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Insights into the genetic diversity of *Angiostrongylus* spp. causing human angiostrongyliasis and implications for molecular identification and diagnosis

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ABSTRACT

Angiostrongylus cantonensis and *Angiostrongylus costaricensis* are known human pathogens responsible for eosinophilic angiostrongyliasis and abdominal angiostrongyliasis, respectively. Humans are accidental hosts, where infection occurs through the consumption of the infective larva stage 3 in intermediate or paratenic hosts. The proven method for abdominal angiostrongyliasis diagnosis is the histological examination through tissue biopsy, while the diagnosis of eosinophilic angiostrongyliasis is the detection of larva in the cerebrospinal fluid. As there is molecular evidence of cryptic species within *A. cantonensis* and *A. costaricensis* lineages, along with morphological similarities within both lineages, accurate species identification and disease diagnosis may be challenging. Moreover, species within the lineages share similar intermediate and definitive hosts and geographic distribution. For example, both *A. cantonensis* and *Angiostrongylus malaysiensis* (a closely related species in *A. cantonensis* lineage) overlap in their geographic distribution in Southeast Asia. Additionally, variations in the molecular makeup of *A. costaricensis* and *A. cantonensis* lineages may impact the pathogenicity, infectivity, and disease severity of angiostrongyliasis. Understanding of the genetic diversity of both lineages is a cornerstone for improved diagnosis and disease intervention, especially in a changing global environment. To shed light and provide insights into the genetic diversity of the *Angiostrongylus* lineages causing human angiostrongyliasis, we aim to present an up-to-date review of the studies conducted and genetic markers used for *A. costaricensis* and *A. cantonensis* lineages. The implications for accurate molecular identification and diagnosis of human angiostrongyliasis are also discussed.

1. Introduction

Human angiostrongyliasis, caused by the nematode *Angiostrongylus cantonensis* (Chen, 1935) and *Angiostrongylus costaricensis* Morera & Cespedes, 1971, is a zoonotic disease found in the tropics, subtropics, and temperate regions (Pandian et al., 2023; Rojas et al., 2021). These nematodes are responsible for eosinophilic angiostrongyliasis and abdominal angiostrongyliasis, respectively, leading to symptoms of varying severity. Both species have similar life cycles involving gastropods (e.g., snails and slugs) as intermediate hosts and rodents as definitive hosts. Humans become dead-end hosts by consuming infective larva stage 3 in intermediate or

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paratenic hosts. *Angiostrongylus costaricensis* was first discovered in humans in Costa Rica and has since been reported in the Caribbean and other countries in South America (Valente et al., 2018). *Angiostrongylus cantonensis*, initially found in rodents in China, has expanded its range and is now cosmopolitan, present in Southeast Asia, East Asia, South Asia, Australia, North and South America, Europe, and Africa (Barratt et al., 2016).

Cryptic species, defined as morphologically similar taxa but representing distinct genetic lineages, complicate the identification of *Angiostrongylus*. Previous studies have hinted at the possibility of cryptic species within the *A. cantonensis* lineage, which includes the closely related *Angiostrongylus malaysiensis* (Bhaibulaya and Cross, 1971) and *Angiostrongylus mackerrasae* Bhaibulaya, 1968 (Dusitsittipon et al., 2017; Dusitsittipon et al., 2018). Although the geographical range of *A. mackerrasae* is confined to Australia, *A. cantonensis* and *A. malaysiensis* overlap in Southeast Asia (Barratt et al., 2016). The overlap of geographic distribution, sharing of similar intermediate and definitive host species, and close genetics exacerbate the risk for morphological misidentification, phenotypic plasticity, and potential hybridization events. Additionally, the co-occurrence of both *A. cantonensis* and *A. malaysiensis* DNA was detected in snail hosts and human cerebrospinal fluid (CSF) in Thailand, suggesting the possibility of *A. malaysiensis* as a human pathogen (Jakkul et al., 2021; Watthanakulpanich et al., 2021). For *A. costaricensis*, molecular evidence suggests the possibility of sibling species between the isolates obtained from Costa Rica and Brazil (Rojas et al., 2021; Yong et al., 2015a).

As morphological similarities and molecular evidence of cryptic species within the *Angiostrongylus* lineages are evident, molecular methods are crucial for accurate identification and diagnosis. The presumptive diagnosis for both species are clinical symptoms, travel, and food consumption history. For definitive diagnosis, the current method for abdominal angiostrongyliasis caused by *A. costaricensis* is the histological examination of worms after tissue biopsy (Rodriguez et al., 2023). Serology methods like the enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay (IFA), and immunochromatographic tests have also been developed (Geiger et al., 2001; Graeff-Teixeira et al., 2020). For eosinophilic angiostrongyliasis due to *A. cantonensis* infection, the definite diagnosis is the detection of larva in the CSF (Ansdell et al., 2021). However, the larva is not often found in CSF samples, and if the larva is present, *A. cantonensis* cannot be morphologically differentiated from *A. malaysiensis*. Serological assays using the 31-kDa and 29-kDa antigen for immunoblot, ELISA, and immunochromatographic tests have been developed for serum and CSF samples with high sensitivity (Dekumyoy et al., 2000; Eamsobhana et al., 2018a; Sawanyawisuth et al., 2011; Somboonpatarakun et al., 2020). However, cross-reactivity with other helminthiasis remains challenging for specific diagnosis.

DNA-based methods can enhance the diagnosis of *A. costaricensis* and *A. cantonensis*. Species-level identification is possible using various sample types (e.g., larva, adult, host tissue, and CSF) (Qvarnstrom et al., 2013; Watthanakulpanich et al., 2021). Additionally, DNA-based methods can help understand genetic diversity and the detection of cryptic species within *A. costaricensis* and *A. cantonensis* lineages. Various genetic markers from the nuclear and mitochondrial DNA have been utilized for *Angiostrongylus* molecular identification, diagnosis, and genetic diversity (Dusitsittipon et al., 2018; Lv et al., 2017; Qvarnstrom et al., 2013;). Understanding the genetic diversity and molecular makeup of *A. costaricensis* and *A. cantonensis* lineages is a cornerstone for improved diagnosis and disease intervention, especially in a rapidly changing global landscape due to climate changes and globalization. In the Atlantic Island of Tenerife (Canary Islands), *A. cantonensis* were found only in the northern part of the island, which has a warmer and more humid climate than other regions (Martín-Carrillo et al., 2021). Changes in climate may potentially increase the range of both definitive and intermediate hosts, aiding disease transmission and altering the distribution of *Angiostrongylus.* Moreover, the impact of globalization on the transmission of *Angiostrongylus* can be seen in the increase in angiostrongyliasis in non-endemic countries (Cowie et al., 2022). Here, to shed light and provide insights into the genetic diversity of the *Angiostrongylus* lineages causing human angiostrongyliasis, we present an up-to-date review of the studies conducted and genetic markers used for *A. costaricensis* and *A. cantonensis* lineages, The implications for accurate molecular identification and diagnosis of human angiostrongyliasis are also discussed.

2. Genetic diversity of *A. cantonensis* and *A. costaricensis* cryptic lineages

2.1. Angiostrongylus costaricensis cryptic lineage

Angiostrongylus costaricensis was first discovered in Costa Rica in 1952 in a child presenting symptoms of severe abdominal pain. To date, *A. costaricensis* distribution ranges from parts of South, Central, and North America, and Costa Rica remains endemic. At least 18 species of terrestrial mollusks are the experimental or natural intermediate hosts of *A. costaricensis*, and four out of the 18 mollusk species were found naturally infected with *A. cantonensis* (Carvalho et al., 2012; Rojas et al., 2021). Although the hispid cotton rat *Sigmodon hispidus* is the natural definitive host, other rodents (e.g., *Rattus norvegicus* and *Rattus rattus*) and mammals such as dogs, raccoons, opossums, and non-human primates have also been implicated as potential definitive hosts (Alfaro-Alarcón et al., 2015; Miller et al., 2006).

The genetic diversity of *A. costaricensis* is not widely studied. Currently, there are only a handful of *A. costaricensis* nucleotide sequences in the National Center for Biotechnology Information (NCBI) database for the nuclear 18S ribosomal RNA (rRNA) gene, 28S rRNA gene, internal transcribed spacer 2 region (ITS2), mitochondrial cytochrome *c* oxidase I (*COI*) gene, and whole mitochondrial genome. Using the two mitochondrial genome sequences obtained from *A. costaricensis* from Costa Rica and Brazil, genetic distances between the two isolates suggest that they are genetically distinct. The genetic distances obtained using the 36 mitochondrial genes and the 12 mitochondrial protein genes were 15.3% and 16.1%, respectively (Yong et al., 2015a). Similarly, Eamsobhana et al. (2010a) also observed a genetic distance of 11.7% with the mitochondrial *COI* gene between the isolates from Costa Rica and Brazil (Eamsobhana et al., 2010a).

Additionally, the genetic distance values obtained with the two isolates were similar to the genetic distance obtained between *A. cantonensis* and *A. malaysiensis* (9.8% and 11.9% with *COI* and whole mitochondrial genomes, respectively) (Chan et al., 2020; Yong

et al., 2016). The nuclear ITS2 region also supports that the isolates were phylogenetically distinct, with genetic distances of 7.4% to 9.1% (Jefferies et al., 2009). Moreover, intraspecies genetic distances for other *Angiostrongylus* spp. using ITS2 region are generally small to none. With molecular evidence, the *A. costaricensis* isolates from Costa Rica and Brazil possibly represents a cryptic lineage. Evolutionary forces like the diversity of the intermediate and definitive hosts for *A. costaricensis* and the parasite's distribution range could drive its genetic diversity. As there are currently few studies on the genetic diversity of *A. costaricensis*, more data from various localities are needed for taxonomic delimitation to elucidate its status. Moreover, the elucidation of species status and increased molecular information for *A. costaricensis* is crucial for the search of candidate proteins for treatment targets for abdominal angiostrongyliasis (da Silva et al., 2022; da Silva and Morassutti, 2021; Rebello et al., 2011).

2.2. *Angiostrongylus cantonensis* cryptic lineage

The *Angiostrongylus cantonensis* cryptic lineage consists of *A. cantonensis*, *A. malaysiensis*, and *A. mackerrasae*. Of them, *A. cantonensis* is a human pathogen, while *Angiostrongyluus malaysiensis* has been implicated as a potential pathogen, but more substantial studies are needed to elucidate its pathogenicity (Watthanakulpanich et al., 2021). The life cycles of the three species are similar, with *A. cantonensis* and *A. malaysiensis* sharing intermediate and definitive host species, while the main definitive host of *A. mackerrasae* is *Rattus fuscipes*, found in Australia (Valentyne et al., 2020). Although *A. mackerrasae* has been found in non-native rodents, rodents native to Australia (e.g. *R. fuscipes*, *Rattus lutreolus*, and *Melomys cervinipes*) are more susceptible to *A. mackerrasae* infection (Tian et al., 2023). Regarding distribution, *A. cantonensis* and *A. mackerrasae* are present in Australia, while *A. cantonensis* and *A. malaysiensis* overlap widely in Thailand, Lao People's Democratic Republic (PDR), and Myanmar. Although morphological differences exist among adults, accurate identification may still be challenging. Using molecular-based methods, studies have mainly been performed in countries where *A. cantonensis* is prevalent (e.g., in Southeast Asia, East Asia, and Hawaii) using the mitochondrial *COI* and Cytochrome b (*Cytb*) genes and the nuclear ITS1 and ITS2 regions (Apichat et al., 2016; Dusitsittipon et al., 2017; Lv et al., 2017; Rodpai et al., 2016). The origin of *A. cantonensis* is hypothesized to be from South China or the Indochinese peninsula as it was first reported in 1935 from Guangzhou Province, China, in the bronchi of *Rattus norvegicus* and *Rattus rattus* (Chen, 1935). Both rodent species are generalists, live near humans, and can thrive in various environments (Feng and Himsworth, 2013). The intermediate host *Achatina fulica* has expanded its distribution range and was introduced as an invasive species in many countries (Celis-Ramírez and Quintero-Angel, 2022). Currently, *A. cantonensis* is distributed globally, and its spread can be attributed to the spread of the definitive rodent and intermediate snail hosts, which is, in turn, influenced by human activity.

Outside of Asia and Southeast Asia, the genetic diversity of *A. cantonensis* is low. Červená et al. (2019) revealed that the genetic distance using the mitochondrial *COI* gene between the *A. cantonensis* isolate from Sydney, Australia, and Tenerife, Spain, was 0.06%, while there was no genetic difference between the isolates from Hawaii, USA, and Fatu Hiva, French Polynesia (Červená et al., 2019). Similarly, a recent study by Álava et al. (2023) conducted in 11 provinces in Ecuador revealed no genetic differences in the *A. cantonensis* sampled (Álava et al., 2023). Complete mitochondrial genome phylogenies also support the genetic differentiation between the isolates from Asia and outside Asia, where the isolates from China and Thailand each formed their subclade while the isolates from Australia, Spain, the USA, French Polynesia, and Taiwan formed another subclade (Červená et al., 2019). The genetic diversity of *A. cantonensis* in Asia is comparatively higher, with 5% genetic distances between *A. cantonensis* isolates from China and Japan using mitochondrial genomes, while those between China and Thailand were 3.8% using the *COI* gene (Song et al., 2018; Tokiwa et al., 2012). Also, five subclades of *A. cantonensis* were observed using the mitochondrial *COI* gene, with subclades 1 and 3 solely from Thailand, subclade 4 from Southeast Asia and Japan, while subclades 2 and 5 are the most global as they contain isolates from Southeast Asia, the Americas, East Asia, and Australia (Dusitsittipon et al., 2018; Červená et al., 2019). The Thailand isolates were found in subclades 1, 2, and 3, revealing their high genetic diversity. Additionally, a recent study using complete mitochondrial genomes revealed that the haplotype diversity of *A. cantonensis* in Southeast and East Asia were significantly higher than those from other regions, corroborating with previous studies (Tian et al., 2023).

Moreover, genetic variability may impact the pathogenicity and infectivity of *A. cantonensis* in hosts and may be a factor influencing the severity of angiostrongyliasis in humans. In the laboratory setting, the pathogenicity and infectivity of the Hualien and Pingtung lineages of *A. cantonensis* in Taiwan has been investigated. Infection in rats and mice with the Hualien lineage resulted in lower infectivity and milder pathology as compared to the Pingtung lineage (Lee et al., 2014).

2.3. *Angiostrongylus cantonensis* cryptic lineage in Thailand

In Thailand, early studies revealed high genetic diversity with low gene flow between *A. cantonensis* populations, indicative of local fixation, and that the dispersal of *A. cantonensis* is likely due to human activity. Using eight *A. cantonensis* populations in Thailand (Chiang Mai, Nong Khai, Khon Kaen, Chanthaburi, Kanchanaburi, Lop Buri, Prachuap Khiri Khan, and Narathiwat provinces), two groups with statistically significant differentiation resulted using the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) method (Thaenkham et al., 2012). Moreover, the low gene flow, high genetic diversity between populations, and low genetic differences within populations suggest local fixation within their own populations. Support for high genetic diversity and low gene flow across geographic localities was also seen with the mitochondrial *COI* and *Cytb* genes, with seven haplotypes from seven provinces (Ranong, Samut Prakan, Phitsanulok, Trat, Chantaburi, Nakhon Phanom, and Kanchanaburi) and 11 haplotypes from eight provinces (Nan, Bangkok, Kanchanaburi, Lop Buri, Mahasarakam, Nakhon Sri Thammarat, Prachuap Khiri Khan, and Phitsanulok), respectively (Dusitsittipon et al., 2015; Eamsobhana et al., 2017). Additionally, four phylogenetic clusters were observed, with a maximum of 3.1% intraspecies genetic distance using the *Cytb* gene.

Aside from *A. cantonensis*, the closely related *A. malaysiensis* was also found in Thailand, where only *A. cantonensis* was initially thought to be present. *Angiostrongylus malaysiensis* was first discovered in *Rattus tiomanicus* from Malaysia, and it was known as the Malaysian lineage of *A. cantonensis* (Bhaibulaya and Cross, 1971). Currently, the distribution of *A. malaysiensis* ranges from Malaysia, Thailand, Myanmar, and Lao PDR, where overlapping countries with *A. cantonensis* are Thailand, Myanmar, and Lao PDR. Using the nuclear 18S rRNA gene, ITS2 region, and the mitochondrial *COI* gene, Rodpai et al. (2016) found *A. malaysiensis* in Phrae and Phatthalung provinces, while *A. cantonensis* was found in Khon Kaen and Surat Thani provinces (Rodpai et al., 2016). Sampling from 16 provinces across Thailand, Dusitsittipon et al. (2017) also uncovered the presence of both *A. cantonensis* and *A. malaysiensis* using the mitochondrial *Cytb* gene and microsatellite markers (Dusitsittipon et al., 2017). *Angiostrongylus cantonensis* was found in nine provinces – Nan, Nakhon Phanom, Phitsanulok, Mahasarakham, Lop Buri, Kanchanaburi, Bangkok, Prachuap Khiri Khan, and Nakhon Si Thammarat, while *A. malaysiensis* was found in ten provinces – Chiang Mai, Nong Khai, Khon Kaen, Lop Buri, Bangkok, Chanthaburi, Prachuap Khiri Khan, Ranong, Phang Nga, and Narathiwat. Additionally, of the 16 provinces, both species of *Angiostrongylus* were present in Bangkok, Lop Buri, and Prachuap Khiri Khan provinces. These results confirmed the widespread distribution of *A. malaysiensis* in Thailand and the occurrence of both species in the same province. Additionally, *A. malaysiensis*'s status as a distinct species from *A. cantonensis* is evidenced and supported by both nuclear and mitochondrial genetic markers (Chan et al., 2020). The interspecies genetic distance between these two species in *A. cantonensis* cryptic lineage ranged from 10.9% to 12.1%, 9.8 to 16.4%, and 15.1 to 15.7% with the *Cytb* gene, *COI* gene, and ITS2 region, respectively.

Besides having both *A. cantonensis* and *A. malaysiensis*, molecular evidence using the *Cytb* gene further revealed coinfection of both species in the same snail host. In a survey done in Bangkok parks, 5.97% of *A. fulica* were coinfected with both *Angiostrongylus* spp. (Jakkul et al., 2021). Moreover, all *A. fulica* that were infected with *A. cantonensis* were consistently found to be coinfected with *A. malaysiensis*. In an angiostrongyliasis outbreak area in Kalasin Province in 2019, *Pila virescens* was found coinfected with both *Angiostrongylus* spp. (Watthanakulpanich et al., 2021). Moreover, the DNA of *A. malaysiensis* and *A. cantonensis* were detected in human CSF samples. With evidence revealing both species coinfecting in one intermediate snail host and in humans, along with their close genetics, hybrids may occur between *A. cantonensis* and *A. malaysiensis* in nature. Supporting this hypothesis, one F1 hybrid female was identified in Bangkok using microsatellite markers (Dusitsittipon et al., 2017). Recently, using the nuclear ITS2 region, 8.2% of the *Angiostrongylus* specimens sampled were identified as hybrids (Kaenkaew et al., 2024). Also, experimental crosses conducted among *A. cantonensis* x *A. malaysiensis* and *A. cantonensis* x *A. mackerrasae* revealed that the F1 hybrid females were able to produce eggs, while the F1 males were sterile (Bhaibulaya, 1974).

Hybridization can result in the introgression and transfer of genetic material, with genes affecting parasite transmission, pathogenicity, adaptation, drug resistance, and phenotypic characteristics. Evidence of hybridization has been observed in nature between *Haemonchus contortus* and *Haemonchus placei* and can consequently lead to interspecies introgression of anthelmintic resistance alleles from one species to another (Chaudhry et al., 2015; Santos et al., 2019). Hybrids were also detected among the sibling species of *Anisakis simplex* and *Anisakis pegreffii*, where hybrid species complicate accurate identification using morphology (Roca-Geronès et al., 2021). Consequently, the detection and evidence of hybrids occurring between *A. cantonensis* and *A. malaysiensis* has significant implications in the epidemiological, identification, and diagnostic aspects, further complicating the cryptic species status of *A. cantonensis* lineage in Thailand. Morphological similarities, overlapping characters, and no reliable diagnostic character to differentiate both species and their hybrid forms further complicate accurate identification. For treatment, although studies have shown no difference between treatments (corticosteroid only or combination of corticosteroid and anthelminthic drugs) administered for eosinophilic meningitis, the pathogenicity of infection may be different among *A. cantonensis*, *A. malaysiensis*, or their hybrids (Chotmongkol et al., 2009). Thus, the effectiveness and treatment regime may potentially be affected. However, more studies are needed to elucidate this hypothesis.

3. Genetic markers used for molecular diagnosis and identification of *Angiostrongylus*

The advancement of molecular techniques greatly aided in accurately diagnosing and identifying pathogens, including

Table 1
Summary of molecular studies conducted on *A. costaricensis*, including genetic marker, molecular method, sample type, host, and location of study.

Genetic marker	Method	Sample type/ Host/ Locality	Type of study	Reference
COI	cPCR	Adult/ Rodent/ Costa Rica	Identification, phylogenetics	(Eamsobhana et al., 2010a)
	cPCR	Adult, larva/ White-nosed coati/ Costa Rica	Identification	(Santoro et al., 2016)
	PCR-RFLP	Adult, larva/ Rodent, mollusk/ Brazil	Identification, assay development	(Caldeira et al., 2003)
	Secondary dataset	NA	Genetic diversity, phylogenetics	(Song et al., 2018)
12S	cPCR	Adult, larva/ Dog/ Costa Rica	Diagnosis	(Alfaro-Alarcón et al., 2015)
Mt genome	Long-PCR	Adult/ Rodent/ Brazil	Mt genome characterization	(Lv et al., 2012)
	Illumina	Adult/ Rodent/ Costa Rica	Mt genome characterization	(Yong et al., 2015a)
	Secondary dataset	NA	Mt genome characterization	(Song et al., 2018)
18S	cPCR	Adult, larva/ White-nosed coati/ Costa Rica	Identification	(Santoro et al., 2016)
		Adult, larva/ Dog/ Costa Rica	Diagnosis	(Alfaro-Alarcón et al., 2015)
66-kDa	cPCR	Adult/ Rodent/ Costa Rica	Phylogenetics	(Eamsobhana et al., 2010b)
		Paraffin embedded (FFPE) tissue / Human/ Brazil	Diagnosis	(Rodriguez et al., 2014)
		Adult, serum/ Rodent, human/ Brazil	Diagnosis	(Amaral et al., 2023)
		Serum/ Human/ Brazil	Diagnosis	(da Silva et al., 2003)

Table 2
Summary of molecular studies conducted on *A. cantonensis*, including genetic marker, molecular method, sample type, host, and location of study.

Genetic marker	Method	Sample type/ Host/ Locality	Type of study	Reference
COI	cPCR	Larva/ Human/ Thailand	Identification	(Boonroumkaew et al., 2020)
		Adult/ Hedgehog/ Spain	Identification	(Delgado-Serra et al., 2022)
		Adult/ Rodent/ Australia	Identification	(Valentyne et al., 2020)
		Adult/ Rodent/ China	Genetic diversity	(Lv et al., 2017)
		CSF/ Human/ Hawaii	Identification	(Qvarnstrom et al., 2016)
		Larva/ Mollusk/ Thailand	Identification	(Apichat et al., 2016)
		Adult, larva/ Rodent, mollusk/ Japan, China, Taiwan, Thailand	Identification, phylogenetics	(Tokiwa et al., 2012)
		Larva/ Mollusk/ Brazil	Identification, phylogenetics	(Monte et al., 2012)
		Adult, larva/ Rodent, mollusk/ Brazil	Identification	(Moreira et al., 2013)
		Adult, larva/ Rodent, mollusk/ Thailand, Hawaii, China	Identification, phylogenetics	(Eamsobhana et al., 2010a)
		Adult/ Rodent/ French Polynesia, Spain, Hawaii, Australia	Genetic diversity	(Červená et al., 2019)
		Larva/ Mollusk/ Thailand	Genetic diversity	(Dumidae et al., 2019)
		Adult/ Rodent/ Japan	Identification, genetic diversity	(Tokiwa et al., 2013)
		Adult/ Opossum, armadillo/ Greece, America	Identification	(Dalton et al., 2017)
		Adult/ Rodent/ Thailand	Genetic diversity, phylogenetics	(Eamsobhana et al., 2018b)
		Adult/ Rodent/ Spain	Identification	(Galán-Puchades et al., 2022)
		Larva/ Mollusk/ Thailand, Lao PDR, Myanmar	Identification, phylogenetics	(Rodpai et al., 2016)
		Larva/ Mollusk/ Taiwan	Identification	(Lee et al., 2014)
	PCR-RFLP	Adult, larva/ Rodent, mollusk/ Japan	Identification, assay development	(Caldeira et al., 2003)
	qPCR	CSF/ Dog/ Australia	Identification	(Mallaiyaraj et al., 2021)
	Secondary dataset	NA	NA	(Dusitsittipon et al., 2018)
		NA	Genetic diversity, phylogenetics	(Song et al., 2018)
NAD1	cPCR	Adult/ Rodent/ China	Genetic diversity	(Lv et al., 2017)
Cytb	cPCR	Larva/ Mollusk/ China, Taiwan, Lao PDR	Genetic diversity	(Peng et al., 2017)
		Adult/ Rodent/ Thailand, Hawaii, China	Genetic diversity, phylogenetics	(Yong et al., 2015b)
		Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dusitsittipon et al., 2015)
		Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dumidae et al., 2019)
		Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dusitsittipon et al., 2017)
	qPCR	Larva, CSF/ Mollusk, human/ Thailand	Diagnosis, identification	(Watthanakulpanich et al., 2021)
		Larva, CSF/ Mollusk, human/ Thailand	Assay development, identification	(Jakkul et al., 2021)
		Adult/ Rodent/ Thailand	Identification	(Kaenkaew et al., 2024)
	Secondary dataset	NA	Genetic diversity, phylogenetics	(Dusitsittipon et al., 2018)
12S	cPCR	Adult/ Rodent/ Thailand	Identification	(Chan et al., 2020)
16S	cPCR	Adult/ Rodent/ Thailand	Identification	(Chan et al., 2020)
Mt genome	Long-PCR	Adult/ Rodent/ Australia	Mt genome characterization	(Aghazadeh et al., 2015)
		Adult/ Rodent/ Australia	Mt genome characterization	(Valentyne et al., 2020)
		Adult/ Rodent/ China	Mt genome characterization	(Lv et al., 2012)
		Adult/ Rodent/ China	Mt genome characterization, phylogenetics	(Tian et al., 2023)
	Long-PCR, Illumina	Adult/ Rodent/ French Polynesia, Spain, Hawaii, Australia	Genetic diversity	(Červená et al., 2019)
		Adult/ Rodent/ Thailand	Mt genome characterization, genetic diversity	(Yong et al., 2016)
		NA	Genetic diversity, phylogenetics	(Song et al., 2018)
18S	cPCR	Larva/ Human/ Thailand	Identification	(Boonroumkaew et al., 2020)
		Larva/ Mollusk/ French Polynesia	Identification	(Fontanilla and Wade, 2012)
		Larva/ Mollusk/ Spain	Identification	(Martin-Alonso et al., 2015)
		Larva/ Mollusk/ Philippines	Identification	(Fontanilla and Wade, 2008)
		Adult/ Rodent/ Thailand, Hawaii, Japan, China,	Phylogenetics	(Eamsobhana et al., 2015)
		Larva, adult/ Mollusk, rodent/ Japan, China, Taiwan, Thailand	Identification, Phylogenetics	(Tokiwa et al., 2012)
		Larva/ Mollusk/ Philippines	Identification	(Constantino-Santos et al., 2014)
		Larva/ Mollusk/ Thailand	Identification	(Dumidae et al., 2019)
		Adult/ Rodent/ Japan	Identification	(Tokiwa et al., 2013)
		Larva/ Mollusk/ Thailand, Lao PDR, Myanmar	Identification, phylogenetics	(Rodpai et al., 2016)

(*continued on next page*)

Table 2 (*continued*)

Genetic marker	Method	Sample type/ Host/ Locality	Type of study	Reference
		Lung tissue/ Fox/ Brazil	Identification	(Caprioli et al., 2019)
		Larva, mollusk tissue/ Mollusk/ Hawaii	Assay development	(Qvarnstrom et al., 2007)
		Larva/ Human/ Vietnam	Identification	(Van De et al., 2015)
		Mollusk tissue/ Mollusk/ Australia	Identification	(Chan et al., 2015)
	LAMP	Larva/ Mollusk/ China	Assay development	(Chen et al., 2011)
		Larva/ Mollusk/ Spain	Identification	(Martin-Alonso et al., 2015)
ITS1	cPCR	Adult/ Rodent/ China	Genetic diversity	(Lv et al., 2017)
		Adult/ Rodent/ China	Assay development	(Liu et al., 2011)
		Larva/ Mollusk/ Colombia	Identification	(Giraldo et al., 2019)
	qPCR	Mollusk tissue/ Mollusk/ USA	Identification	(Iwanowicz et al., 2015)
		Adult, mollusk tissue, CSF/ Rodent, mollusk, human/ USA	Assay development	(Sears et al., 2021)
		CSF/ Human/ Hawaii	Identification	(Qvarnstrom et al., 2016)
		Larva/ Mollusk/ Hawaii	Assay development	(Jarvi et al., 2012)
		Adult, larva, feces/ Rodent/ USA	Identification	(Stockdale-Walden et al., 2017)
		Blood, rodent tissue/ Rodent/ Hawaii	Identification	(Jarvi et al., 2015)
		Larva, mollusk tissue/ Mollusk/ USA	Identification	(Stockdale-Walden et al., 2015)
		Fox tissue/ Fox/ Brazil	Identification	(Caprioli et al., 2019)
		Larva, mollusk secretion/ Mollusk/ Hawaii	Assay development	(Qvarnstrom et al., 2010)
		Larva, mollusk tissue/ Mollusk/ USA	Identification	(Smith et al., 2015)
		Mollusk tissue/ Mollusk/ Australia	Identification	(Chan et al., 2015)
		Larva/ Mollusk/ Colombia	Identification	(Giraldo et al., 2019)
	LAMP	Adult/ Rodent/ China	Assay development	(Liu et al., 2022)
ITS2	cPCR	Larva/ Human/ Thailand	Identification	(Boonroumkaew et al., 2020)
		Adult/ Rodent/ China	Genetic diversity	(Lv et al., 2017)
		Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dumidae et al., 2019)
		Larva/ Mollusk/ Thailand, Lao PDR, Myanmar	Identification, phylogenetics	(Rodpai et al., 2016)
		Larva/ Mollusk/ Taiwan	Identification	(Lee et al., 2014)
		Larva/ Mollusk/ Brazil	Identification	(Cardoso et al., 2020)
	PCR-RFLP	Adult/ Rodent/ Japan	Identification	(Caldeira et al., 2003)
		Larva/ Mollusk/ Brazil	Identification	(Caldeira et al., 2007)
		Adult/ Rodent/ Thailand	Identification	(Kaenkaew et al., 2024)
	qPCR	CSF/ Dog/ Australia	Identification	(Mallaiyaraj et al., 2021)
		Larva, feces/ Rodent/ China	Assay development	(Fang et al., 2012)
66-kDa	cPCR	Adult/ Rodent/ Thailand, China, Japan	Phylogenetics	(Eamsobhana et al., 2010b)
		Adult/ Rodent/ Thailand, China, Japan, Hawaii	Phylogenetics, genetic diversity	(Eamsobhana et al., 2019)
		CSF/ Human/ Thailand	Diagnostics	(Eamsobhana et al., 2013)
		Adult, larva/ Rodent, mollusk/ Thailand	Molecular identification	(Dumidae et al., 2022)
Repetitive sequences	qPCR	Adult, mollusk tissue, CSF/ Rodent, mollusk, human/ USA	Assay development	(Sears et al., 2021)
	RPA	CSF/ Human/ Hawaii	Assay development	(Sears et al., 2021)
	RAPD	Larva/ Mollusk/ Thailand	Genetic diversity	(Thaenkham et al., 2012)
Microsatellite		Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dusitsittipon et al., 2017)
Whole genome	Shot-gun sequencing	Adult/ Rodent/ China	Genomics	(Xu et al., 2019)
	Illumina	Adult/ Rodent/ Thailand	Genomics	(Yong et al., 2015d)
	NGS	CSF/ Human/ China	Diagnostics	(Xie et al., 2019)
		CSF/ Human/ China	Diagnostics	(Liu et al., 2022)
		CSF/ Human/ China	Diagnostics	(Zou et al., 2020)
		CSF/ Human/ China	Diagnostics	(Feng et al., 2020)

Angiostrongylus. Tables 1 to 4 present the summary of genetic markers used for molecular-based studies of *A. costaricensis* and *A. cantonensis* cryptic lineages.

Based on our literature search, there were only 14 molecular studies specifically for *A. costaricensis*. Two of these used secondary datasets, and two focused on characterizing *A. costaricensis* mitochondrial genome. The genetic marker most utilized for diagnosis is the 66-kDa gene, where abdominal angiostrongyliasis was confirmed in human serum and paraffin-embedded tissue samples (Amaral et al., 2023; da Silva et al., 2003; Rodriguez et al., 2014). Adults and larva stages 1 and 3 were also identified in various host species, including dogs, white-nosed coati, and rodents using the nuclear 18S rRNA gene, ITS2 region, and the mitochondrial *COI* and 12S rRNA genes (Alfaro-Alarcón et al., 2015; Eamsobhana et al., 2010a; Santoro et al., 2016). Additionally, a PCR-restriction fragment length polymorphism (PCR-RFLP) using the ITS2 region and *COI* gene was developed by Caldeira et al. (2003) to differentiate between *A. costaricensis*, *A. cantonensis* and *A. vasorum* (Caldeira et al., 2003). With molecular evidence suggesting that the Brazil and Costa Rica isolates of *A. costaricensis* are possibly sibling species, there is an increasing need to determine the differences in pathogenicity and

Table 3
Summary of molecular studies conducted on *A. malaysiensis*, including genetic marker, molecular method, sample type, host, and location of study.

Genetic marker	Method	Sample type/Host/Locality	Type of study	Reference
COI	cPCR	Adult, larva/ Rodent, mollusk/ Thailand, Malaysia	Identification, phylogenetics	(Eamsobhana et al., 2010a)
		Larva/ Mollusk/ Thailand	Genetic diversity	(Dumidae et al., 2019)
		Larva/ Mollusk/ Thailand	Identification	(Chaisiri et al., 2019)
		Adult/ Rodent/ Thailand	Genetic diversity, phylogenetics	(Eamsobhana et al., 2018b)
		Larva/ Mollusk/ Thailand, Lao PDR, Myanmar	Identification, phylogenetics	(Rodpai et al., 2016)
	Secondary dataset	NA	Genetic diversity, phylogenetics	(Dusitsittipon et al., 2018)
		NA	Genetic diversity, phylogenetics	(Song et al., 2018)
Cytb	cPCR	Adult/ Rodent/ Malaysia	Genetic diversity, phylogenetics	(Yong et al., 2015b)
		Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dumidae et al., 2019)
		Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dusitsittipon et al., 2017)
	qPCR	Larva, CSF/ Mollusk, human/ Thailand	Diagnosis, identification	(Watthanakulpanich et al., 2021)
		Larva, CSF/ Mollusk, human/ Thailand	Assay development	(Jakkul et al., 2021)
		Adult/ Rodent/ Thailand	Identification	(Kaenkaew et al., 2024)
	Secondary dataset	NA	Genetic diversity, phylogenetics	(Dusitsittipon et al., 2018)
12S	cPCR	Adult/ Rodent/ Thailand	Identification	(Chan et al., 2020)
16S	cPCR	Adult/ Rodent/ Thailand	Identification	(Chan et al., 2020)
Mt genome	Illumina	Adult/ Rodent/ Thailand	Mt genome characterization, genetic diversity	(Yong et al., 2015c)
		Adult/ Rodent/ Malaysia	Mt genome characterization	(Yong et al., 2016)
	Secondary dataset	NA	Genetic diversity, phylogenetics	(Song et al., 2018)
18S	cPCR	Adult/ Rodent/ Malaysia	Phylogenetics	(Eamsobhana et al., 2015)
		Larva/ Mollusk/ Thailand	Identification	(Dumidae et al., 2019)
		Larva/ Mollusk/ Thailand, Lao PDR, Myanmar	Identification, phylogenetics	(Rodpai et al., 2016)
ITS2	cPCR	Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dumidae et al., 2019)
		Larva/ Mollusk/ Thailand, Lao PDR, Myanmar	Identification	(Rodpai et al., 2016)
	PCR-RFLP	Adult/ Rodent/ Thailand	Identification	(Kaenkaew et al., 2024)
66-kDa	cPCR	Adult/ Rodent/ Malaysia	Phylogenetics	(Eamsobhana et al., 2010b)
		Adult/ Rodent/ Malaysia	Phylogenetics, genetic diversity	(Eamsobhana et al., 2019)
		Adult, larva/ Rodent, mollusk/ Thailand	Molecular identification	(Dumidae et al., 2022)
Microsatellite		Larva/ Mollusk/ Thailand	Genetic diversity, phylogenetics	(Dusitsittipon et al., 2017)

Table 4
Summary of molecular studies conducted on *A. mackerrasae*, including genetic marker, molecular method, sample type, host, and location of study.

Genetic marker	Method	Sample type/Host/Locality	Type of study	Reference
COI	cPCR	Adult/ Rodent/ Australia	Identification	(Valentyne et al., 2020)
	Secondary dataset	NA	Genetic diversity, phylogenetics	(Song et al., 2018)
Mt genome	Long PCR	Adult/ Rodent/ Australia	Mt genome characterization	(Valentyne et al., 2020)
	Secondary dataset	NA	Genetic diversity, phylogenetics	(Song et al., 2018)

transmission among them. Moreover, diagnostic tools for abdominal angiostrongyliasis will have to be refined, ensuring the accuracy for detecting DNA of both isolates as substantial differences in genetic variation could lead to lowered sensitivity of the genetic markers and assays for molecular diagnosis.

For *A. cantonensis* lineage, various genetic markers have been used for molecular identification. These genetic markers include the nuclear 18S rRNA gene, ITS regions, mitochondrial *Cytb*, *COI*, 12S and 16S rRNA genes, and the 66-kDa gene. Various molecular techniques have also been developed to detect *A. cantonensis* lineage in different sample types, e.g., mollusk tissue, human serum and CSF, and rodent fecal samples. Of note, the TaqMan quantitative real-time PCR assay developed by Qvarnstrom et al. (2010) employing the ITS1 region is widely used for *A. cantonensis* detection (Qvarnstrom et al., 2010). The assay has been employed in detecting *A. cantonensis* DNA in human CSF samples and environmental samples (mollusk tissue), showing high sensitivity at a detection limit of 10 plasmid copies (Qvarnstrom et al., 2010, 2016). Fang et al. (2012) also developed a copro-detection assay using qPCR and revealed that their assay using the ITS2 region could detect a single L1 *A. cantonensis* larva in rodent fecal samples (Fang et al., 2012). Another species-specific qPCR assay targeting *A. cantonensis* and *A. malaysiensis* was developed using the mitochondrial *Cytb* gene, showing its ability to detect a single larva at approximately 10^{-4} ng (Jakkul et al., 2021). Next-generation sequencing (NGS) techniques are also gaining traction to aid in diagnosis. Employing metagenomic NGS, *A. cantonensis* DNA was detected in pediatric patients with

eosinophilic meningitis (Liu et al., 2022; Xie et al., 2019). Also, the high sensitivity and usefulness of NGS was shown by Zou et al. (2020), where the patient presenting eosinophilic meningitis was ELISA negative for *A. cantonensis*, but NGS results revealed the presence of *A. cantonensis* in the CSF (Zou et al., 2020).

Despite the effectiveness of molecular genetic markers for the molecular diagnosis and identification of *Angiostrongylus* spp., molecular techniques may not be available in resource-limited settings and for detection in the field. For example, majority of the molecular-based studies utilized either conventional PCR or qPCR, and only four studies have utilized the field-based loop-mediated isothermal amplification (LAMP) technique or recombinase polymerase amplification (RPA) (Chen et al., 2011; Liu et al., 2011; Martin-Alonso et al., 2015; Sears et al., 2021). Additionally, the selection of an appropriate genetic marker is essential for species discrimination of closely related species, especially within cryptic lineages. For example, low sequence variation (0 to 1% genetic distance) was observed between *A. cantonensis* and *A. malaysiensis* with the nuclear 18S rRNA gene (Chan et al., 2020). With the occurrence of hybrids between *A. cantonensis* and *A. malaysiensis*, possible introgression, morphological similarities, the overlapping distribution of both species, and the unclear potential of *A. malaysiensis* as a human pathogen, appropriate genetic markers with sufficient genetic variation must be employed to successfully differentiate between *A. cantonensis*, *A. malaysiensis*, and their hybrid forms, especially in areas where they live in sympatry. Moreover, the utilization of appropriate genetic markers can aid in the accurate diagnosis of eosinophilic angiostrongyliasis.

4. Conclusion

In conclusion, we highlight the need to consider the genetic variability among *A. costaricensis* and *A. cantonensis* cryptic lineages to improve molecular diagnosis further. As organisms continue to evolve and adapt in this rapidly changing environment, understanding the genetic diversity of the *Angiostrongylus* spp. causing human angiostrongyliasis is essential for accurate identification and has significant implications for diagnosis.

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Authors' contributions

UT and AC conceived the study, wrote, and reviewed the manuscript draft. CK and WP reviewed and revised the manuscript. All authors approved the final manuscript.

CRediT authorship contribution statement

Abigail Hui En Chan: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Chanisara Kaenkaew:** Formal analysis, Writing – review & editing. **Wallop Pakdee:** Formal analysis, Writing – review & editing. **Urusa Thaenkham:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests of personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material

All data generated or analysed during this study are included in this published article.

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