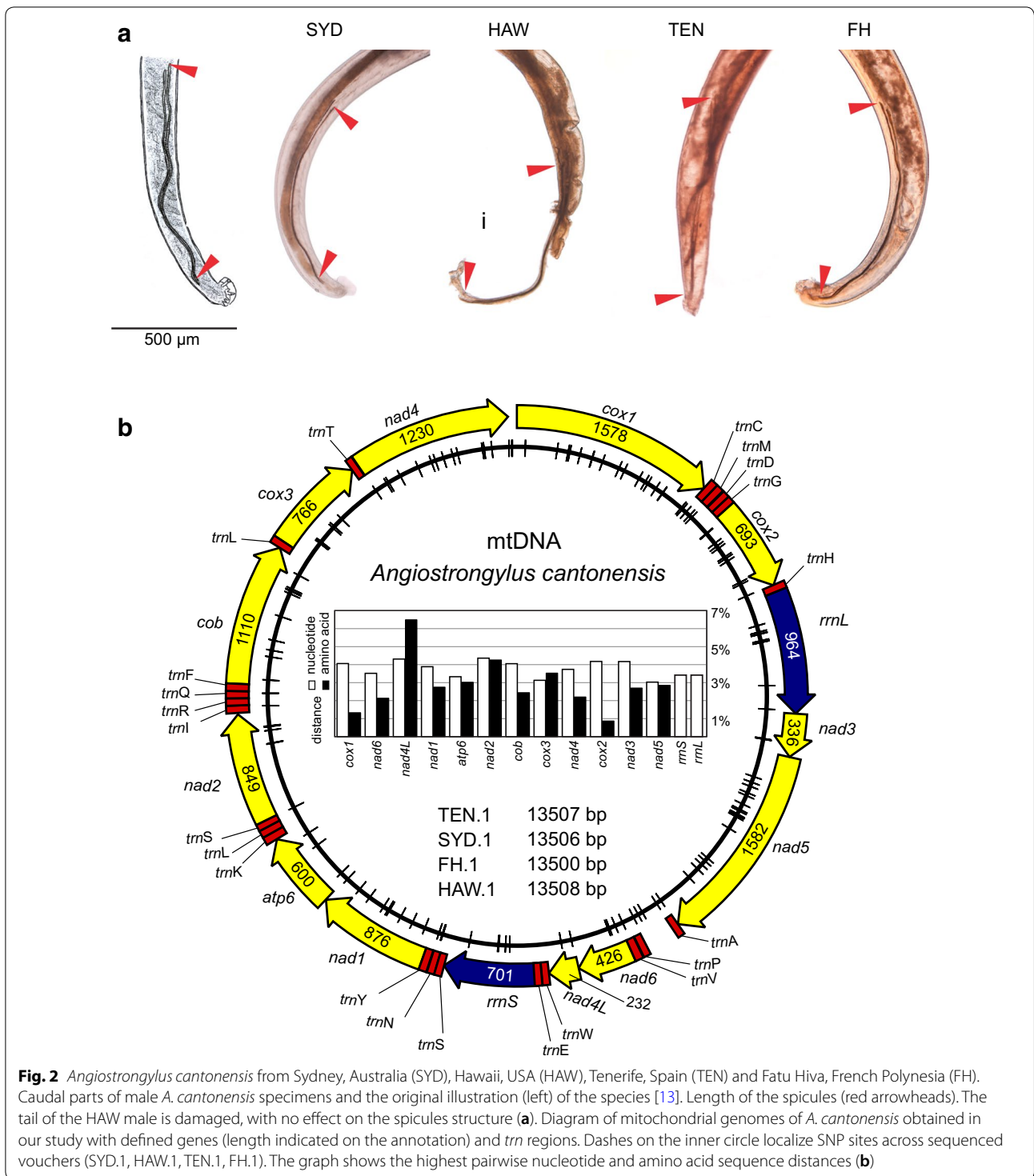


Fig. 2 *Angiostrongylus cantonensis* from Sydney, Australia (SYD), Hawaii, USA (HAW), Tenerife, Spain (TEN) and Fatu Hiva, French Polynesia (FH). Caudal parts of male *A. cantonensis* specimens and the original illustration (left) of the species [13]. Length of the spicules (red arrowheads). The tail of the HAW male is damaged, with no effect on the spicules structure (a). Diagram of mitochondrial genomes of *A. cantonensis* obtained in our study with defined genes (length indicated on the annotation) and *trn* regions. Dashes on the inner circle localize SNP sites across sequenced vouchers (SYD.1, HAW.1, TEN.1, FH.1). The graph shows the highest pairwise nucleotide and amino acid sequence distances (b)

Short overlapping *cox1* sequence limits characterization of rat lungworm *Angiostrongylus cantonensis*

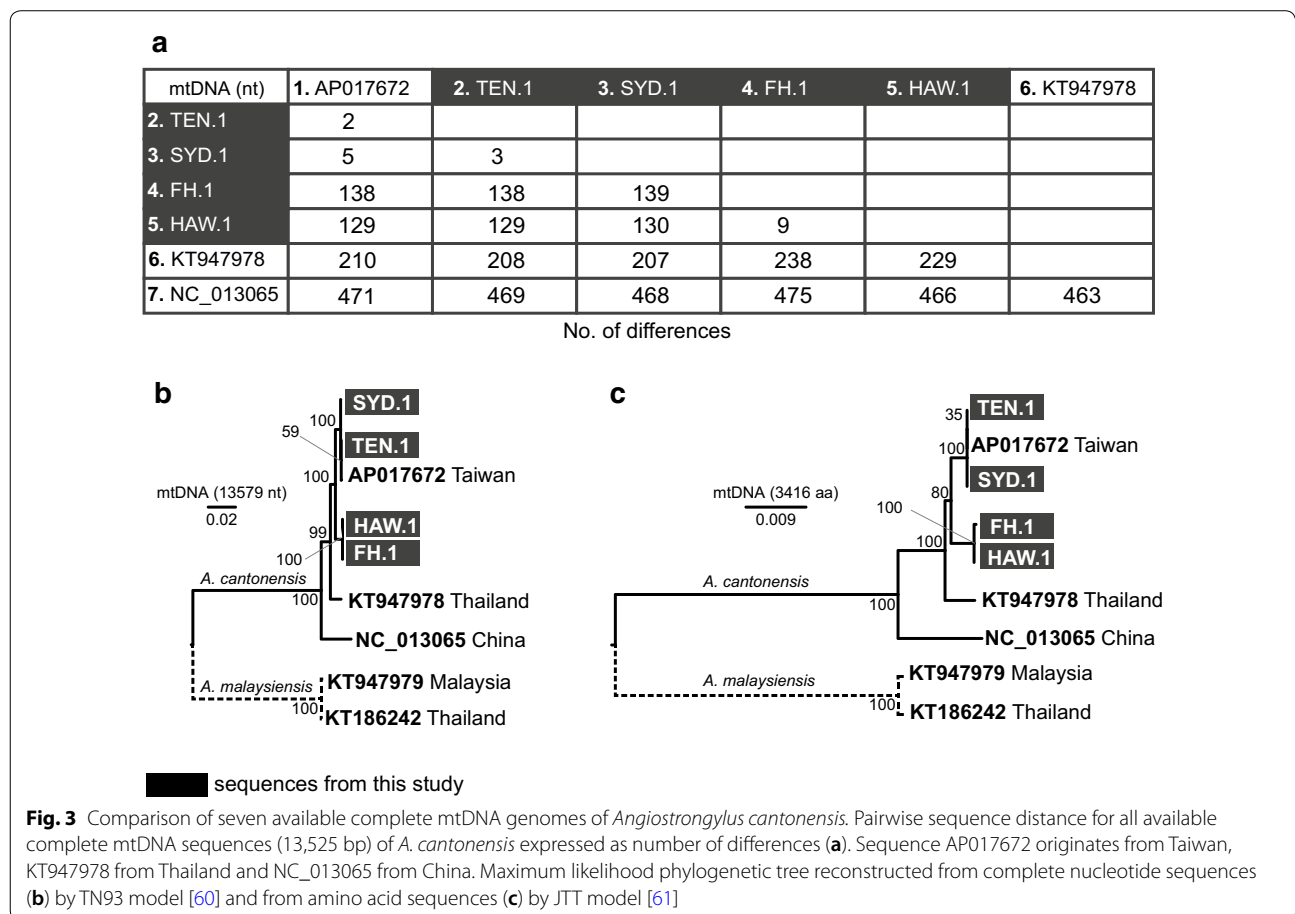
Currently, there are 86 *A. cantonensis* (including 3 complete mtDNA) and 13 *A. malaysiensis* (including 2 complete mtDNA) *cox1* sequences available in

public DNA repositories. Six sequences of *A. cantonensis* (GU138106–11) were excluded from further analyses because they included internal stop codons. The complete coding sequences of *cox1* (1578 bp; 525 aa + stop codon) extracted from newly obtained complete

Table 1 Whole genome next-generation sequencing of *Angiostrongylus cantonensis* raw data summary

Identifier	Sequence ID	DNA amount (ng)	Total read bases (G bp)	Total reads (mil)	G-C content (%)	A-T content (%)	Q30 (%)
SYD.1	JS4458	43	2.0	20.2	41.39	58.61	92.28
HAW.1	JS4459	36	5.4	53.5	41.38	58.62	89.49
TEN.1	6967	57	2.3	22.7	41.81	58.19	92.72
FH.1	R23-F	50	1.4	13.8	41.56	58.44	92.99

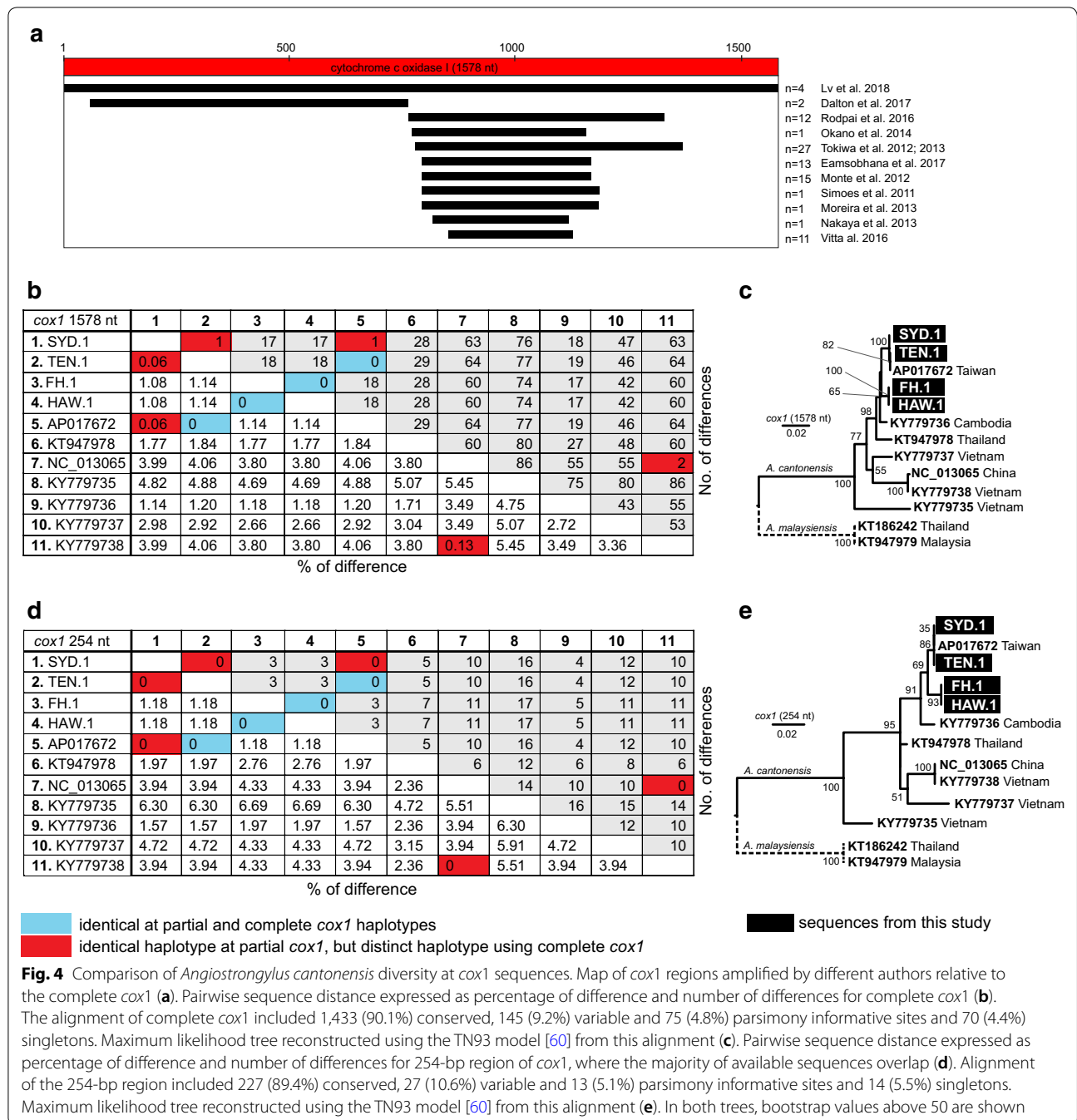
Note: Paired end 101-bp Illumina sequencing of Nextera XT DNA library. Q refers to Phred Quality Score which is calculated with $-10\log_{10}P$, where P is probability of erroneous base call. Q30 stands for 1 incorrect base call in 1000



mtDNA were aligned with available sequences, demonstrating no gaps and no variation in length (Additional files 1, 2, 3, 4, 5). There were 13 complete *cox1* sequences (11 for *A. cantonensis*, 2 for *A. malaysiensis*) in the final alignment (Fig. 4a, b, c). The remaining sequences were distributed across ten different fragments of *cox1* depending on the primer sets used in the respective studies (Fig. 4a). The majority (98%, 95/97) of the *A. cantonensis cox1* sequences overlapped in 254-bp region (positions 847–1101). The *cox1* sequences

MF000735-MF000736 (*A. cantonensis* from mainland USA) were outside this 254-bp region (Fig. 4a).

The comparison between PSD_N for 11 available complete *cox1* and restricted to the 254-bp fragment of the same sequences demonstrated a decrease in the absolute number of polymorphic nucleotide residues (Wilcoxon matched pair test, *P*-value < 0.0001) (Fig. 4b, d). While sequences SYD.1, TEN.1 and AP017672 represented different haplotypes of complete *cox1*, in this 254-bp region, they appeared identical. Similarly, sequences KY779738



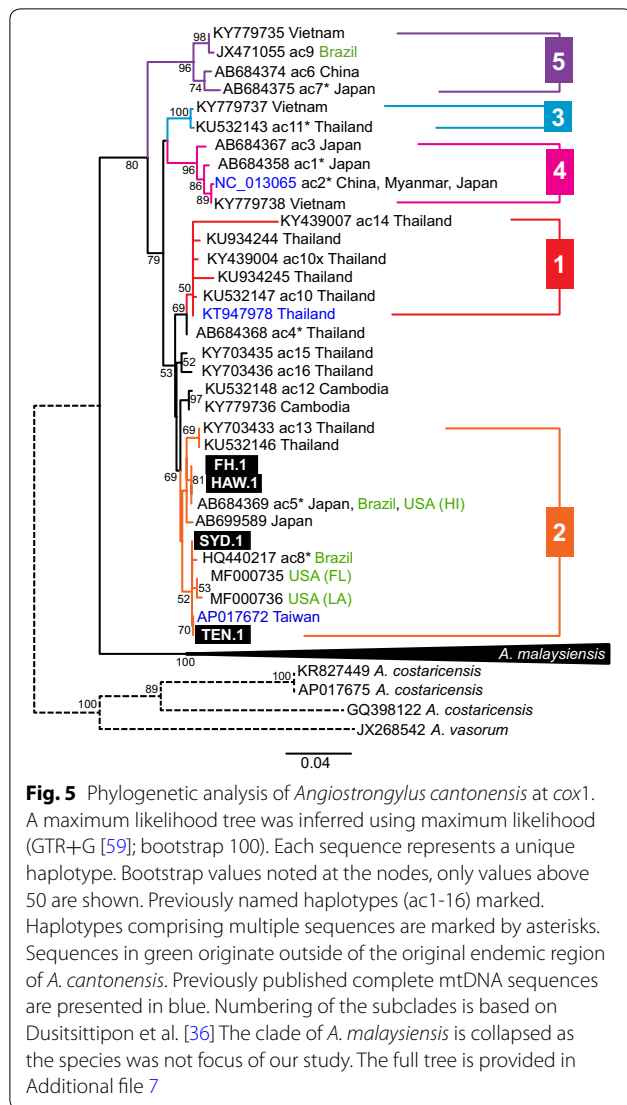
identical at partial and complete *cox1* haplotypes
 identical haplotype at partial *cox1*, but distinct haplotype using complete *cox1*

sequences from this study

Fig. 4 Comparison of *Angiostrongylus cantonensis* diversity at *cox1* sequences. Map of *cox1* regions amplified by different authors relative to the complete *cox1* (a). Pairwise sequence distance expressed as percentage of difference and number of differences for complete *cox1* (b). The alignment of complete *cox1* included 1,433 (90.1%) conserved, 145 (9.2%) variable and 75 (4.8%) parsimony informative sites and 70 (4.4%) singletons. Maximum likelihood tree reconstructed using the TN93 model [60] from this alignment (c). Pairwise sequence distance expressed as percentage of difference and number of differences for 254-bp region of *cox1*, where the majority of available sequences overlap (d). Alignment of the 254-bp region included 227 (89.4%) conserved, 27 (10.6%) variable and 13 (5.1%) parsimony informative sites and 14 (5.5%) singletons. Maximum likelihood tree reconstructed using the TN93 model [60] from this alignment (e). In both trees, bootstrap values above 50 are shown

and NC_013065 were not detected as different haplotypes based on this 254-bp region. The phylogenetic tree reconstructed from the 254-bp alignment of *cox1* (Fig. 4e) showed lower resolution of internal relationships within the *A. cantonensis* clade compared to the tree reconstructed from complete *cox1* (Fig. 4c). To show relationships between our four newly sequenced isolates and previously published data, the

initial dataset was narrowed down to 56 unique *cox1* sequences (*A. cantonensis*, *n*=40; *A. malaysiensis*, *n*=12; outgroup, *n*=4) and used to construct a maximum likelihood phylogenetic tree. The alignment consisted of 1581 characters with 1094 (69.1%) conserved, 484 (30.6%) variable and 316 (20%) parsimony informative sites and 168 (10.6%) singletons. Tree topology (Fig. 5) showed separation of *A. malaysiensis* and *A.*



cantonensis as two distinct clades. Eight sequences named as *A. cantonensis* downloaded from GenBank clustered within the *A. malaysiensis* clade (Additional files 6 and 7). These sequences originated from Thailand ($n = 7$) and Taiwan ($n = 1$).

All of our four *A. cantonensis* isolates represented by *cox1* clustered together with sequence AP017672 (extracted from mtDNA) to the Clade 2 *sensu* Dusitsittipon et al. [36]. This clade also contained sequences originating from Brazil, Hawaii and continental USA (Florida and Louisiana). Further subdivision of the subclade was not supported (bootstrap < 50%).

Systematic literature review shows lack of complete characterization of experimentally studied *Angiostrongylus cantonensis* strains

The Sydney and Fatu Hiva *A. cantonensis* strains are currently maintained in experimentally infected laboratory rats and snails (Table 2). The literature search yielded 412 articles on *A. cantonensis* published after 2011, with 104 articles describing experimental work (Additional file 8). The exact origin of *A. cantonensis* used for the experimental studies was specified in 46% of studies (48/104) and 4% (2/48) provided *cox1* to confirm the identity of the parasite (Table 3).

Discussion

To demonstrate how whole genome sequencing can assist routine nematode characterization, we analysed complete mtDNA genomes of the invasive metastrongylid *A. cantonensis* originating from four geographically distant areas well outside the endemic range of the parasite in Southeast Asia. Dusitsittipon et al. [36] attempted to synthesize published data on *A. cantonensis* mtDNA and concluded that detailed morphological and genetic characterization is urgently needed. Our data not only provide complete mtDNA of morphologically identified *A. cantonensis* vouchers, but the sequence data represent a resource from which other target genes sequences can be extracted.

The circular mtDNA of our *A. cantonensis* includes two ribosomal subunits, 12 protein-coding genes and 22 *trn* coding regions, concordant with published results [43, 44]. The generally low mtDNA genetic distance amongst invasive *A. cantonensis* isolates studied is in stark contrast to the genetic distances observed between Southeast Asian isolates, suggesting that only a limited number

Table 2 Summary of *Angiostrongylus cantonensis* used in this study

Identifier	Locality of origin, host	Date of collection for analysis	Laboratory strain (laboratory host, year of isolation)
FH.1	Fatu Hiva, Marquesas Islands, French Polynesia; <i>Rattus exulans</i>	February 2017	University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic; Wistar rat, <i>Rattus norvegicus</i> , 2017
TEN.1	Tenerife, Canary Islands, Spain; <i>Rattus norvegicus</i>	April 2018	–
HAW.1	Hawaii Island, Hawaii, USA; <i>Rattus exulans</i>	May, 2018	–
SYD.1	Mosman (near Taronga Zoo, Sydney), NSW, Australia; <i>Rattus norvegicus</i>	December, 2017	Westmead Hospital, NSW, Australia; Wistar rat, <i>Rattus norvegicus</i> , 1997

Table 3 Review of identification of *A. cantonensis* strains used in experimental studies

	Total	Vertebrate host				Invertebrate host	
		Diagnostics	Pathology	Physiology	Treatment	Pathology	Physiology
Number of articles	104	3	46	31	12	11	1
Origin of the strain specified	48	1	14	17	5	10	1
Helminths sequenced	2	0	1	1	0	0	0
cox1 haplotype determined	1	0	0	1	0	0	0

of mtDNA haplotypes had the capacity to emerge as globally invasive *A. cantonensis* [36, 41].

The isolates of *A. cantonensis* from tropical Pacific islands of Fatu Hiva and Hawaii are almost identical. Similarly, two localities which are almost 20,000 km apart (Sydney and Tenerife) differ by only 3 nucleotides across the entire 13.5 kbp mtDNA. *Angiostrongylus cantonensis* was first detected in eastern Australia in 1950s, while Tenerife, Canary Islands has only recently been invaded [24, 45, 46]. It would be too speculative to suggest an Australian origin for the recent Tenerife invasion, because we do not know the extent of *A. cantonensis* diversity in other regions that could have equally been the source population, and there is no plausible historical connection between Australia and the Canary Islands (Fig. 1). Indeed, the Tenerife and Sydney isolates are almost identical to the Taiwanese isolate (AP017672) from BioProject PRJEB493. We speculate that the rapid spread of *A. cantonensis* in the Pacific region was a consequence of naval operations during and/or after the World War II, because troop and supply ships could readily permit spread of the *A. cantonensis* infected rats and/or snails. In a telling analogy, *A. cantonensis* repeatedly colonised Japan during the 20th century, spreading from southern islands to the north resulting in a presence of several different haplotypes from three clades [22].

The phylogeny inferred from partial *cox1* sequences corresponds to the tree topography described by Dusitsittipon and colleagues [36]. Numerous haplotypes from different clades detected in China, Taiwan, Thailand, Vietnam, Myanmar and Cambodia support the theory that *A. cantonensis* originated historically from South-east Asia in the same manner as its *Rattus* hosts [10, 32–34, 47]. In contrast, all but one sequence originating from outside of the endemic range of *A. cantonensis* cluster within the Clade 2 *sensu* Dusitsittipon et al. [36], confirming the trend of low diversity for invasive *A. cantonensis* isolates. The situation in Hawaii might have experienced more complicated scenario, because *cob* sequences KP721454–55 are distinct from our HAW.1 and exhibit high similarity to the Chinese isolate NC_013065 (Additional file 9). Unfortunately, no detailed information is available on the origin of the previously

submitted *A. cantonensis* (e.g. the particular island) from Hawaii.

The genotypic diversification of a parasite is commonly associated with morphological and biological diversification, enabling the organism to acquire traits that permit invasion of new territories and hosts [48, 49]. The complexity of haplotypes of *A. cantonensis* suggests variability in its biological traits, but this is yet to be established experimentally [36]. The review of 104 experimental studies involving *A. cantonensis* revealed that only two studies identified their *A. cantonensis* genetically using, for example, *cox1* [41, 50]. Nevertheless, Lee et al. [50] while comparing infectivity and pathogenicity between what they considered two *A. cantonensis* (named strain P and H) inadvertently worked with two distinct *Angiostrongylus* species, because *cox1* (strain H; KF591126) clusters within the *A. malaysiensis* clade in our analyses (Additional file 7).

In the past, obtaining the complete mtDNA of *A. cantonensis* was accomplished with laborious PCR and primer walking [43, 51]. With the accessibility of next-generation sequencing services, it is logical to consider this methodology for any *A. cantonensis* laboratory-maintained strain. Such resource can be further mined for other genes of interest, including those under Darwinian selection and therefore informing on phenotypic characterization.

Conclusions

Using whole genome next-generation sequencing methods, we assembled and analysed complete mtDNA of four *A. cantonensis* isolates originating from four geographically disparate localities outside of the parasite's endemic range. The observed uniformity of invasive strains implies that only certain *A. cantonensis* genetic lineages have the capacity to become globally invasive. Our review of published experimental studies demonstrated a need of improved consistency in reporting the identity and origin of tested *A. cantonensis*. We encourage researchers working with *A. cantonensis* in laboratories to provide thorough information on their strains, including the origin (country, region, host species) and genetic characterization represented

by sharing raw data, or mtDNA sequence or minimally, provide a sequence of complete *cox1*. Having a resource with standardised good quality information will provide a basis for future studies focused on phenotypic traits of *A. cantonensis*.

Methods

Collection of adult rat lungworms *Angiostrongylus cantonensis*

The adults of *A. cantonensis* were collected from *Rattus* spp. in four geographically distant localities in the Pacific and Atlantic region and donated for the study as vouchers in 90% ethanol (Fig. 1a, Table 2).

Morphological determination of adult rat lungworms *Angiostrongylus cantonensis*

All the adult specimens were examined under the light microscope (Olympus BX53 and BX60) equipped with Nomarski interference contrast optics. The morphology was compared to descriptions of *A. cantonensis*, *A. mackerrasae* and *A. malaysiensis* [13, 42, 52, 53]. In male worms, the structure of the copulatory bursa was observed, and spicules were measured as the spicules varying in length from 1.0–1.46 mm, postero-lateral ray significantly shorter than medio-lateral ray and separation of the ventro-ventral from the latero-ventral ray at the point about distal one third of the common trunk are typical features for distinguishing *A. cantonensis*. Females of *A. cantonensis* are determined by the length of the vagina in the range 1.5–3.25 mm (2.1 mm on average), by the vulva located 0.16 mm from the anus and finally, by the absence of a minute terminal projection at the tip of the tail [42, 52].

Several specimens from each series were deposited to the helminthological collection of the Institute of Parasitology, Biology Center, České Budějovice (Fatu Hiva and Tenerife material; accession number IPCAS N-260) and to the Australian National Wildlife Collection, CSIRO, Canberra, Australian Capital Territory, Australia (Hawaii and Sydney material; accession numbers: W/L HC# N5703–N5711)

Isolation of genomic DNA from tissue segment of adult rat lungworms *Angiostrongylus cantonensis*

DNA was isolated from dried c.0.5 cm segment of the mid-body using the Nucleospin Tissue XS (Macherey-Nagel, Düren, Germany) or Isolate II Genomic DNA Kit (Bioline, Alexandria, Australia) according to manufacturers' instructions and eluted in 100 µl of Tris buffer (pH = 8.5). DNA was stored at –20 °C.

Amplification of *cox1* mtDNA from rat lungworms

Angiostrongylus cantonensis

Partial sequence of the cytochrome *c* oxidase subunit 1 gene (*cox1*) was amplified using primers LCO1490 (forward) and HCO2198 (reverse) [54]. PCR mixtures were prepared using 15 µl of MyTaq™ Red Mix Kit (Bioline, Alexandria, Australia), 10.5 µl of PCR water, 1.25 µl of each of the primers (10 µM) and 2 µl of the template DNA. The PCR protocol was as follows: initial denaturation at 95 °C for 1 min; 35 cycles of 15 s at 95 °C, 15 s at 55 °C and 10 s at 72 °C; and a final elongation at 72 °C for 7 min. The PCR products were sequenced in Macrogen Inc. (Amsterdam, Netherlands and Seoul, South Korea), the quality of sequences was assessed in CLC Genomic Workbench 6.9.1 (<https://www.qiagenbioinformatics.com/>) and the sequences were searched through BLAST [55] (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm the identity of the worms.

Sequencing and assembly of the rat lungworms

Angiostrongylus cantonensis mtDNA from the whole genome sequencing data

Isolated DNA (36–57 ng; Table 1) was used for Illumina Nextera XT library construction followed by the next-generation sequencing using 100 bp paired end Illumina HiSeq 2500 sequencing systems utilizing at the depth of 1Gb of raw sequence data (Macrogen, Seoul, Republic of Korea). All four specimens used for the whole genome sequencing were females. The complete mitochondrial genome (mtDNA) was assembled from FastQ data using the MITObim pipeline [56] available at <https://github.com/chrishah/MITObim> with the sequence of *A. cantonensis* complete mtDNA (NC_013065) as bait. The assembly was repeated three times with varying percentage of the raw FastQ sequence data used (2–10%), keeping mtDNA coverage at 60–100×. The obtained mtDNA was annotated with the aid of MITOS Web Server [57] available at <http://mitos.bioinf.uni-leipzig.de/> and aligned with available *Angiostrongylus* spp. complete mtDNA sequences in CLC Genomics Workbench 6.9.1. for manual validation.

Pairwise sequence distance of complete mtDNA sequences

Newly obtained mtDNA sequences were aligned with published mtDNA sequences of *A. cantonensis* (AP017672, KT947978, NC_013065) and pairwise sequence distance (PSD) expressed as number of differences was calculated in CLC Genomics Workbench 6.9.1. Another mtDNA sequence (KT186242) labelled as *A. cantonensis* was not included in our analyses as Dusittipon et al. [36] showed that the sequence clusters within the *A. malaysiensis* clade. To assess intraspecific

diversity of individual genes and thus their suitability for phylogenetic studies, all 12 mitochondrial genes were separately translated to amino acids and PSD was calculated for both nucleotide (PSD_N) and amino acid (PSD_{AA}) sequences. The PSD_N was calculated for both mitochondrial rRNA gene subunits. As *cox1* is the most commonly used gene for studying diversity and phylogeography of *Angiostrongylus cantonensis*, we included *cox1* sequence extracted from complete mtDNA of *A. malaysiensis* (KT947979) for PSD_{AA} calculation, aiming to compare the intra- and interspecific variability. The maximum likelihood trees were constructed from complete nucleotide mtDNA sequences and from amino acid sequences of the 12 protein-coding genes in MEGA X [58] using sequences of *A. malaysiensis* (KT186242, KT947979) as outgroup. The model was selected by model test implanted in MEGA X.

Analysis of *cox1* alignment and maximum likelihood phylogenetic tree of *cox1* sequences

Complete sequences of *cox1* were extracted from available *A. cantonensis* mtDNA ($n=7$) and all *cox1* sequences labelled as *A. cantonensis* were downloaded from GenBank. The geographical location of all isolates was recorded. All sequences were aligned in CLC Genomics Workbench 6.9.1 and the alignment was manually checked for gaps and stop-codons. As we determined that the majority of sequences overlapped in a short fragment (254 nt) of *cox1*, we decided to compare the information contained in this fragment with the information from complete *cox1*. The PSD_N expressed as number of differences and percentage of difference was calculated for 11 available complete *cox1* sequences (7 extracted from complete mtDNA and 4 sequences KY779735–KY779738) and for the overlapping 254-bp fragment of the same 11 sequences. Paired values were compared using Wilcoxon matched pair test (GraphPad Prism 7.02, GraphPad, CA) at significance level P -value 0.05. Number of singletons, conserved, variable and parsimony informative sites were recorded, and a maximum likelihood tree was constructed in MEGA X [58] from both alignments using *cox1* sequences of *A. malaysiensis* (KT186242, KT947979) as the outgroup. The GTR+G model was chosen by model test integrated in MEGA X [58] software. The phylogenetic relationships of the taxa were tested by 100 replicates of bootstrap. To provide corroborating evidence using an alternative gene, we analysed *cob* of *A. cantonensis* (Additional file 9).

To compare diversity of all available *A. cantonensis* *cox1* sequences, a maximum likelihood tree was constructed. The alignment comprised one sequence representing each recognised *A. cantonensis* haplotype. As there are apparently sequences (including a mitochondrial genome

accession number KT186242), labelled as *A. cantonensis*, which are in fact *A. malaysiensis* (see Dusitsittipon et al. [58]), sequences of *A. malaysiensis* ($n=13$) were added to the analysis as the *A. cantonensis* sibling species and for the verification of the GenBank sequences taxonomic identity. Complete *cox1* sequences extracted from mtDNA of *A. costaricensis* (AP017675, GQ398122, KR827449) and *A. vasorum* (JX268542) were used as outgroups. A maximum likelihood tree was constructed in MEGA X [58] by the GTR+G model [59] chosen by the model test implemented within the software. The phylogenetic relationships of the taxa were tested using 100 replicates of bootstrap.

Systematic review of *A. cantonensis* literature published between 2011–2019

On January 9, 2019, we searched the Web of Science database using the keywords “*Angiostrongylus*” AND “*cantonensis*”. Because the first *cox1* sequence of *A. cantonensis* was published in 2011 [40], we limited the search to articles published between 2011 and 2019. The next step was identification of experimental studies using *A. cantonensis*. The inclusion criteria were: (i) the authors infected any vertebrate or invertebrate host in the laboratory irrespective of which aspect of the infection was being studied, including investigations concerning biological traits of the parasite or even just aspects of the life-cycle of the parasite; and (ii) experiments where *in vitro* cultivation of any parasite stage was the objective of the study. Phylogeographic studies, case reports, reviews or reports of new occurrence were not evaluated as experimental. The decision as to whether the study was or was not experimental was based on reading the abstracts, or the full-text (if it was not clear from the abstract).

Experimental studies were further classified based on the type of the host investigated (vertebrate or invertebrate) and objectives of the study (diagnostics, pathology, physiology, treatment) based on reading the abstract or full-text. As our aim was to determine the amount of information provided on laboratory-maintained strains of *A. cantonensis*, full-texts were manually searched for any information on how the authors obtained the helminths for the experiment(s), including reference to a previous publication, whether the isolate comes from nature/other laboratory or if the isolate is maintained in the laboratory on a long-term basis, etc., and also, if any DNA characterization of the strain was attempted. Thus, the results were tabulated in categories: (i) origin of the helminths specified; (ii) any DNA marker sequenced; (iii) *cox1* haplotype determined.

Additional files

Additional file 1: Alignment S1. Alignment of complete mtDNA sequences of all available *A. cantonensis* and *A. malaysiensis* in FASTA format.

Additional file 2: Alignment S2. Alignment of complete mtDNA sequences of all available *A. cantonensis* and *A. malaysiensis* in CLC format including annotations.

Additional file 3: Table S1. Overview of *Angiostrongylus cantonensis* *cox1* sequences from GenBank. Sequences in bold represent the given haplotype in the *cox1* phylogenetic tree (Fig. 5). *Abbreviation:* mtDNA, sequence was extracted from complete mtDNA.

Additional file 4: Alignment S3. Alignment of complete *cox1* sequences used for PSD table and maximum likelihood tree in Fig. 4b, c.

Additional file 5: Alignment S4. Alignment of 254-bp fragment of *cox1* sequences used for PSD table and maximum likelihood tree in Fig. 4d, e.

Additional file 6: Alignment S5. Alignment of *cox1* sequences used for construction of maximum likelihood tree in Fig. 5.

Additional file 7: Tree S1. Full maximum likelihood tree of *cox1* from Fig. 5 where the *A. malaysiensis* clade is not collapsed.

Additional file 8: Table S2. List of publications used in our literature review.

Additional file 9: Text S1. Analysis of diversity of *cob* in invasive lineages of *A. cantonensis*.

Abbreviations

aa: amino acid; bp: base pairs; *cox1*: gene for subunit 1 of cytochrome *c* oxidase; *cox2*: gene for subunit 2 of cytochrome *c* oxidase; *cob*: gene for cytochrome *b*; mtDNA: mitochondrial DNA; *nad2*: gene for subunit 2 of NADH dehydrogenase; *nad5*: gene for subunit 5 of NADH dehydrogenase; *nad4L*: gene for subunit 4L of NADH dehydrogenase; NGS: next-generation sequencing; nt: nucleotide; PSD: pairwise sequence distance; PSD_N: pairwise sequence distance of nucleotide sequences; PSD_{AA}: pairwise sequence distance of amino acid sequences; SNP: single nucleotide polymorphism.

Acknowledgements

We thank 'Excmo. Cabildo Insular' of Tenerife for allowing us to conduct the field study. We thank the Sydney School of Veterinary Science, Faculty of Science, The University of Sydney for hosting BČ during her postdoctoral fellowship. We thank Dave Spratt (CSIRO) for discussions and insight into the *Angiostrongylus* biology.

Funding

The work in Tenerife was supported by "Proyectos I + D de la Consejería de Economía, Industria, Comercio y Conocimiento de la Comunidad Autónoma de Canarias" and FEDER 2014–2020 [grant number ProID2017010092]; Red de Investigación de Centros de Enfermedades Tropicales-RICET [grant number RD16/0027/0001]; ISCIII-Subdirección General de Redes y Centros de Investigación Cooperativa RETICS, Ministry of Health and Consumption, Spain (PF, AM). This study is outcome of project International Collaboration in ecological and evolutionary biology of vertebrates (CZ02.2.69/0.0/0.0/16_027/000802 7) within operational program Research, Development and Education controlled by the Ministry of Education, Youth and Sports of the Czech Republic funded by the European Structural and Investing Funds which supported the postdoctoral internship of BČ at the University of Sydney. Maintenance of Fatu Hiva isolates at UVPS was supported by grant of the Internal Grant agency of UVPS Project No. 123/2018/FVL (DM, BF). KH has been financially supported by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601).

Availability of data and materials

Sequence data were deposited at SRA NCBI BioProject: PRJNA533181. The complete mtDNA genome sequences were submitted to GenBank under the accession numbers MK570629 (TEN.1), MK570630 (HAW.1), MK570631 (SYD.1), MK570632 (FH.1). Vouchers from Hawaii (HAW) and Sydney (SYD)

were deposited in the Australian National Wildlife Collection, CSIRO, Canberra, Australian Capital Territory, Australia under the accession numbers: W/L HC# N5703–N5711. Vouchers from Fatu Hiva (FH) and Tenerife (TEN) were deposited in the helminthological collection of Institute of Parasitology, Biology Center, České Budějovice, under the accession number IPCAS N-260.

Authors' contributions

BČ carried out bioinformatics and data analysis, results interpretations and drafted the manuscript; BF provided systematic literature analysis. BČ, BF isolated DNA and carried out PCR analysis and morphological determination. RM, JW contributed insight into the historical emergence and clinical disease consequences. DM, KH, PF, AMA, RL, JW, CNN collected and contributed material. JŠ, DM supervised the study and provided resources. JŠ, DM conceived and coordinated the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All isolates were obtained according to local animal ethics guidelines including appropriate permits adhering to local regulations. In Hawaii, USA, wild rats (*R. exulans*) were live-trapped in May 2018 and humanely euthanised in a CO₂ chamber and frozen at –20 °C. The necropsy was performed in July 2018 and worms were extracted from pulmonary artery of a single male rat. All animal procedures were conducted according to the Guidelines of the American Society of Mammalogists for the use of wild mammals in research and following the approved Institutional Animal Care and Use Committee protocols (QA-2835; US Department of Agriculture, National Wildlife Research Center). In Tenerife, Spain, wild rats (*R. rattus* and *R. norvegicus*) were live-trapped in April 2018 in the forested area of Parque Rural de Anaga. Captured animals were sacrificed by barbiturate overdose in the laboratories of the Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias and necropsied. Worms were extracted from pulmonary artery of a single adult *R. norvegicus*. All the animal procedures were performed in accordance with the guidelines of animal welfare in experimental science and the European Union legislation (Directive 86/609/EEC). In Sydney, adult worms were harvested from the lungs of laboratory Wistar rats experimentally infected with *A. cantonensis*. This parasite culture originated from a wild rat (*Rattus norvegicus*) caught around the Taronga Park Zoological Gardens in Sydney about 30 years ago. A live culture of these worms has been maintained in the laboratory (Western Sydney Local Health District Animal Ethics approval number 8002.02.13). Adult worms were frozen at –20 °C until extracted and sequenced. In Fatu Hiva, French Polynesia, the rats (co-occurring *R. rattus*, *R. exulans*) were trapped in snap traps in February 2017 at the edge of Omoa village, the worms used in the current study were extracted from the pulmonary artery of a single male *R. exulans*. All the animal procedures were performed in accordance with the guidelines of animal welfare in experimental science and the European Union legislation (Directive 86/609/EEC).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Sydney School of Veterinary Science, University of Sydney, Sydney, NSW 2006, Australia. ² Department of Pathology and Parasitology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého třída 1946/1, 612 42 Brno, Czech Republic. ³ Institute of Vertebrate Biology, Czech Academy of Sciences, Květná 8, 603 65 Brno, Czech Republic. ⁴ Institute of Parasitology, Biology Center of the Czech Academy of Sciences, Branišovská 1160/31, 370 05 České Budějovice, Czech Republic. ⁵ CEITEC VFU, University of Veterinary and Pharmaceutical Sciences Brno, Palackého třída 1946/1, 612 42 Brno, Czech Republic. ⁶ Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, C/Astrofísico F Sanchez, s/n, Tenerife, 38203 La Laguna, Canary Islands, Spain. ⁷ Department Obstetricia y Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad de La Laguna, 38203 San Cristóbal de La Laguna, Canary Islands, Spain. ⁸ Westmead Clinical School, University of Sydney, Sydney, NSW 2145, Australia. ⁹ Marie Bashir Institute for infectious Diseases and Biosecurity, University of Sydney, Sydney, NSW 2006, Australia. ¹⁰ USDA-APHIS-WS, National Wildlife Research Center, Hawaii Field Station, PO Box 10880, Hilo, HI 96721, USA. ¹¹ Centre for Veterinary Education, University

of Sydney, Sydney, NSW 2006, Australia. ¹² Present Address: Manaaki Whenua-Landcare Research, PO Box 69040, Lincoln 7608, New Zealand.

Received: 10 March 2019 Accepted: 6 May 2019

Published online: 16 May 2019

References

- Pysek P, Richardson DM. Invasive species, environmental change and management, and health. *Annu Rev Env Resour.* 2010;35:25–55.
- Simberloff D, Martin JL, Genovesi P, Maris V, Wardle DA, Aronson J, et al. Impacts of biological invasions: what's what and the way forward. *Trends Ecol Evol.* 2013;28:58–66.
- Hulme PE. Trade, transport and trouble: managing invasive species pathways in an era of globalization. *J Appl Ecol.* 2009;46:10–8.
- Pejchar L, Mooney HA. Invasive species, ecosystem services and human well-being. *Trends Ecol Evol.* 2009;24:497–504.
- Hoberg EP. Invasive processes, mosaics and the structure of helminth parasite faunas. *Rev Sci Tech.* 2010;29:255–72.
- Ehrenfeld JG. Ecosystem consequences of biological invasions. *Annu Rev Ecol Evol S.* 2010;41:59–80.
- Audoin-Rouzeau F, Vigne J-D. La colonisation de l'Europe par le rat noir (*Rattus rattus*). *Rev Paléobiol.* 1994;13:125–45.
- Morand S, Bordes F, Chen HW, Claude J, Cosson JF, Galan M, et al. Global parasite and *Rattus* rodent invasions: the consequences for rodent-borne diseases. *Integr Zool.* 2015;10:409–23.
- Kosoy M, Khlyap L, Cosson JF, Morand S. Aboriginal and invasive rats of genus *Rattus* as hosts of infectious agents. *Vector-Borne Zoonotic Dis.* 2015;15:3–12.
- Barratt J, Chan D, Sandaradura I, Malik R, Spielman D, Lee R, et al. *Angiostrongylus cantonensis*: a review of its distribution, molecular biology and clinical significance as a human pathogen. *Parasitology.* 2016;143:1087–118.
- Rosen L, Chappell R, Laqueur GL, Wallace GD, Weinstein PP. Eosinophilic meningoencephalitis caused by a metastrongylid lung-worm of rats. *JAMA.* 1962;179:620–4.
- Rosen L, Laigret J, Bories S. Observations on an outbreak of eosinophilic meningitis on Tahiti, French Polynesia. *Am J Hyg.* 1961;74:26–42.
- Chen HT. Un nouveau nématode pulmonaire, *Pulmonema cantonensis* n.g., n.sp. des rats de Canton. *Ann Parasitol Hum Comp.* 1935;13:312–7.
- Beaver PC, Rosen L. Memorandum on the first report of *Angiostrongylus* in man, by Nomura and Lin, 1945. *Am J Trop Med Hyg.* 1964;13:589–90.
- Bhaibulaya M. Comparative studies on the life history of *Angiostrongylus mackerrasae* Bhaibulaya, 1968 and *Angiostrongylus cantonensis* (Chen, 1935). *Int J Parasitol.* 1975;5:7–20.
- Wallace GD, Rosen L. Studies on eosinophilic meningitis. II. Experimental infection of shrimp and crabs with *Angiostrongylus cantonensis*. *Am J Epidemiol.* 1966;84:120–31.
- Kim JR, Hayes KA, Yeung NW, Cowie RH. Diverse gastropod hosts of *Angiostrongylus cantonensis*, the rat lungworm, globally and with a focus on the Hawaiian Islands. *PLoS ONE.* 2014;9:e94969.
- Lunn JA, Lee R, Smaller J, MacKay BM, King T, Hunt GB, et al. Twenty two cases of canine neural angiostrongylosis in eastern Australia (2002–2005) and a review of the literature. *Parasit Vectors.* 2012;5:70.
- Ma GM, Dennis M, Rose K, Spratt D, Spielman D. Tawny frogmouths and brushtail possums as sentinels for *Angiostrongylus cantonensis*, the rat lungworm. *Vet Parasitol.* 2013;192:158–65.
- Walker AG, Spielman D, Malik R, Graham K, Ralph E, Linton M, et al. Canine neural angiostrongylosis: a case-control study in Sydney dogs. *Aust Vet J.* 2015;93:195–9.
- Alicata JE. Biology and distribution of the rat lungworm, *Angiostrongylus cantonensis*, and its relationship to eosinophilic meningoencephalitis and other neurological disorders of man and animals. *Adv Parasitol.* 1965;3:223–48.
- Tokiwa T, Hashimoto T, Yabe T, Komatsu N, Akao N, Ohta N. First report of *Angiostrongylus cantonensis* (Nematoda: Angiostrongylidae) infections in invasive rodents from five islands of the Ogasawara Archipelago, Japan. *PLoS ONE.* 2013;8:e70729.
- Wallace GD, Rosen L. Studies on eosinophilic meningitis. I. Observations on the geographic distribution of *Angiostrongylus cantonensis* in the Pacific Area and its prevalence in wild rats. *Am J Epidemiol.* 1965;81:52–62.
- Foronda P, Lopez-Gonzalez M, Miquel J, Torres J, Segovia M, Abreu-Acosta N, et al. Finding of *Parastrongylus cantonensis* (Chen, 1935) in *Rattus rattus* in Tenerife, Canary Islands (Spain). *Acta Trop.* 2010;114:123–7.
- Mugisha L, Bwangamoi O, Cranfield MR. *Angiostrongylus cantonensis* and other parasites infections of rodents of Budongo Forest Reserve, Uganda. *AJABS.* 2012;7:91–5.
- York EM, Creecy JP, Lord WD, Caire W. Geographic range expansion for the rat lungworm in North America. *Emerg Infect Dis.* 2015;21:1234–6.
- Dusitsittipon S, Criscione CD, Morand S, Komalamisra C, Thaenkham U. Cryptic lineage diversity in the zoonotic pathogen *Angiostrongylus cantonensis*. *Mol Phylogenet Evol.* 2017;107:404–14.
- Dusitsittipon S, Thaenkham U, Watthanakulpanich D, Adisakwattana P, Komalamisra C. Genetic differences in the rat lungworm, *Angiostrongylus cantonensis* (Nematoda: Angiostrongylidae), in Thailand. *J Helminthol.* 2015;89:545–51.
- Eamsobhana P, Lim PE, Solano G, Zhang HM, Gan XX, Sen Yong H. Molecular differentiation of *Angiostrongylus* taxa (Nematoda: Angiostrongylidae) by cytochrome c oxidase subunit I (COI) gene sequences. *Acta Trop.* 2010;116:152–6.
- Eamsobhana P, Song SL, Yong HS, Prasartvit A, Boonyong S, Tungtrongchitr A. Cytochrome c oxidase subunit I haplotype diversity of *Angiostrongylus cantonensis* (Nematoda: Angiostrongylidae). *Acta Trop.* 2017;171:141–5.
- Lv S, Guo YH, Nguyen HM, Sinuon M, Sayasone S, Lo NC, et al. Invasive *Pomacea* snails as important intermediate hosts of *Angiostrongylus cantonensis* in Laos, Cambodia and Vietnam: Implications for outbreaks of eosinophilic meningitis. *Acta Trop.* 2018;183:32–5.
- Rodpai R, Intapan PM, Thanchomnang T, Sanpool O, Sadaow L, Laymanivong S, et al. *Angiostrongylus cantonensis* and *A. malaysiensis* broadly overlap in Thailand, Lao PDR, Cambodia and Myanmar: a molecular survey of larvae in land snails. *PLoS ONE.* 2016;11:e0161128.
- Tokiwa T, Harunari T, Tanikawa T, Komatsu N, Koizumi N, Tung KC, et al. Phylogenetic relationships of rat lungworm, *Angiostrongylus cantonensis*, isolated from different geographical regions revealed widespread multi-ple lineages. *Parasitol Int.* 2012;61:431–6.
- Vitta A, Srisongcram N, Thiproaj J, Wongma A, Polsut W, Fukruksa C, et al. Phylogeny of *Angiostrongylus cantonensis* in Thailand based on cytochrome c oxidase subunit I gene sequence. *Southeast Asian J Trop Med Public Health.* 2016;47:377–86.
- Yong HS, Eamsobhana P, Song SL, Prasartvit A, Lim PE. Molecular phylogeography of *Angiostrongylus cantonensis* (Nematoda: Angiostrongylidae) and genetic relationships with congeners using cytochrome b gene marker. *Acta Trop.* 2015;148:66–71.
- Dusitsittipon S, Criscione CD, Morand S, Komalamisra C, Thaenkham U. Hurdles in the evolutionary epidemiology of *Angiostrongylus cantonensis*: pseudogenes, incongruence between taxonomy and DNA sequence variants, and cryptic lineages. *Evol Appl.* 2018;11:1257–69.
- Dalton MF, Fenton H, Cleveland CA, Elsmo EJ, Yabsley MJ. Eosinophilic meningoencephalitis associated with rat lungworm (*Angiostrongylus cantonensis*) migration in two nine-banded armadillos (*Dasypus novemcinctus*) and an opossum (*Didelphis virginiana*) in the southeastern United States. *Int J Parasitol Parasites Wildl.* 2017;6:131–4.
- Monte TCC, Simoes RO, Oliveira APM, Novaes CF, Thiengo SC, Silva AJ, et al. Phylogenetic relationship of the Brazilian isolates of the rat lungworm *Angiostrongylus cantonensis* (Nematoda: Metastrongylidae) employing mitochondrial COI gene sequence data. *Parasit Vectors.* 2012;5:248.
- Moreira VLC, Giese EG, Melo FTV, Simoes RO, Thiengo SC, Maldonado A, et al. Endemic angiostrongyliasis in the Brazilian Amazon: natural parasitism of *Angiostrongylus cantonensis* in *Rattus rattus* and *R. norvegicus*, and sympatric giant African land snails, *Achatina fulica*. *Acta Trop.* 2013;125:90–7.
- Simões RO, Monteiro FA, Sanchez E, Thiengo SC, Garcia JS, Costa-Neto SF, et al. Endemic angiostrongyliasis, Rio de Janeiro, Brazil. *Emerg Infect Dis.* 2011;17:1331–3.
- Monte TC, Gentile R, Garcia J, Mota E, Santos JN, Maldonado A. Brazilian *Angiostrongylus cantonensis* haplotypes, ac8 and ac9, have two different biological and morphological profiles. *Mem Inst Oswaldo Cruz.* 2014;109:1057–63.

42. Bhaibulaya M. A new species of *Angiostrongylus* in an Australian rat *Rattus fuscipes*. *Parasitology*. 1968;58:789–99.
43. Lv S, Zhang Y, Zhang L, Liu Q, Liu HX, Hu L, et al. The complete mitochondrial genome of the rodent intra-arterial nematodes *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*. *Parasitol Res*. 2012;111:115–23.
44. Yong HS, Song SL, Eamsobhana P, Lim PE. Complete mitochondrial genome of *Angiostrongylus malaysiensis* lungworm and molecular phylogeny of metastrongyloid nematodes. *Acta Trop*. 2016;161:33–40.
45. Mackerras MJ, Sandars DF. The life history of the rat lung-worm, *Angiostrongylus cantonensis* (Chen) (Nematoda: Metastrongylidae). *Aust J Zool*. 1955;3:1–25.
46. Prociw P, Carlisle MS. The spread of *Angiostrongylus cantonensis* in Australia. *Southeast Asian J Trop Med Public Health*. 2001;32:126–8.
47. Verneau O, Catzeffis F, Furano AV. Determining and dating recent rodent speciation events by using L1 (LINE-1) retrotransposons. *Proc Natl Acad Sci USA*. 1998;95:11284–9.
48. Maldonado Junior A, Zeitone BK, Amado LA, Rosa IF, Machado-Silva JR, Lanfredi RM. Biological variation between two Brazilian geographical isolates of *Echinostoma paraensei*. *J Helminthol*. 2005;79:345–51.
49. Gharamah AA, Rahman WA, Azizah MNS. Morphological variation between isolates of the nematode *Haemonchus contortus* from sheep and goat populations in Malaysia and Yemen. *J Helminthol*. 2014;88:82–8.
50. Lee JD, Chung LY, Wang LC, Lin RJ, Wang JJ, Tu HP, et al. Sequence analysis in partial genes of five isolates of *Angiostrongylus cantonensis* from Taiwan and biological comparison in infectivity pathogenicity between two strains. *Acta Trop*. 2014;133:26–34.
51. Yong HS, Eamsobhana P, Lim PE, Razali R, Aziz FA, Rosli NSM, et al. Draft genome of neurotropic nematode parasite *Angiostrongylus cantonensis*, causative agent of human eosinophilic meningitis. *Acta Trop*. 2015;148:51–7.
52. Bhaibulaya M, Cross JH. *Angiostrongylus malaysiensis* (Nematoda: Metastrongylidae), a new species of rat lung-worm from Malaysia. *Southeast Asian J Trop Med Public Health*. 1971;2:527–33.
53. Yokogawa S. A new species of nematode found in the lungs of rats *Haemostrongylus ratti* sp. nov. *Trans Nat Hist Soc Formosa*. 1937;27:247–50.
54. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol*. 1994;3:294–9.
55. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;215:403–10.
56. Hahn C, Bachmann L, Chevreur B. Reconstructing mitochondrial genomes directly from genomic next-generation sequencing reads—a baiting and iterative mapping approach. *Nucl Acids Res*. 2013;41:e129.
57. Bernt M, Donath A, Juhling F, Externbrink F, Florentz C, Fritzsch G, et al. MITOS: improved *de novo* metazoan mitochondrial genome annotation. *Mol Phylogenet Evol*. 2013;69:313–9.
58. Kumar S, Stecher G, Li M, Niyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018;35:1547–9.
59. Nei M, Kumar S. *Molecular evolution and phylogenetics*. New York: Oxford University Press; 2000.
60. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*. 1993;10:512–26.
61. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci*. 1992;8:275–82.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

