Parasitology

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Analysis of the mitochondrial genome to determine the origins and pathways of entry of *Angiostrongylus cantonensis* in continental Europe (Valencia, Spain)

Mercedes Gómez-Samblás[1], Beatriz Navarro-Dominguez[2], Sandra Sáez-Durán[3], Antonio Osuna[1], Rubén Bueno-Marí[3,4], María Teresa Galán-Puchades[3] and Màrius V. Fuentes[3]

[1]Department of Parasitology, Biochemical and Molecular Parasitology Group CTS-183, and Institute of Biotechnology, University of Granada, 18071 Granada, Spain; [2]Genetics Department, Science Faculty, University of Granada, 18071 Granada, Spain; [3]Parasites & Health Research Group, Department of Pharmacy, Pharmaceutical Technology and Parasitology, Faculty of Pharmacy and Food Sciences, University of Valencia, Burjassot 46100, Valencia, Spain and [4]Laboratorios Lokímica and European Centre of Excellence in Vector Control, Rentokil Initial, Paterna, Valencia, Spain

Research Article

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Corresponding author:
Mercedes Gómez-Samblás;
Email: msambla@ugr.es

Abstract

Angiostrongylus cantonensis, the rat lungworm, is a zoonotic parasite mainly of rats which act as definitive hosts. If humans become accidentally infected, the nematode is capable of migrating to the brain causing meningoencephalitis. Intermediate hosts are snails and slugs. Although originating from mainland China, *A. cantonensis* has now spread to various countries and continents. The precise timing of its departure from mainland China remains uncertain although it is often associated with significant historical events or migratory movements. The exit of *A. cantonensis* from mainland China is believed to have occurred in a singular event, followed by its divergence into 2 distinct clades: clade I, originating from mainland China, and clade II, representing global spread. *Angiostrongylus cantonensis* was first identified in continental Europe in 2021, specifically in Valencia, Spain. Illumina genome sequencing of 7 individuals isolated from rats captured in 2 different districts in the city of Valencia was carried out. The complete mitochondrial genome was assembled and compared with published *A. cantonensis* mitochondrial genomes through Bayesian phylogenetic analysis, both for complete mitochondrial genomes and for the cytochrome c oxidase I gene, given its widespread use for identification of the species. The findings revealed the presence of 2 different *A. cantonensis* haplotypes in the rats studied in Valencia, both belonging to clade II. In 2 rats both clades were present.

Introduction

The detection of the zoonotic parasite *Angiostrongylus cantonensis*, the rat lungworm, in the city of Valencia (Spain) in 2021 marked a significant milestone (Galán-Puchades *et al.*, 2022). However, the review conducted in the same year by Gonzálvez and Ruiz de Ybáñez (2022) did not report this finding. Its significance cannot be overstated as it marks the initial documented occurrence of this zoonotic parasite in continental Europe. Previous observations of the parasite in European countries were limited to the Spanish islands of Tenerife in the Canary Islands (Foronda *et al.*, 2010) and Mallorca in the Balearic Islands (Paredes-Esquivel *et al.*, 2019).

The expansion of *A. cantonensis* beyond its presumed native region in Southeast Asia is attributed to global phenomena, notably international trade and travel (Kliks and Palumbo, 1992; Gippet *et al.*, 2023). Valencia, with a major port located less than 10 km from its city centre, plays a crucial role in this dynamic. This port is the 4th busiest in Europe and the 2nd largest in Spain and the entire Mediterranean region. It plays a pivotal role as a strategic maritime hub facilitating the flow of goods between Asia and Europe (Autoridad Portuaria de Valencia, 2024).

The life cycle of *A. cantonensis* revolves primarily around rats, in which adult parasites inhabit the pulmonary arteries and the right ventricle. However, in this part of its life cycle, the parasite usually produces only mild symptoms in rats (Morgan *et al.*, 2021). It completes its cycle in molluscs (snails and slugs) (Cowie *et al.*, 2022), which act as intermediate hosts, and uses crustaceans, planarians and frogs, among other taxa, as paratenic hosts (Turck *et al.*, 2022). Domestic animals such as dogs (Cowie *et al.*, 2023) and wildlife can become accidentally infected through ingestion of 3rd-stage larvae (L_3). In the specific case of humans, the L_3 migrate to the brain, often leading to severe, sometimes fatal, neuroangiostrongyliasis that could produce eosinophilic meningitis or meningoencephalitis (Galán-Puchades *et al.*, 2023). The first association of *A. cantonensis* with eosinophilic meningitis (neuroangiostrongyliasis) in humans was documented by Beaver and Rosen (1964), based on a case in Taiwan in 1944.

To date, the pathogenicity and pathophysiology of the disease remain poorly understood (Chang *et al.*, 2024). In the European continent, a case of neuroangiostrongyliasis was confirmed through a parasite antigen test in Paris in 2016 (Nguyen *et al.*, 2017), although the exact route of infection remains unknown. Various authors have warned about the global risk of neuroangiostrongyliasis emergence in countries where it had not been previously reported, designating it as an emerging parasitic disease (Cowie *et al.*, 2022; Gippet *et al.*, 2023). *Angiostrongylus cantonensis* has now spread to many parts of the tropics and subtropics, and most recently to more temperate locations, not only to the Canary Islands (Spain) (Foronda *et al.*, 2010), the Balearic Islands (Spain) (Paredes-Esquivel *et al.*, 2019) and mainland Spain (Galán-Puchades *et al.*, 2022), but also to Uganda (Mugisha *et al.*, 2012), the USA, namely Oklahoma and Georgia (York *et al.*, 2015, Gottdenker *et al.*, 2023) and Argentina (Hancke *et al.*, 2024). Cases of neuroangiostrongyliasis are likely to increase as the parasite spreads further. It is important to make medical practitioners more aware of this disease.

However, the precise timing and pathways of these invasions remain largely unknown. Several hypotheses have been proposed regarding the parasite's introduction to South America and the Canary Islands, its origins and the chronology of its arrival. It may have occurred through a single event, with a direct introduction from Asia to the Canary Islands, or through a series of intermediate steps involving Asia, other parts of the world, including other islands, such as Hawaii (Červená *et al.*, 2019; Tian *et al.*, 2023). Furthermore, little was known until 2015 (Martin-Alonso *et al.*, 2015) about whether the parasite was completing its life cycle in Europe (political Europe) and which intermediate hosts were involved. In 2024, Fuentes *et al.* (2024) amplified the *A. cantonensis* genome in snails collected in several locations in Valencia. These snails serve as intermediate hosts in the parasite's life cycle, confirming the complete establishment of the nematode in this Spanish city. This conclusion was bolstered by the findings of a Mallorcan group (Jaume-Ramis *et al.*, 2023) and Martin-Carrillo *et al.* (2023) from the Canary Islands, who also amplified the *A. cantonensis* genome in intermediate hosts. These investigations concluded that the snails *Theba pisana* and *Cornu aspersum* act as intermediate hosts for *A. cantonensis* in mainland Spain and Mallorca, while *Cernuella virgata* has been identified as a candidate host in mainland Spain only. *Limacus flavus*, *Milax gagates*, *Insulivitrina emmersoni* and *Insulivitrina oromii* were found infected by *A. cantonensis* in the Canary Islands of La Gomera and Gran Canaria.

Various genetic lineages of *A. cantonensis* have been identified across different endemic regions, although data on genetic and phenotypic diversity within invaded areas are limited (Červená *et al.*, 2019). Lee *et al.* (2014) noted the potential variability in the pathogenicity of *A. cantonensis* across distinct genetic lineages. Additionally, it remains unknown whether a single host can be parasitized by 1 or multiple lineages, and, if so, whether these lineages would share a common origin.

Compared to other parasites, such as protozoans (e.g. *Plasmodium falciparum* has around 450 sequences in the GenBank), there are few *A. cantonensis* genome sequences available for phylogenetic and evolutionary studies. Partial sequences of the mitochondrial genes internal transcribed spacer (ITS)-1 and ITS-2 are the most numerous. However, it appears that researchers are increasingly recognizing the parasite's dispersal and zoonotic potential, which has led to an increase in research on the biology of *A. cantonensis*. For instance, Tian *et al.* (2023) compiled and analysed all existing complete mitochondrial sequences, and several other partial mitochondrial and ITS markers of *A. cantonensis* published in various databases. Although Červená *et al.* (2019), Jefferies *et al.* (2009), Liu *et al.* (2011) and Rodpai *et al.* (2016) found that ITS1 and ITS2 are not suitable markers for resolving evolutionary relationships within *A. cantonensis* clades, as their low nucleotide diversity hinders phylogenetic reconstruction. Additionally, *A. cantonensis* has a notably low diversity mitochondrial genome, and hence longer sequences of the complete mitochondrial genome are needed for accurate phylogenetic resolution (Červená *et al.*, 2019).

By using Illumina sequencing and assembly, the current study aims to provide insights into the temporal and geographical origins of *A. cantonensis* found for the first time in continental Europe, as well as to elucidate if the rat population studied in Valencia since 2021 is parasitized by 1 or more lineages of *A. cantonensis*.

Materials and methods

Collection of A. cantonensis

The *A. cantonensis* specimens used for sequencing in this study were collected from rats captured in Valencia between June 2021 and June 2022, specifically from rats trapped in 2 districts, one of them associated with orchards near the port (district 19-Pobles del Sud), and the other in the sewer system almost 15 km from the port (district 16-Benicalap), as described by Galán-Puchades *et al.* (2022). The infected rats selected for the study were randomly chosen, consisting of 2 *Rattus norvegicus* and 2 *Rattus rattus* individuals (Table S1). *Angiostrongylus cantonensis* adults and subadults were extracted from the rat pulmonary arteries and brain, respectively, and identified based on morphological and molecular data as described by Galán-Puchades *et al.* (2022). Upon examination of the nematode genitalia, 1 male and 1 female were selected from each of the 2 *R. norvegicus* and 1 *R. rattus*, and 1 male was selected from one of the *R. rattus* specimens (Table S1) for Illumina sequencing.

For this study, specimen codes were assigned as follows: Acan (*A. cantonensis*) RVNN (where RV represents *Rattus* Valencia and NN denotes rat number), followed by M (male) or F (female) to indicate the sex of the nematode (Table S1).

Isolation of genomic DNA from tissue segments of A. cantonensis *adults*

Genomic DNA (gDNA) from the 4 male and 3 female *A. cantonensis* was obtained using the Nucleospin Tissue Kit protocol (Macherey-Nagel, Düren, Germany, catalogue number 740952.250) with modifications. Considering the tough cuticle (Gasser *et al.*, 1993) of nematodes, a mechanical disruption was performed by homogenizing the worms in tubes containing beads (MN Bead Tubes Type D, Macherey-Nagel, Düren, Germany, catalogue number 740814.5) and 200 µL of phosphate-buffered saline in a Precellys 24-Dual instrument (Bertin Technologies, Montigny-le-Bretonneux, France, catalogue number 03119-200-RD010) at 6500 rpm twice for 30 s. Subsequently, 200 µL of T1 buffer and 25 µL of proteinase K were added to each sample, mixed by vortexing, incubated overnight at 56°C and shaken every 3–4 h.

The following day, 200 µL of T3 buffer were added and incubated for 10 min at 70°C. DNA precipitation and washing steps were performed following the kit protocol, and elution was performed using 50 µL of MilliQ water. The quality and concentration of gDNA were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Waltham, Massachusetts, United States). The samples were stored at −20°C until use.

Whole genome sequencing of the rat lungworms

Library preparation and sequencing were performed by Biomarker Technologies GmbH (BMKGENE). Isolated gDNA

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(7.72–37.8 ng) was used for Illumina Reseq-M library preparation, followed by Illumina sequencing using Novaseq 6000 sequencing systems (150 bp, paired-end). The *A. cantonensis* genome size is estimated to be 290 Mb (Xu *et al.*, 2019). We obtained around 3 Gb data per individual to achieve 10× coverage. Prior to further analyses, we performed a quality trimming with Trimmomatic (Bolger *et al.*, 2014), using the options 'ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:28 TRAILING:28 SLIDINGWINDOW: 10:30 MINLEN:100'. After trimming, estimated coverage ranged between 7.5 and 9.6× (Table S1). Illumina reads are available in NCBI (Bioproject PRJNA1102741; SRA accession numbers in Table S1).

Assembly and phylogenetic analysis of the mitogenome

The full mitogenome of the 4 males and 3 females of *A. cantonensis* was assembled using 1 000 000 paired reads per species with the MITObim assembler (Hahn *et al.*, 2013); see Table S1 for GenBank accession numbers. For the phylogenetic analysis of mitogenomes, 10 sequences of *A. cantonensis* were retrieved from GenBank (Table S2), and also 8 mitogenomes assembled and available as supplementary material from Tian *et al.* (2023), listed in Table S2, were used. Sequences of *Angiostrongylus costaricensis* and *Angiostrongylus malaysiensis* were added to be used as outgroups for phylogenetic reconstruction (Table S2). COX-1 sequences were extracted from all mitogenomes, and 82 additional ones were obtained from GenBank (Table S3).

Sequences were aligned using mafft with the LINSI option (Katoh and Standley, 2013). Phylogenetic trees were constructed under Bayesian inference, performed in MrBayes version 3.2.7 (Huelsenbeck and Ronquist, 2001), with the GTR substitution model and Gamma rate variation (GTR-G), as in Tian *et al.* (2023). The posterior probabilities were estimated using Markov chain Monte Carlo simulations. Haplotypes and minimum spanning trees were constructed using the R package pegas (Paradis, 2010). Number of nucleotide differences and *P*-distances were estimated with MEGA11 (Tamura *et al.*, 2021).

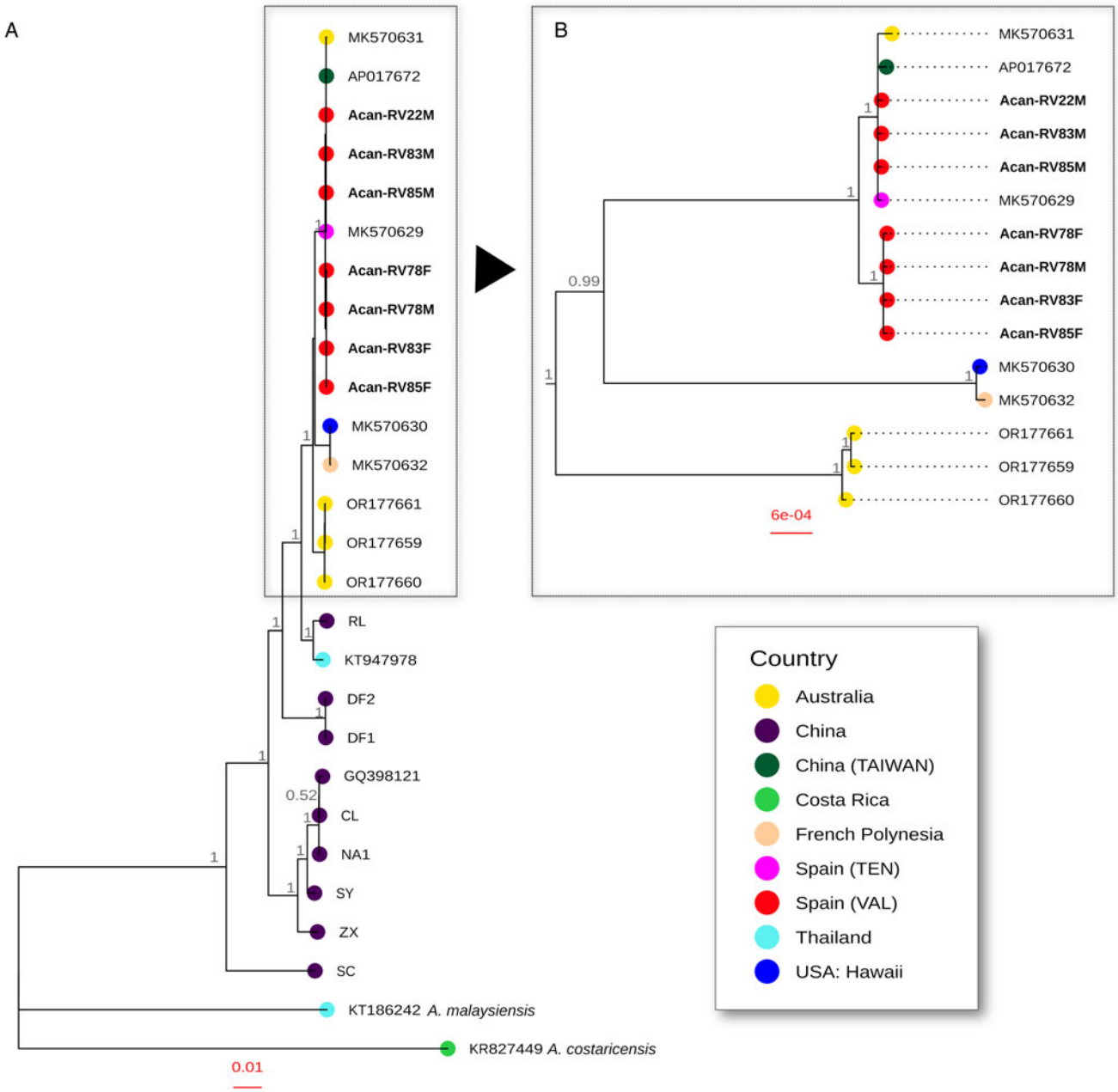

Figure 1. (A) Phylogenetic tree of complete mtDNA sequences, built using MrBayes. Numbers in the nodes indicate posterior probabilities. (B) Valencia samples branch shown at a smaller scale for better visualization.

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Results

Two different haplotypes of A. cantonensis *mtDNA from Valencia*

Our mtDNA assemblies are collinear with those reported by Červená *et al.* (2019). Length (13 505–13 509 bp) and gene content are also alike, with 12 protein-coding genes, 22 tRNA regions and 2 ribosomal subunits (12S and 16S).

We found 2 different haplotypes of mtDNA in the samples of *A. cantonensis* from Valencia (Fig. 1A, B). Both haplotypes belong to clade II, defined by Tian *et al.* (2023), which includes sequences from French Polynesia, Hawaii, Tenerife (Spain) and Sydney (Australia), sequenced by Červená *et al.* (2019), and from Taiwan (Kikuchi *et al.*, 2016) (Table S2).

Haplotypes from different locations in mainland China show a larger genetic distance among them than among haplotypes from elsewhere, even though the latter come from widely separated places (e.g. Spain and Australia; see Fig. 2).

The sequences of the individuals found in Valencia were split into 2 groups: 3 of them (Val-I) are very similar (0–2 nucleotide changes) to the Sydney (MK570631), Tenerife (MK570629) and Taiwan (AP017672) isolates; and the other 4 sequences (Val-II) group together in a different branch, with none of the sequences from other countries (Fig. 1B).

Val-I is identical to the haplotype found in Tenerife (Fig. 2). There were more differences between Val-I and Val-II (14 nucleotide changes) than between Val-I and one of the haplotypes found in Sydney (AUS-I, 2 nucleotide changes) and Taiwan (1 nucleotide change). There is almost no variation within the Val-I and Val-II haplotypes, and neither were any intermediate haplotypes found.

In each of the 2 rats, RV83 (*R. norvegicus*) and RV85 (*R. rattus*), we found *A. cantonensis* individuals of 2 different mitochondrial haplotypes (Table S1, Fig. 1).

Cytochrome c oxidase I (COI) indicates the origin of the Val-II haplotype in Brazil

A phylogenetic analysis using 107 sequences of *A. cantonensis* from various locations, plus 1 *A. costaricensis* and 1 *A. malaysiensis* as outgroup taxa (Fig. 3) indicates that the Val-I sequences (Valencia, Tenerife, Hawaii, Taiwan and Sydney) are the same as those in Mallorca (Paredes-Esquivel *et al.*, 2019) and New Orleans (Rael *et al.*, 2018).

The Val-II sequences form a cluster with 16 sequences from different locations in Brazil (Fig. 3), belonging to the one defined as the ac8 haplotype by Monte *et al.* (2012), which groups sequences from the states of São Paulo, Espirito Santo and Rio de Janeiro (southeastern Brazil), Pará (northern Brazil) and Pernambuco (northeastern Brazil) (Table S2) (Simões *et al.*, 2011; Monte *et al.*, 2012; Moreira *et al.*, 2013).

Discussion

This study is the first comprehensive sequencing of the complete mitochondrial genome of *A. cantonensis* from 7 individuals derived from continental Europe. Červená *et al.* (2019) aimed to elucidate the global geographic movements of *A. cantonensis*

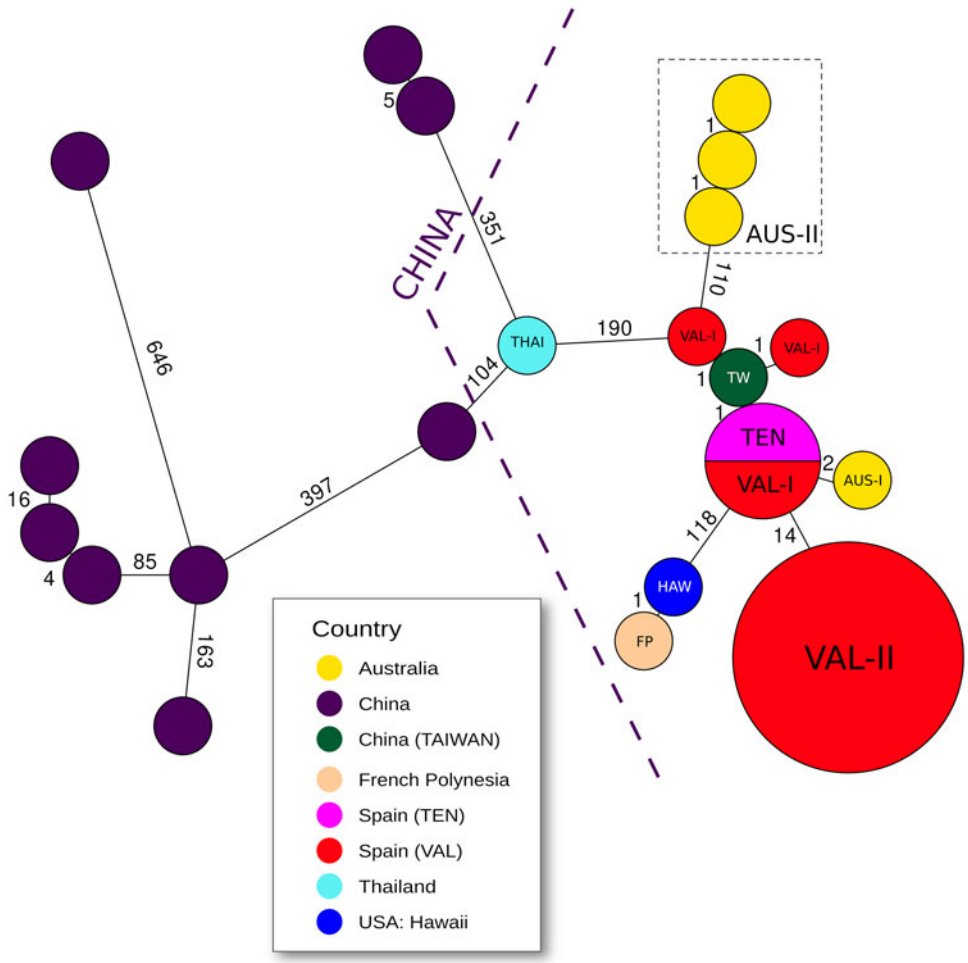

Figure 2. Minimal spanning network of complete mtDNA sequences. Numbers indicate the number of nucleotide differences between haplotypes.

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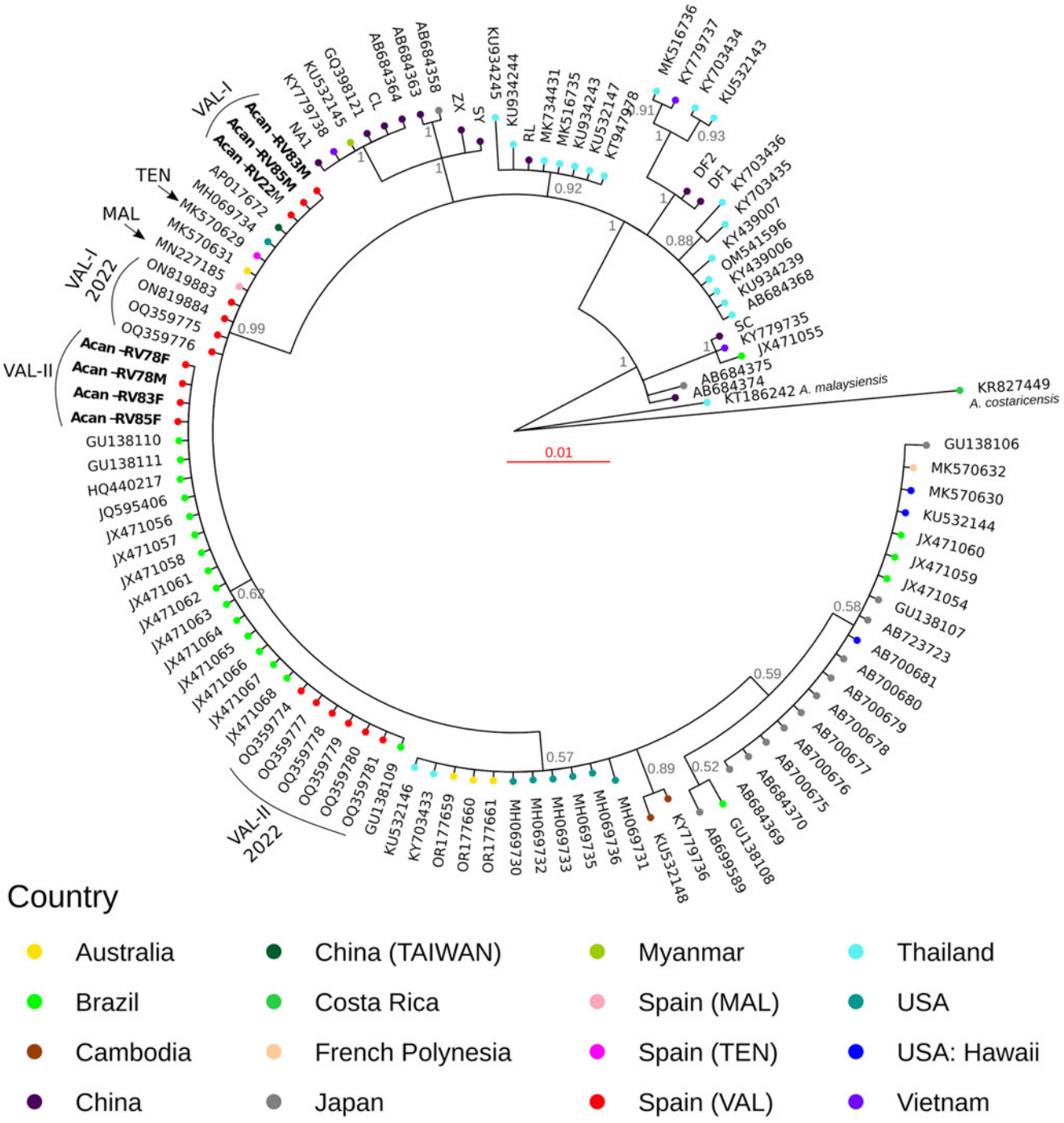

Figure 3. COI sequence phylogenetic analyses performed using MrBayes. Numbers on the nodes indicate posterior probabilities.

and recognized that, until recently, sequencing the mitochondrial genome was both complex and expensive. Even now, the number of published *A. cantonensis* sequences remains low. In their investigation, Červená *et al.* (2019) examined 4 individuals, each originating from geographically distant countries. According to our results, the genome assembly obtained mirrors the characteristics and features outlined by Červená *et al.* (2019) in their study.

Previous studies on *A. cantonensis* mtDNA revealed a greater nucleotide diversity among individuals in Asia compared with the mere 3 nucleotides differing across the entire mtDNA in individuals isolated from geographically distant regions such as Sydney and Tenerife. Our study corroborates this phenomenon when analysing all sequences isolated from China, compiled by Tian *et al.* (2023). As suggested by Červená *et al.* (2019), it appears that only a few individuals have managed to migrate from mainland China, and the genetic variability observed across different and distant regions of the world is likely to stem from a single invasion event from mainland China.

Among the 7 individuals sequenced, we identified 2 distinct haplotypes (Figs 1 and 2). All of our individuals belong to clade II, as defined by Tian *et al.* (2023). This clade includes most of the sequences that occur outside mainland China, encompassing sequences from French Polynesia (MK570632), Hawaii (MK570630), Tenerife (MK570629), Sydney (MK570631) and Taiwan (AP017672), among others.

Additionally, 3 of our individuals from 3 different rats (Acan-RV22M, Acan-RV83M and Acan-RV85M) correspond to haplotype Val-I, clustering with specimens isolated from Australia, Tenerife and Taiwan. Meanwhile, 4 individuals isolated from 3 different rats (Acan-RV78F, Acan-RV78M, Acan-RV83F

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and Acan-RV85F), among which 1 harboured both male and female nematodes, were grouped into haplotype Val-II, closely related to specimens isolated from Brazil.

This study is the first to demonstrate the coexistence of 2 distinct strains of *A. cantonensis* within a single host. Specifically, we found 2 rats with different haplotypes: RV83 parasitized by Acan-RV83M (Val-I) and Acan-RV83F (Val-II), and RV85 parasitized by Acan-RV85M (Val-I) and Acan-RV85F (Val-II). Such an observation was made possible through comprehensive mitochondrial genome sequencing, an approach seldom employed in previous investigations. Our findings underscore that the haplotype of *A. cantonensis* is not contingent upon the host species, as evidenced by the presence of the same haplotype parasitizing different rat species (i.e. Acan-RV22 isolated from *R. rattus* and Acan-RV83 from *R. norvegicus*). Notably, this phenomenon has been observed in districts near the maritime port.

The notion that these 2 haplotypes diverged from a common origin upon arrival in Valencia is refuted when considering the genetic distances between them. Val-I aligns with the haplotype found in Tenerife, whereas there are more differences between Val-I and Val-II (14 nucleotide changes) than between Val-I and the haplotypes found in Sydney and Taiwan. As there was almost no variation within the Val-I and Val-II haplotypes, and intermediate haplotypes were not observed, it is unlikely that these haplotypes diverged *in situ* in Valencia.

Our hypothesis regarding the invasion and distribution of *A. cantonensis*, associated with its first detection in continental Europe, suggests that haplotype Val-I (clade II) originated from Asia *via* Taiwan (clade II), aligning with the hypothesis of Červená *et al.* (2019). In our case, this may be attributed to the influx of cargo ships arriving on a daily basis at the port of Valencia, carrying containers that may also introduce rats from Asia. Although *A. cantonensis* originated from the Chinese mainland (clade I), our minimum spanning network analysis (Fig. 2) suggests that its outward spread was initially *via* Thailand (clade I), which are the sequences belonging to clade I that are molecularly closest to clade II. Still, haplotypes from Thailand (clade I) have 190 nucleotide differences compared to the clade II group of samples from Spain (Tenerife and Valencia), French Polynesia, Hawaii, Sydney and Taiwan. However, it is difficult to infer a clear timeline from Thailand because of both incomplete sampling and low differentiation among haplotypes in clade II.

Regarding the haplotype Val-II (clade II), our results from the analysis of COI sequences suggest that it entered continental Spain (Valencia) from Brazil. The absence of polymorphisms among individuals of the Val-II haplotype in Valencia, coupled with a divergence of 14 mutations from the closest Spanish haplotype (Tenerife and Val-I from Valencia), supports this inference. The exact time of arrival in Valencia could not be determined due to the lack of complete sequences from specimens isolated in Brazil, the potential source of introduction.

However, Val-I is more widely spread among European places frequently connected by boat (Valencia, Mallorca, Tenerife) and its sequences are also more variable, which supports the inference that Val-I was the first haplotype to enter Spain, and the arrival of Val-II from Brazil must have been more recent.

This study concludes that the *A. cantonensis* specimens isolated from rats in the European continent so far, specifically in the city of Valencia, belong to clade II as defined by Tian *et al.* (2023). This clade encompasses the other *A. cantonensis* specimens spread worldwide outside mainland China. Additionally, in Valencia, 2 haplotypes are found, differing by 14 nucleotides. Our results also indicate that the same rat, regardless of the species (*R. norvegicus* or *R. rattus*), can be parasitized by both haplotypes. Val-I is mostly identical to the specimens described from Tenerife and has been found both in rats captured near the port and in a city location 15 km away from the port. Val-II, a haplotype seemingly originating from Brazil, has only been found in rats captured near the port. Therefore, sequencing individuals from all sampled districts in Valencia with infected rats, as well as from other locations further from the coast, would be necessary to determine the true extent of this Val-II haplotype. This approach would enable the correlation of haplotype distribution with specific sampling locations relative to the port, which probably serves as the most important point of entry of the parasite to mainland Europe. Additionally, it would be worthwhile to obtain mitogenomes from *A. cantonensis* individuals found in Mallorca and to extend the study to other relevant ports in Spain, such as Algeciras, Spain's number 1 port, as the rat lungworm was not found in Barcelona (Galán-Puchades *et al.*, 2018), the 3rd busiest port in Spain after Valencia.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182024001318.

Data availability statement. All sequencing data generated in this study have been deposited in GenBank. Accession numbers for all the generated data are provided in the supplementary tables. All the information required to replicate the analyses presented in this study are included within the main text and supplementary materials.

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Author contributions. M. G.-S., B. N.-D. and A. O. conceived and designed the study and conducted data gathering. M. T. G.-P., M. V. F. and S. S.-D. conducted experimental work, collected the samples and identified parasites. R. B.-M. reviewed the draft. M. G.-S., B. N.-D., A. O., M. T. G.-P. and M. V. F. wrote the article. M. Gómez-Samblás and B. Navarro-Dominguez contributed equally to this work.

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Competing interests. None.

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