# Epidemiology of *Angiostrongylus cantonensis* and eosinophilic meningitis in the People's Republic of China

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*To my family*

# Table of contents

## 7. Population genetics of invasive snail *Pomacea* spp. in the People's Republic of China: multiple and secondary introductions 131

## 8. The emergence of angiostrongyliasis in the People's Republic of China: the interplay of invasive snails, climate change and transmission dynamics 151

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# Summary

***Background:*** Human eosinophilic meningitis is mainly caused by the rat lungworm *Angiostrongylus cantonensis*, especially in tropical and subtropical regions. Since the mid-1940s, more than 2800 cases of eosinophilic meningitis have been reported in at least 30 countries. Of note, *A. cantonensis* is one of a few helminths that can cause an outbreak within a short time period (usually within 2 weeks). Fatal cases, particular among young children, have been reported in heavily infected subjects. Eosinophilic meningitis is paradoxically an emerging infectious disease in the People's Republic of China (P.R. China). Indeed, while the rat lungworm was discovered in Guangzhou (formerly Canton) in 1933, there were only few cases reported until the mid 1990s. However, the biological invasion of two exotic snail species, namely the African land snail *Achatina fulica* and the South American freshwater snail *Pomacea* spp., drove the emergence of this disease. Thus far, more than three-quarter of human cases could definitely be related to the consumption of these snail species, particularly *Pomacea* spp. The seven outbreaks that occurred between 1997 and 2006 in P.R. China were all attributed to these two invasive snail species.

Due to the emergence of eosinophilic meningitis and the issue of outbreaks that occurred at a growing frequency in P.R. China, there as a felt need to deepen our understanding of the epidemiology and control of this disease. Although several small-scale surveys pertaining to *A. cantonensis* had been carried out in P.R. China, the exact distribution of the parasite, and the invasive snail species that act as intermediate hosts remained to be determined.

***Goal and objectives:*** The overarching goal of this thesis is to improve our knowledge on the epidemiology of *A. cantonensis* and eosinophilic meningitis in P.R. China. There are six specific and interlinked objectives: (i) to identify the control priorities in eosinophilic meningitis by observing and analyzing outbreaks; (ii) to reveal the geographical distribution of *A. cantonensis* and to identify the major intermediate hosts; (iii) to characterize the mitochondrial (mt) genome of *A. cantonensis* and its close relative *A. costaricensis* in order to identify genetic marker that might give rise to novel diagnostic assays and population genetic studies; (iv) to evaluate the intraspecific differentiation of

*A. cantonensis* and explore the potential dynamic scenarios in P.R. China; (v), to assess the genetic diversity of the invasive snail species *Pomacea* spp. and explore potential spread scenarios; and (vi) to assess the interplay between invasive snails, climate change and transmission dynamics.

***Methodology and principal findings:*** In 2008, we had the opportunity to study an outbreak of angiostrongyliasis that occurred in Dali due to the consumption of *Pomacea* spp. We found a prolonged period (8 months) of this outbreak with the peak occurring in February 2008, owing to a traditional festival. Only 11 out of 33 patients with complete data records were categorized into clinically diagnosed cases, whereas the others were grouped into suspected cases according to the existing diagnosis criteria. None of the patients was parasitologically diagnosed. Some important information for diagnosis was missing, which reflected the ignorance of clinicians on this disease. The existing diagnosis criteria for clinically diagnosed case requested an elevated eosinophil count both in peripheral blood and in cerebrospinal fluid (CSF), which was rather restrict. Interestingly, not all patients simultaneously experienced a marked change in eosinophil count, both in peripheral blood and CSF. Our findings called for further standardization of diagnosis and generalization of the clinical criteria. Surveillance system in endemic areas should be established for both security of snail food and human cases in hospitals.

We had access to data obtained from the first national survey pertaining to *A. cantonensis* which was implemented using a geographical grid sampling approach. Our results showed that the rat lungworm was endemic in 59 of the 164 surveyed counties (36.0%), which represented seven south provinces in the mainland of P.R. China. Two of these provinces were newly confirmed. Two invasive snail species were identified as the key intermediate hosts. On average, the prevalence of *A. cantonensis* among *A. fulica* and *Pomacea* spp. was 13.4% and 6.8%, respectively. The prevalence among other terrestrial mollusks range from 0.3% (snail) to 6.5% (slugs), while that among freshwater snails was only 0.05%. *Pomacea* snails were found in 11 provinces, whereas *A. fulica* was encountered in six provinces.

The complete mt genomes of *A. cantonensis* and *A. costaricensis* are 13,497 bp and 13,585 bp in length, respectively. Hence, these two closely related nematodes have the smallest mt genomes in the class of Chromadorea characterized thus far. Overall

nucleotide identity of these two mt genomes was 81.6%, with a range from 77.7% to 87.1% in individual gene pairs. These findings provide a sound basis for screening potential genetic markers for diagnosis and further in-depth population genetic studies. The mt genome-wide analysis identified three major gene arrangement patterns from 48 nematode mt genomes. The gene arrangement, coupled with a phylogenetic tree based on concatenated amino acid sequence, supported a closer relationship between Ascaridida and Strongylida rather than Spirurida, which is inconsistent with findings of previous studies according to the nuclear small ribosomal subunit DNA.

*A. cantonensis* specimens obtained from 33 collection sites were used to study the intraspecific differentiation based on the mitochondrial *nad1* gene. A total of 73 haplotypes of *A. cantonensis* were identified from 143 sequences, which resulted in seven distinctive clades (Mainland, Hainan, Sanya, Tiane, Nanao, Zixing and Thailand). Geographical distance and natural isolation played a role in the spatial distribution of these clades, which supported the Southeast Asian origin from a molecular point of view. A considerable haplotype invasion was noted, which indicated the impact of human activities on biodiversity. The potential invasion routes for the clades Hainan, Sanya and Tiane were inferred based on a network analysis. The conflict between the presumptive origin of the clades Hainan and Tiane and those of the clades Nanao and Sanya call for further research.

A total of 523 sequences of mt gene *cox1* of *Pomacea* spp. were obtained from 56 collection sites. Twenty-five haplotypes were identified with an overall diversity of 0.702, which was higher than those observed in previous studies. From a global point of view, only five out of 98 haplotypes, which were determined by the currently 228 available sequences in GenBank as well as the 523 sequences described in the present thesis, were shared between introduced (Southeast Asia) and native (South America) ranges. The 98 haplotypes were clustered into 10 groups. Six groups occurred in the mainland of P.R. China, among only two can be traced back to the South Americas, whereas were two also occurred in other countries in Southeast Asia. The remaining two groups only occurred in P.R. China. A phylogenetic analysis showed that two species (i.e. *P. canaliculata* and *P. insularum*) coexist in the mainland of P.R. China, although the phylogenetic position of group B is still pending. The definite spread route of this snail species was not determined, but the increased diversity in single collection sites indicated multiple and secondary introductions.

Finally, we developed a biology-driven model to determined potential impacts of climate change on the distribution of *Pomacea* spp., and hence the transmission of *A. cantonensis*. Mean January temperature and snail generation intensity (generation number) were identified as key factors determining the distribution of *Pomacea* spp.. The model predicted an increase of 56.9% for the ‘spread’ and a decrease of 40.9% for the ‘establishment’ regions (‘spread’ and ‘establishment’ defined according to the aforementioned national sampling survey) by the 2030s relative to the present day. Key determinants of *A. cantonensis* transmission were identified as the generation intensity in the intermediate host, the longevity of *A. cantonensis*-infected rats and the dormant period of *Pomacea* spp. Importantly, transmission of *A. cantonensis* occurs only in areas where the snail’s dormant period is below 173.2 days. The potential endemic area of *A. cantonensis* was predicted to double by the 2030s relative to the present day.

***Conclusions/significance***: Outbreaks of eosinophilic meningitis are of particular and growing concern in P.R. China. Standardization and generalization of diagnosis and treatment are therefore urgently required to be better equipped for future outbreaks. The first national survey deepened our understanding of the distribution of *A. cantonensis* and two invasive snail species that have been identified as the key intermediate hosts. Our in-depth population genetic studies of *A. cantonensis* revealed that human activities changed the original distribution and might have facilitated long-distance dispersal. The well divergent clades implied that the mt genes are promising candidates for novel diagnostic markers and population genetic studies. Our research also showed that two species of *Pomacea* coexist in P.R. China. The current distribution indicated multiple and secondary introductions. Although the definite role of these snail in the current distribution pattern of *A. cantonensis* is not clear, the potential impact is considerable in a future warmer P.R. China.

# Zusammenfassung

***Hintergrund:*** Eosinophile Meningitis beim Menschen wird hauptsächlich in tropischen und subtropischen Regionen durch den Ratten-Lungenwurm *Angiostrongylus cantonensis* verursacht. Seit Mitte der 1940er, wurden in mindestens 30 Ländern mittlerweile über 2800 Fälle von eosinophiler Meningitis gemeldet. *A. cantonensis* ist eine weniger Helminthenarten, welche in einer äußerst kurzen Zeitspanne (normalerweise innerhalb von zwei Wochen) einen Ausbruch verursachen kann. Bei Schwerinfizierten, insbesondere bei Kindern, wurden tödliche Fälle dokumentiert. In China ist die eosinophile Meningitis paradoxerweise eine neu auftauchende Infektionskrankheit, obwohl der Ratten-Lungenwurm erstmals schon 1933 in Guangzhou (früher Canton) entdeckt wurde. Bevor der Mitte der 90er wurden nur sehr wenige Fälle dokumentiert. Allerdings wurde das vermehrte Auftreten der Krankheit durch die biologische Invasion von zwei exotischen Schneckenarten, der afrikanischen Landschnecke *Achatina fulica* und der südamerikanischen Frischwasserschnecke *Pomacea* spp., begünstigt. Bis anhin, konnten über drei Viertel der Fälle auf den Verzehr dieser Schneckenarten, insbesondere *Pomacea* spp., zurückgeführt werden. Die sieben Ausbrüche, die sich zwischen 1997 und 2006 ereignet hatten, sind auf diese beiden invasiven Schneckenarten zurückzuführen.

Auf Grund des vermehrten Auftretens der eosinophilen Meningitis, insbesondere die mit höherer Frequenz auftretenden Ausbrüche in der Volksrepublik China (VR China), ist es uns ein Bedürfnis die grundlegenden Probleme von Diagnose, Behandlung und Kontrolle aufzuarbeiten und dadurch die Kontrollstrategien zu verbessern. Trotz mehreren kleinräumig angelegten Studien über *A. cantonensis* in der VR China bleibt die genaue Verteilung des Parasiten sowie die der als Zwischenwirt fungierenden invasiven Schneckenarten grösstenteils unbekannt.

***Zielsetzung:*** Das Hauptziel dieser Doktorarbeit ist, das Verständnis der Epidemiologie von *A. cantonensis* und der eosinophilen Meningitis in der VR China zu verbessern. Im Folgenden wird auf die sechs spezifischen und ineinandergreifenden Ziele eingegangen: (i) die Kontrollprioritäten der eosinophilen Meningitis durch Beobachtung und Analyse von Ausbrüchen zu identifizieren; (ii) die geographische Verbreitung von *A. cantonensis* und die wichtigsten Zwischenwirte und Vektoren zu bestimmen; (iii) das mitochondriale (mt) Genom von *A. cantonensis* und von dessen naher Verwandter *A. costaricensis* zu sequenzieren um einen potentiellen, genetischen Marker für eine neue Diagnostikmethode

und genetische Populationsstudien zu identifizieren; (iv) die innerartlichen Differenzierung von *A. cantonensis* zu evaluieren und die potentiellen dynamischen Szenarien in der VR China zu erforschen; (v) die genetische Diversität der invasive Schneckenart *Pomacea* spp. zu bestimmen und die Ausbreitungsszenarien zu beurteile; und (vi) das Zusammenspiel von invasiven Schnecken, Klimawandel und Transmissionsdynamik zu beurteilen.

***Methoden und Hauptergebnisse:*** 2008 hatten wir die Gelegenheit einen Angiostrongylasis-Ausbruch in Dali, hervorgerufen durch den Verzehr von *Pomacea* spp., zu untersuchen. Wir stellten einen langanhaltenden Ausbruch (8 Monate) mit Höchststand im Februar 2008 fest, welcher auf ein traditionelles Fest zurückgeführt werden konnte. Nur 11 der 33 Patienten mit vollständigen Datensätzen wurden klinisch diagnostiziert, während die restlichen Patienten nach bestehenden Diagnosekriterien als Verdachtsfälle eingestuft wurden. Bei keinem Patienten wurden parasitologische Untersuchungen durchgeführt. Das Fehlen von wichtigen Informationen für die Diagnose spiegelt die verbreitete Unkenntnis dieser Krankheit unter Ärzten wider. Die bestehenden klinischen Diagnosekriterien beinhalten einen erhöhten Eosinophilenwert im peripheren Blut sowie in der Cerebrospinalflüssigkeit (CSF). Interessanterweise ergab die Analyse unsere Laborergebnisse, dass nicht alle Patienten gleichzeitig einen deutlichen Anstieg des Eosinophilenwert im peripheren Blut sowie in der CSF aufwiesen. Unsere Resultate weisen auf eine dringend benötigte Standardisierung der Diagnose und Generalisierung der klinischen Kriterien hin. Überwachungssysteme für Krankheitsfälle und für Schnecken als Lebensmittel sollten vor allem in endemischen Gebieten eingerichtet werden.

Wir hatten Zugang zu den Daten der nationalen *A. cantonensis* Erhebung, die mit Hilfe eines geographischen Raster-Samplings durchgeführt wurde. Der Ratten-Lungenwurm war endemisch in 59 der 164 untersuchten Bezirke, was wiederum sieben Provinzen auf dem chinesischen Festland entsprach, in zwei dieser sieben Provinzen zum ersten Mal. Der Anteil der invasiven Schneckenarten in unserer Studie war nicht unerheblich; die durchschnittliche Prävalenz von *A. cantonensis* bei *A. fulica* und *Pomacea* spp. lag bei 13.4% bzw. bei 6.8%. Die Prävalenzen bei anderen Landschnecken reichten von 0.3% (Schnecken) bis 6.5% (Nacktschnecken), während die bei den Frischwasserschnecken nur 0.05% betrug. *Pomacea* Schnecken wurden in elf Provinzen gefunden, hingegen trafen wir in nur sechs Provinzen auf *A. fulica.*

Die kompletten mt Genome waren zwischen 13,497 Basenpaare (bp) bzw. 13,585 bp lang, die bis anhin Kleinsten in der Unterklasse der Chromadorea. Die

Nukleotidübereinstimmung der zwei mt Genome lag bei 81.6% mit Abweichungen von 77.7% bis 87.1% bei einzelnen Genpaaren. Diese Ergebnisse dienen als eine solide Grundlage für das Screening der potenziellen Marker für die Diagnose und weiteren tiefergehenden populationsgenetischen Studien. Die mt-Genom-weite Analyse ergab hauptsächlich drei Genanordnungen von 48 Nematoden mt Genomen. Die Genanordnung, verbunden mit dem Stammbaum basierend auf der verketteten Aminosäuresequenz, unterstützt eine engere Verwandtschaft von Ascaridida zu Strongylida anstatt Spirurida. Diese Resultate stehen im Wiederspruch zu bisherigen Studien, welche die DNA der kleinen ribosomalen Untereinheit verwendeten.

Die an 33 Standorten gesammelten Proben von *A. cantonensis* wurden verwendet, um die innerartliche Differenzierung anhand des mitochondrialen Gens *nad1* zu studieren. 73 Haplotypen von *A. cantonensis* wurden aus insgesamt 143 Sequenzen identifiziert, welche in sieben unterscheidbare phylogenetische Stämme unterteilt werden konnten (Festland, Hainan, Sanya, Tiane, Nanao, Zixing and Thailand). Geographische Distanz und natürliche Isolation spielten ein Rolle bei der räumlichen Verbreitung der Stämme, welche von einem molekularen Standpunkt aus einen südostasiatischen Ursprung unterstützt. Eine erhebliche Invasion von Haplotypen wurde festgestellt, höchstwahrscheinlich zurückzuführen auf die Auswirkungen menschlicher Aktivitäten auf die Biodiversität. Mögliche Invasionswege der Hainan-, Sanya- und Tiane-Stämme wurden mit Hilfe einer Netzwerkanalyse ermittelt. Die Kontroverse um den Ursprung der Hainan- und Tiane-Stämme und dem der Nanao- und Sanya-Stämme erfordern weitere Untersuchungen.

Im Ganzen erhielten wir 523 Sequenzen des mitochondrialen *cox1* Gens von *Pomacea* spp. von 56 Standorten. 25 Haplotypen wurden bestimmt und diese wiesen eine Diversität von 0.702 auf, welche höher ist als in vorangegangenen Studien. Insgesamt traten nur fünf der 98 Haplotypen, welche mit Hilfe der gegenwärtig 228 Sequenzen umfassenden „GenBank“ und den eigenen 523 Sequenzen bestimmt wurden, bei den eingeführten (Südostasien) sowie den heimischen (Südamerika) Schnecken auf. Die 98 Haplotypen konnten in 10 Gruppen unterteilt werden. Sechs Gruppen kommen auf dem chinesischen Festland vor, wovon nur gerade zwei Gruppen auch in Südamerika und zwei weitere in anderen südostasiatischen Ländern vorkommen. Die verbleibenden zwei Gruppen wurden bisher nur in der VR China gefunden. Die phylogenetische Analyse zeigte, dass zwei Arten (d.h. *P. canaliculata* und *P. insularum*) auf dem Festland koexistieren, obwohl die phylogenetische Stellung der Gruppe B weiterhin unklar bleibt.

Die Ausbreitungsroute der Schneckenart konnte zwar nicht endgültig bestimmt werden, jedoch weist die erhöhte Diversität der einzelnen Standorte auf mehrfache und sekundäre Ausbreitungswellen hin.

Schlussendlich haben wir ein Biologie-basiertes Modell entwickelt, um die potenziellen Auswirkungen des Klimawandels auf die Verbreitung von *Pomacea* spp. und folglich auf die Übertragung von *A. cantonensis* abzuschätzen. Durchschnittstemperaturen im Januar und die Intensität der Schneckengenerationen (Anzahl Generationen) stellten sich als wichtigste Faktoren für die Verbreitung von *Pomacea* spp. heraus. Bis 2030 sagt unser Modell eine Zunahme von 56.9% für die „spread"- und eine Abnahme von 40.9% für die „establishment"-Regionen („spread" und „establishment" wurden nach den Richtlinien der zuvor erwähnten nationalen Enerhebung definiert) voraus. Als Schlüsselfaktoren der *A. cantonensis* Transmission wurden die Intensität der Zwischenwirtgeneration, die Lebensdauer der *A. cantonensis*-infizierten Ratten und die Ruheperiode der *Pomacea* spp. ausgemacht. Es ist wichtig zu erwähnen, dass die Übertragung von *A. cantonensis* nur in Regionen erfolgt in welcher die Ruhephase der Schnecken weniger als 173.2 Tage beträgt. Vermutlich wird sich das heutige endemische Gebiet von *A. cantonensis* bis 2030 auf die doppelte Fläche ausdehnen.

***Schlussfolgerungen/Bedeutung:*** Ausbrüche der eosinophilen Meningitis sind von besonderem und wachsendem Interesse für die VR China. Im Angesicht kommender Ausbrüche sollten die Standardisierung und Generalisierung von Diagnose und Behandlung angepackt und verbessert werden. Das Wissen über die Verbreitung und die wichtigsten Vektoren wurde mit Hilfe der ersten nationalen Erhebung vergrössert. Unsere tiefergehenden populationsgenetischen Studien von *A. cantonensis* zeigen klar, dass menschliche Aktivitäten die Verbreitung beeinflussen und deuten auf eine erleichterte Langstrecken-Ausbreitung hin. Die verschiedenen, unterschiedlichen Stämme sind eine vielversprechende Voraussetzung, um die mt Gene in Zukunft für neue Diagnostikmethoden und populationsgenetische Studien zu verwenden. Unsere Forschung belegt desweiteren die Koexistenz von zwei *Pomacea* Arten in der VR China. Das derzeitige Verbreitungsmuster weist auf mehrfache und sekundäre Ausbreitungswellen hin. Obwohl die eindeutige Rolle dieser Schnecken im derzeitigen Verbreitungsmuster von *A. cantonensis* unklar bleibt, wird der Einfluss in einer durch den Klimawandel erwärmten VR China erheblich grösser.

# Figure index

# Table index

# 1. Introduction

## 1.1. Life cycle of *Angiostrongylus cantonensis*

*Angiostrongylus cantonensis* (rat lungworm) was discovered during the routine examination of wild rats in Guangzhou (formerly known as Canton), the Poeple's Republic of China (P.R. China) in 1933 and described as *Pulmonema cantonensis* (Chen, 1935). The same species has also been reported as *Haemostrongylus ratti* in Taiwan (Yokogawa, 1937). The binomial species was reappraised by Dougherty as *Angiostrongylus cantonensis* in 1946 (Dougherty, 1946). The life cycle was elucidated in the mid-1950s (Mackerras and Sandars, 1955), although the species used in this milestone experiment probably was *A. mackerrasae* (Bhaibulaya, 1975), which is resemble that of *A. cantonensis*. Another species virtually identical to *A. cantonensis* is *A. malaysiensis*, which is mainly endemic in Malaysia (Bhaibulaya and Cross, 1971). Thus far, only *A. cantonensis* has been conclusively involved in human ill-health and, to date, this species is widely recognised as the major pathogen of human and wildlife eosinophilic meningitis (Prociv et al., 2000).

Adult *A. cantonensis* worms parasitize the pulmonary arteries and cardiac cavities of rats (Figure 1.1). The female worm can reach 36.8 ± 0.9 mm in length. Male worms are much smaller and measure 26.0 ± 0.4 mm (Kino, 1984). However, the size of adult worms is related to the age and probably varies according to geography. Adult worms release their eggs into the bloodstream and the eggs concentrate in the capillary of lung tissue. After hatching, the larvae penetrate the capillary walls and enter the airways. The first-stage larvae ($L_1$) in the rat sputum are then swallowed and pass the intestine with the faeces. The exact number of eggs released by a single female worm per day is difficult to estimate because it is the hatched first-stage larvae ($L_1$) rather than eggs that are passed out with rat faeces. However, a well-designed experiment predicted more than 50,000 larvae/female/day at the egg-releasing peak time, e.g. 15-45 weeks post-infection (Kino, 1984). The average duration of larval output by female worms is 414.2 days and the total number of larvae released by a single female worm can reach 18.9 million. Of note, these estimates are influenced by worm density in individual hosts (Kino, 1984).

$L_1$ can be alive for several days inside rat faeces, but they are sensitive to dry conditions and extreme temperature. When the faeces harbouring $L_1$ is ingested by their mollusk intermediate hosts (i.e. snails or slugs), the released $L_1$ migrate and are encapsulated in the tissues of hosts upon which they develop into third-stage infective larvae ($L_3$) after two molts. The larval development within the intermediate host markedly depends on environmental temperature and perhaps host species (Ishii, 1984; Lv et al., 2006). Usually, the accomplishment of two molts will take 2-3 weeks at around 25 °C. The longevity of $L_3$ in intermediate hosts is not easy to observe because of shorter life span of hosts. However, $L_3$ is considered as dauer form in many nematode species, including parasites (Blaxter, 2011). If $L_3$ of *A. cantonensis* can also be dauer form and thus is expected to live for a long time until the death of host.

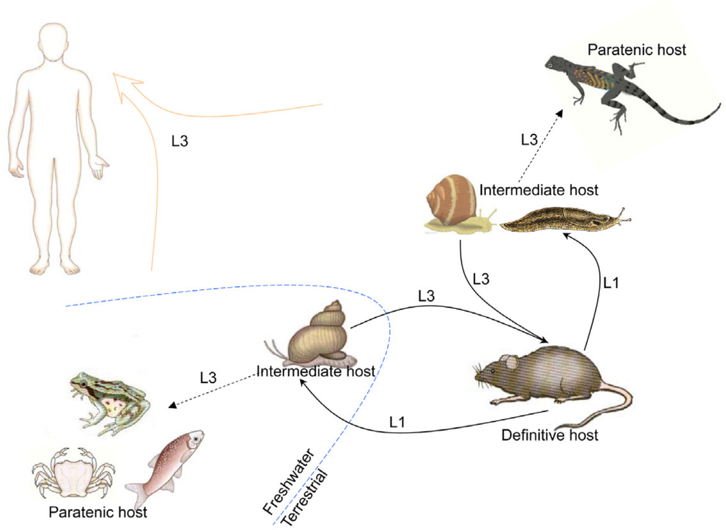

**Figure 1.1** The life cycle of *Angiostrongylus cantonensis*. This figure divides the intermediate and paratenic hosts into terrestrial group and freshwater group, and hence highlights the importance of freshwater fauna in the transmission of *A. cantonensis*. $L_1$ and $L_3$ denote the first-stage and third-stage larvae of *A. cantonensis*.

Various animals (e.g. frogs, shrimps, crabs, fish and lizards) can serve as paratenic hosts in which the infective larvae can live several weeks without further development (Wallace and Rosen, 1966, 1967). $L_3$ can encapsulate themselves in tissues of paratenic hosts, such as frogs, fish and lizards. However, in crustacean the larvae strikingly concentrate in the stomach (Alicata, 1965).

When rats ingest intermediate or paratenic hosts harbouring infective larvae, the larvae penetrate the stomach or intestinal walls and migrate through the host body via bloodstream. *A. cantonensis* is neurotropic, which means the larvae stay in the central nervous system (CNS) where they further grow and develop. Within approximately 4 weeks and after two molts, worms penetrate the cerebral vein and migrate to the heart and pulmonary arteries. Adult worms mate and deposit the first eggs approximately 5 weeks post-infection.

## 1.2. Angiostrongyliasis and eosinophilic meningitis

In particular, humans are non-permissive hosts for *A. cantonensis*. However, the larvae can invade the human CNS via bloodstream, but usually fail to pass through the cerebral vessels and enter the pulmonary arteries. In children, however, the larvae may enter the pulmonary vessels and induce potentially fatal inflammation in the lung, but sexually mature adult worms are seldom found. Infections occur when raw or undercooked freshwater or terrestrial snails and slugs, paratenic hosts or contaminated vegetables are consumed.

### *1.2.1. Clinical manifestation*

*A. cantonensis* induces inflammation in neural tissues and usually increases eosinophil cell counts, a syndrome known as eosinophilic meningitis (the most common clinic entity of angiostrongyliasis). The incubation period of eosinophilic meningitis is variable, ranging from one day to several months. In an angiostrongyliasis outbreak that could be linked to the consumption of the terrestrial snail *Achatina fulica*, typical clinical symptoms appeared within 1-6 days (Kliks et al., 1982). However, in a large outbreak that occurred in Beijing in 2006, involving 160 patients, the incubation period in 128 patients (80%) was 7-36 days with a median of 14 days (He et al., 2007). This outbreak was linked to the consumption of *Pomacea canaliculata*, a freshwater snail species. The estimated mean incubation period among 33 patients in a recent outbreak in Dali, P.R. China, which was due to the consumption of raw *P. canaliculata* snails was 16 days with a range of 3-50 days (Lv et al., 2009a). The latent period was as long as 54 days (standard deviation: 30 days) in an outbreak due to drinking raw vegetable juice (Tsai et al., 2004). These outbreaks indicate that the incubation period might be associated with the number of ingested larvae.

Different clinical manifestations of angiostrongyliasis have been described. Eosinophilic meningitis is the main clinical entity associated with the disease (Sawanyawisuth and Sawanyawisuth, 2008). Other clinical manifestations include eosinophilic meningoencephalitis (Furugen et al., 2006), eosinophilic radiculomyelitis (Schmutzhard et al., 1988), ocular angiostrongyliasis (Sinawat et al., 2008) and, more rarely, nasal (Liu and An, 2000) or pulmonary infections (Li et al., 2001; Lindo et al., 2004). Most infections are self-limiting and recovery is without sequelae (Punyagupta et al., 1975; Yii, 1976). However, illness courses developing into encephalitis may be fatal (Sawanyawisuth and Sawanyawisuth, 2008). Less than 10% of all patients infected with *A. cantonensis* develop encephalitis, but among them a lack of effective treatment results in considerable mortality (Sawanyawisuth and Sawanyawisuth, 2008).

Cerebrospinal inflammation and resulting increased intracranial pressure is the primary pathology of angiostrongyliasis. The most common complaint of patients is acute and deteriorating headache as a result of increased intracranial pressure (Graeff-Teixeira et al., 2009). Headache may be accompanied by nausea and vomiting. Approximately 40% of all patients experience neck stiffness and paraesthesia (Wang et al., 2008), which usually lasts for less than 2 weeks and affects different locations (usually in the extremities). It has been described as hyperesthesia, itching or a sensation of worms crawling under the skin (Wang et al., 2008). Less common symptoms include pain in the trunk or extremities, facial paralysis, blurred vision or diplopia. Fever is mostly absent in adults while pediatric patients are often highly febrile (Hwang and Chen, 1991). Children suffer more often from neck stiffness, nausea, vomiting, somnolence and abdominal pain (Hwang and Chen, 1991).

### *1.2.2. Diagnosis*

The recovery of larvae from cerebrospinal fluid (CSF) or the ocular chamber is the definitive proof for human angiostrongyliasis. However, in a recent review of case reports and outbreaks occurring in the mainland of P.R. China between 1979 and 2006, only 16 patients (4.8%) out of a total of 334 cases were parasitologically diagnosed (Lv et al., 2008). In Thailand, none of the 484 cases reported there revealed parasites in the CSF (Punyagupta et al., 1975). The chance of a parasitological diagnosis is higher in children than in adults; indeed larval recovery rate from CSF by lumbar puncture was as high as 41.5% among pediatric cases in Taiwan (Hwang and Chen, 1991). In another survey involving 125 individuals, eight out of nine parasitologically confirmed cases younger than 8 years were identified (Yii, 1976). A clinical angiostrongyliasis diagnosis is often made based on eating history, clinical symptoms and diagnostic work-up. Specifically, a history of eating freshwater or terrestrial mollusks, paratenic hosts or potentially contaminated vegetables is crucial for clinical diagnosis.

Eosinophilia is an important indicator for helminth infections. An elevated eosinophil cell count (>10% of total white blood cells or >500 cells/µl) in CSF is of specific relevance for angiostrongyliasis due to the frequent involvement of the brain (Punyagupta et al., 1970; Slom et al., 2002). However, not each examination of an angiostrongyliasis case shows eosinophilia in the CSF, since the counts may vary over the course of infection (Punyagupta et al., 1975). Another diagnostic approach is immunological testing for antibodies or specific antigens from *A. cantonensis* in peripheral blood and CSF ( Tsai et al., 2001b; Slom et al., 2002; Dorta-Contreras et al., 2005). Several tests including enzyme-linked immunosorbent assay (ELISA) and immuno-polymerase chain reaction (PCR) have been developed but reported sensitivities and specificities are questionable due to small sample sizes. At present, no commercially available kit for diagnosing angiostrongyliasis is on the market (Wang et al., 2008).

Although *A. cantonensis* is generally regarded as the leading agent for eosinophilic meningitis, other causes must be considered to strenghen differential diagnosis. Hence, thorough diagnostic work-up and differential diagnosis are important in areas such as Southeast Asia and the Far East where special food items (e.g. snails, frogs, snakes and raw fish) are popular and other parasites causing eosinophilic meningitis co-exist. For example, both *A. cantonensis* and *Gnathostoma spinigerum* contributed to an epidemic of eosinophilic meningitis in Thailand (Punyagupta et al., 1975, 1990).

### *1.2.3. Treatment and clinical management*

The severity of human angiostrongyliasis varies from mild and self-limiting to fatal, depending on the involved parts of the brain and worm burden. The clinical management of angiostrongyliasis includes administration of anthelminthic drugs, supportive measures and sometimes even surgery. Repeated lumbar puncture or mannitol transfusion are employed to relieve symptoms such as severe headache and vomiting by decreasing intracranial pressure (Chotmongkol et al., 2000). Surgical removal of worms is the preferred method in the management of ocular angiostrongyliasis ( Toma et al., 2002; Kumar et al., 2005; Malhotra et al., 2006; Sinawat et al., 2008).

The suitability of anthelminthics is controversial due to their potential to exacerbate inflammation (Bowden, 1981; Hidelaratchi et al., 2005). Still, albendazole and mebendazole are widely used against *A. cantonensis* in clinical practice (Sawanyawisuth and Sawanyawisuth, 2008). A trend to lower frequency of headache and a shorter mean duration of headache in the treatment group were reported from a randomised placebo-controlled trial assessing the efficacy of albendazole, but the statistical evidence was weak (Jitpimolmard et al., 2007). Two observational studies showed a satisfactory effect of albendazole in the treatment of children (Hwang and Chen, 1991; Tsai et al., 2003). Levamisole is another anthelminthic drug which has been used to treat children with reportedly good efficacy (Hwang, 1997). Thiabendazole showed no appreciable effect when the drug was administered at a dosage of 50 mg/kg/day for 3 days (Kliks et al., 1982). No clinical trial has assessed the efficacy of mebendazole alone. Corticosteroids have been confirmed to be useful, probably by decreasing inflammation (Chotmongkol et al., 2000; Tsai et al., 2004; Sawanyawisuth and Sawanyawisuth, 2008), but relapses were reported (Sawanyawisuth et al., 2004; Tsai et al., 2004). Currently, a combination of anthelminthic drugs and corticosteroids is recommended for the treatment of angiostrongyliasis patients ( Chotmongkol et al., 2004; Chotmongkol et al., 2006b).

## 1.3. Global distribution and epidemiology

### *1.3.1. The origin*

There are two hypotheses regarding the origin of *A. cantonensis*. The first, herein called as

African origin, was proposed by Alicata who contributed many invaluable work put light on global distribution of this parasite in 1960s and 1970s (Alicata, 1991; Alicata and Jindrak, 1970). After a number of field surveys he realized that the significant association between the distributions of *Achatina fulica* and *A. cantonensis*. The discovery of *A. cantonensis* in Madagascar and offshore islands facilitated the proposal of hypothesis that the nematode species formed after moving to Madagascar from African land, employed the common land snail species (*A. fulica*) as the intermediate host, and then spread eastward with this snail species (Alicata, 1966). Indeed, the global dispersal pattern of *A. fulica* and the remarkable compatibility with *A. cantonensis* support this hypothesis. This proposal was also by the fact that another rat lungworm species, *A. sandarsae*, in Africa might have the common ancestor with *A. cantonensis*. However, the hypothesis was challenged by the latter investigations. For example, other mollusks were also compatible with this parasite. Much higher worm burden in *A. fulica* due to its large size tended to result in death of infected rats. Furthermore, the other more close relatives of *A. cantonensis* were found in Australia (*A. mackerrasae*) and Malaysia (*A. malaysiensis*). Therefore, an alternative hypothesis was proposed.

This hypothesis, herein called as Asian origin, suggests that the three closely related species, i.e., *A. cantonensis*, *A. mackerrasae*, and *A. malaysiensis*, diverged with the evolution of their definitive hosts, *Rattus* spp. in Indo-China peninsula and Australia (Drozdz et al., 1975). *A. cantonensis* might spread following the geographical expansion of *Rattus* spp before the advent of *A. fulica* in the Pacific Basin. The latter probably was only recently involved in the transmission of *A. cantonensis*. The most common definitive host of *A. cantonensis* is *R. norvegicus* and *R. rattus* are dominant species in Southeast Asia, although *A. cantonensis* was also normally discovered from these rat species in other places. Obviously, these rat species in elsewhere were introduced recently, which was facilitated by human activity. According to this hypothesis the presumptive home range might be the southeast part of P.R. China and the offshore islands (Kliks and Palumbo, 1992).

### *1.3.2. Global spread with emphasis on human activities*

The spread of *A. cantonensis* throughout the world indicates the markedly increased frequency of human activities at global scale since 1930s. *Rattus* spp. and *A. fulica* as the vector of *A. cantonensis* also facilitated this process. In the one hundred years between 1800 and 1900 the African land snail was isolated from the African mainland to Madagascar and the offshore islands and later introduced to India. In the early of 20th century the snail was introduced to Malaya peninsula probably from India. The snails were introduced intentionally for food resource or inadvertently with soil or plants in Southeast Asia. However, the essential expansion of *A. fulica* as well as *A. cantonensis* was owned to the Second World War when Japan governed most of countries in Southeast Asia and Pacific Islands (Kliks and Palumbo, 1992). *A. fulica* was not only a snail species at that time; they were often used as a pet in the ornamental gardens by Japanese troops. They were also used for medicine purpose and emergency food source. Both Japanese soldiers and local people especially on the pacific islands consumed African land snails in hard conditions and consequently were attacked by eosinophilic meningitis due to *A. cantonensis*.

Not all the places affected by the war were occupied by *A. fulica* and *A. cantonensis*. A number of cases, e.g., the west coast in the United States and the east coast in Australia, where the land snails once established but were subsequently eradicated, were recorded. In many areas of the Central and the South Americas and Africa the rat lungworm was absent during 1960s when a number of surveys were conducted (Kliks and Palumbo, 1992). However, the parasite and human infections emerged since the 1970s, notably in the Caribbean region (Figure 1.2). One hypothesis for this phenomenon is that the legacy of Cuban adventurism in Indochina, the Americas and southern Africa probably became the successor of the Pacific War to expand the range of *A. cantonensis* (Kliks and Palumbo, 1992). Unlike the Pacific War, rat, perhaps instead of *A. fulica*, played the more important role. As exchange, many agriculture products, certainly with Indochinese rats, were transported to the Caribbean region from Southeast Asia (Kliks and Palumbo, 1992). A similar scenario subsequently reoccurred in Central America and southern Africa, which probably led the parasite again to new sites. Endemic areas of *A. cantonensis* in Central America and southern Africa were expected, but few investigations were actually reported.

The recent emergence of *A. cantonensis* raised a special concern in the South Americas (e.g. Brazil and Ecuador) where another rodent intra-arteiral nematode (*A. costa-*

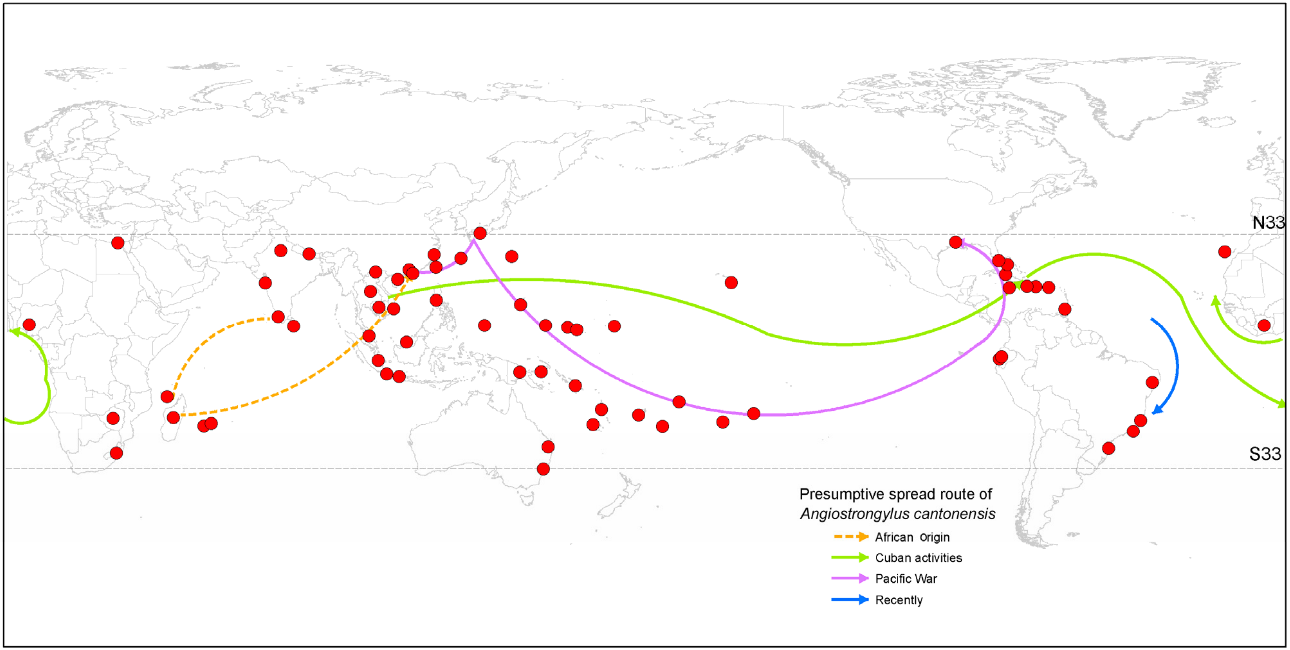

**Figure 1.2** Presumptive spread route of *Angiostrongylus cantonensis* (rat lungworm) in the world. The red points denote the endemic areas where human and animals were locally infected by *A. cantonensis*.

*ricensis*) of importance in human health is endemic (Caldeira et al., 2007; Lima et al., 2009; Pincay et al., 2009; Maldonado et al., 2010). *A. fulica*, the outstanding contributor during the Pacific War, has rampantly expanded in the most of states in Brazil since 1996 or even earlier and is ready for the parasite (Thiengo et al., 2007). The source of *A. cantonensis* presenting in Brazil is unknown, but the neighbourhood Caribbean is more likely to be the origin due to increasing regional trades. The occurrence of *A. cantonensis* in South Africa and the Canary islands of Spain represent an extraordinary expansion in African continent (Foronda et al., 2010; Archer et al., 2011). Unlike in Brazil, the first discovery of *A. cantonensis* in both places was in Southeast Asia rats, i.e. *R. norvegicus* and *R. rattus*, which highlight the role of rats currently.

### *1.3.3. The epidemiology of angiostrongyliasis*

Since 1945 when the first human angiostrongyliasis case was described, over 2800 cases have been documented in the literature; 77% of them in Southeast Asia, PR China and Japan (Wang et al., 2008). More than 30 countries or territories, mainly located in tropical and subtropical regions, have been identified as endemic. All countries in Southeast Asia except Myanmar, Brunei and Timor Leste are considered endemic for *A. cantonensis*. Thailand is the most heavily endemic area; more than 1300 cases have been reported since the 1950s (Wang et al., 2008). Today, hundreds of suspected cases are reported every year, especially from northeastern Thailand (Eamsobhana and Tungtrongchitr, 2005). It is estimated that the annual incidence rate is approximately 2 per 100,000 in Thailand (Suankratay et al., 2001). Other countries including Vietnam, Cambodia, Indonesia and Malaysia sporadically reported cases. A total of 382 cases from nine provinces had been identified in the mainland of P.R. China by the end of 2008 (Deng et al., 2007; Lv et al., 2008; Lv et al., 2009a). Fifty-four cases were described in Japan before 2003 and 61.1% of all cases were found on Okinawa (Nawa et al., 2005). Many of the remaining infections probably originated from Okinawa or outside Japan (Nawa et al., 2005).

The prevalence of angiostrongyliasis in Southeast Asia and the Far East is strongly associated with eating habits deeply rooted in local culture. Freshwater and terrestrial snails, mainly *Pomacea* spp., *Pila* spp. and *A. fulica*, are the most common source of infection in this region (Punyagupta et al., 1970; Yii, 1976; Lv et al., 2008). Since the terrestrial snail *A. fulica* usually has a higher infection rate and worm burden than freshwater snails, the consumption of *A. fulica* often results in more severe manifestations and higher case numbers (Yii, 1976). Isolated cases are often attributed to freshwater snails ( Tsai et al., 2001a; Lv et al., 2008) or terrestrial slugs, frogs and monitor lizards (Chotmongkol et al., 2006a; Liu et al., 2006; Parameswaran, 2006). Living slugs are sometimes swallowed as part of traditional medical treatments and might result in severe meningitis (Chotmongkol et al., 2006a; Liu et al., 2006).

The epidemiological patterns of eosinophilic meningitis due to *A. cantonensis* in Southeast Asia and the south of P.R. China and Japan are changing. For example, the use of snails for rearing domestic animals was widely practiced in Taiwan in the 1970s (Yii, 1976) but is declining, and the importance of slugs in traditional medicine is diminishing. However, the spread of invasive snails, tourism and the consumption of exotic foods have become driving factors in the epidemiology of angiostrongyliasis. The invasion of *P. canaliculata* in P.R. China may serve as an illustration. This freshwater snail was introduced around 1980, and is now well established in southern P.R. China (Lv et al., 2009b). Three quarters of the 382 cases and eight of nine outbreaks (each comprising at least six cases) of cerebral angiostrongyliasis have been attributed to this species (Deng et al., 2007; Lv et al., 2009a; Lv et al., 2008).

There only few cases on the Pacific Islands, where once frequent outbreaks of eosinophilic meningitis due to *A. cantonensis*, were reported since 1980s probably owing to changed eating habits and improved socioeconomic conditions (Kliks and Palumbo, 1992). Instead, in the newly established foci (e.g. Caribbean regions and the South Americas) outbreak and sporadic cases have been reported recently. Some heavily infected or fatal cases were also documented in these new foci (Lima et al., 2009; Pincay et al., 2009). The source of infection is similar to Southeast Asia, but contaminated vegetables or foodstuff are likely contributor to drive the epidemiology.

Unlike the countries or territories mentioned above, in the USA and Australia, animal angiostrongyliasis due to this parasite are more frequently reported, involving more than 10 vertebrate species. In contrast, only few animal species have been found to be affected by *A. cantonensis* in Southeast Asia (Kodama et al., 1981). Obviously, the lack of sophisticated veterinary care and attention to animal health in this region is partially responsible for this observation (Prociv et al., 2000).

Travel is the common route of exposure to *A. cantonensis* for individuals from non-endemic countries (Tsai et al., 2001a; Slom et al., 2002; Deng et al., 2007; Malvy et al., 2008; Maretic et al., 2009). Several cases of angiostrongyliasis were reported in Europe. Most cases reported of traveling to endemic areas. Long-distance food transportation is another important risk factor. The large outbreak in Beijing (non-endemic) in 2006 was attributed to snails imported from an endemic area.

## 1.4. Epidemiology of angiostrongyliasis in P.R. China

### *1.4.1. Emerging angiostrongyliasis with particular consideration to outbreaks and exotic snail species*

In 1979, a case of presumptive angiostrongyliasis was reported in the mainland of P.R. China, but the etiological agent could not be identified (Huang and Xian, 1979). The first parasitologically confirmed case was described in 1984 (He et al., 1984), 50 years later after the discovery of *A. cantonensis* in P.R. China. Only three other cases were recorded over the next 12 years. However, in 1997 an outbreak of angiostrongyliasis, involving 65 people, was recorded in Wenzhou city in the eastern coastal Zhejiang province (Zheng et al., 2001). Since then, several other outbreaks and many case reports have been documented. A total of 334 cases have been recorded in the literatures up to 2006 (Lv et al., 2008) (Figure 1.3). Of all cases, 289 patients were infected during seven outbreaks.

Human angiostrongyliasis cases were reported from at least nine provinces (Heilongjiang, Liaoning, Beijing, Tianjin, Jiangsu, Zhejiang, Fujian, Guangdong, Yunnan) in P.R. China (Figure 1.4). Epidemiological surveys also indicated suppressive or sub-clinical infections in Hainan province (He et al., 1985). The five cities Wenzhou, Fuzhou, Guangzhou, Kunming and Beijing alone accounted for 88.9% of the cases.

The age of the recorded angiostrongyliasis cases ranged from 11 months to 70 years but the majority was middle aged. No significant difference was seen between the number of infections in males and females. Four children (1.2%) were dead, succumbed to the infection (Li et al., 2001; Su et al., 2003). Autopsies revealed adult worms in the pulmonary artery and inflammatory reactions in the lung tissue. Only 16 patients (4.8%)

were etiologically diagnosed and among them, there were three cases of ocular angiostrongyliasis and one case of nasal angiostrongyliasis, the other cases were diagnosed with worms found in cerebrospinal fluid. A 2-year old girl was found with 44 immature worms in the cerebrospinal fluid (Ye et al., 1999). At least 75.3% of all cases were attributed to two snail species, i.e., *P. canaliculata* and *A. fulica*.

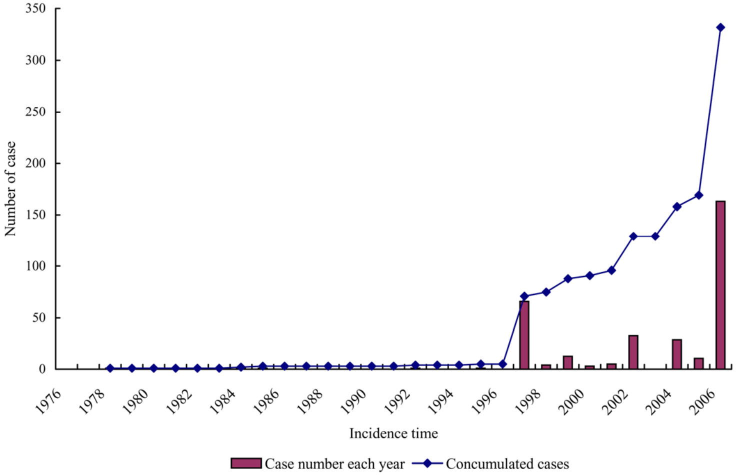

**Figure 1.3** Incidence of angiostrongyliasis in the mainland of P.R. China

*1.4.2. Known endemic areas and host species*

The first investigation of *A. cantonensis* was conducted in Guangzhou in 1933 and resulted in the discovery of this nematode species (Chen, 1933). Subsequent surveys were undertaken in the early 1980s (Ding et al., 1982; Liang et al., 1984). Hence, there was a gap of 50 years between the discovery of this nematode and the series of intentional surveys, mainly explained by the low social and economic development of P.R. China in that period and the little attention paid to this nematode and the disease by the health sector. Indeed, there were far greater public health issues to be added at that time. However, in the late 1980s the surveys were extended to the whole Zhujiang delta (Liang and Pan, 1992). The surveys revealed a high prevalence and infection intensity among intermediate and definitive hosts in the delta region. Subsequently, additional surveys were performed in the provinces of Yunnan, Hainan, Guangxi, Zhejiang and Fujian. Many natural endemic foci were discovered in these provinces. Jinhua city in Zhejiang province is the northernmost natural endemic area discovered thus far. Additionally, infected *A. fulica* snails were reported from a commercial snail breeding unit in Liaoning province in northeast P.R. China where the infected snails might have been imported from southern endemic foci. This observation showed that the parasite could survive and even become established in northern P.R. China.

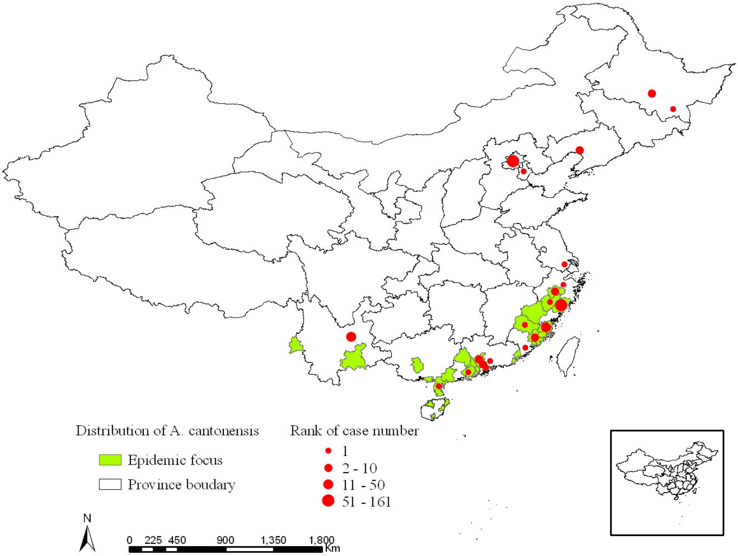

**Figure 1.4** The distribution of angiostrongyliasis cases and endemic areas in the mainland of P.R. China

The *A. cantonensis*-endemic area is probably expanding northward in P.R. China. This is due to three partly interrelated reasons. First, the primary reason is the import of *P. canaliculata* and *A. fulica* and their subsequent rapid establishment in southern P.R. China. The former originated from the South Americas and was imported to the mainland of P.R. China around 1980. The latter originally came from East Africa and was recorded in P.R. China for the first time in 1931. They propagated rapidly and today, they are distributed widely in southern P.R. China. These invasive snail species have both significantly higher

*A. cantonensis* infection prevalence and intensities than native snails, implying a higher compatibility with the parasite. Second, P.R. China offers vast habitats with suitable climatic and geographic conditions. The occurrence of *P. canaliculata* and *A. fulica* depends on the local climatic and geographical conditions, such as the temperature (Zhou et al., 1998; Zhou et al., 2003) and natural or artificial water bodies. Several studies indicate that considerable parts of the suitable habitats in southern P.R. China are not yet colonized, which creates a potential for further expansion of the endemic areas (Zhou et al., 1998; Zhou et al., 2003). Third, the low host-specificity of *A. cantonensis* offers an opportunity for further expansion. According to previous field surveys and laboratory studies, most mollusk and rodent species can serve as hosts of the parasite. Therefore, the life cycle can easily become established in new areas.

In P.R. China, 31 wild mollusk species were examined for infection with *A. cantonensis*. Among them, 21 species were discovered naturally infected by the parasite (Table 1.1). The highest infection prevalence and intensity was recorded among *A. fulica*, followed by *Vaginulus* spp. and *P. canaliculata*. Generally, the prevalence and infection intensity was higher in terrestrial snails and slugs than in freshwater mollusks. Possible reasons include the different conditions for infection. Rat faeces are rapidly dissolved in water and thus the larvae concentration per unit water is generally low which in turn reduces the probability of infection for freshwater mollusks. However, there is at least one freshwater snail species that is important for human infection with *A. cantonensis*, i.e. *P. canaliculata*.

Of 15 investigated wild rodent species, 11 species were found to occasionally harbour *A. cantonensis* in mainland P.R. China (Table 1.2). The rodent fauna shows important variations with regard to the local species composition and their relative frequency across P.R. China. Therefore, the survey results differed significantly from each other, but the infection prevalence and intensity in *R. norvegicus* was usually higher than in other species. Infections were also found among non-human primates and equine as well as canine species where the parasite could even develop into sexually mature adult worms (Mason, 1987; Duffy et al., 2004). The tendency to sexual maturation in a variety of animals increases the life-threatening potential of the parasite to domestic animals and wildlife. The prevalence in domestic animals and non-rodent wildlife has not been systematically investigated and no record about animal angiostrongyliasis is reported in P.R. China by now. The variable pathogenicity of different isolates of *A. cantonensis* is not excluded, but the lack of awareness of animal health probably plays an important role.

**Table 1.1** Intermediate hosts naturally infected with *A. cantonensis* in the mainland of P.R. China

| Intermediate host | First investigation | First positive observation | Highest prevalence |
|---|---|---|---|
| *Achatina fulica* | 1979-1982, Guangzhou | 1979-1980, Guangzhou | 96.8% (Zhongshan) |
| *Ariophantidae* spp. | 1988, Hekou | 1988, Hekou | 12.0% (Hekou) |
| *Bellamya* spp. | 2005, Lianjiang/Nan’an | 2005, Lianjiang/Nan’an | 4.1% (Lianjiang/Nan’an) |
| *Bellamya aeruginosa* | 2004, Minhou/Lianjiang | 2004, Minhou/Lianjiang | 3.8% (Minhou) |
| *Bellamya quadrata* | 1986-1990, Panyu | 1986-1990, Panyu | 2.5% (Panyu) |
| *Bradybaena brevispira* | 1979-1982, Guangzhou | 1979-1980, Guangzhou | 8.3% (Nanhai) |
| *Bradybaena revida* | 2005, Lianjiang/Nan’an | 2005, Lianjiang/Nan’an | 3.1% (Lianjiang/Nan’an) |
| *Bradybaena similaris* | 1980-1982, Guangzhou | 1980-1982, Guangzhou | 3.2% (Guangzhou) |
| *Camaena cicatricose* | 1979-1982, Guangzhou | 1979-1982, Guangzhou | 50.0% (Zhongshan) |
| *Deroceras laeve* | 2005, Lianjiang/Nan’an | 2005, Lianjiang/Nan’an | 23.8% (Lianjiang/Nan’an) |
| *Macrochlamys loana* | 2005, Lianjiang/Nan’an | 2005, Lianjiang/Nan’an | 11.2% (Lianjiang/Nan’an) |
| *Meghimati umbilinestum* | 2005, Lianjiang/Nan’an | 2005, Lianjiang/Nan’an | 5.9% (Lianjiang/Nan’an) |
| *Phiolomycus bilineatus* | 1979-1982, Guangzhou | 1979-1982, Guangzhou | 100% (Guangzhou) |
| *Plectotropis appanata* | 2005, Lianjiang/Nan’an | 2005, Lianjiang/Nan’an | 2.6% (Lianjiang/Nan’an) |
| *Pomacea canaliculata* | 1988, Hekou | 1997, Cangnan | 69.4% (Cangnan) |
| *Trichochloritis rufopila* | 1986-1990, Zhuhai | 1986-1990, Zhuhai | 5.8% (Zhuhai) |
| *T. hungerfordianus* | 1986-1990, Zhuhai | 1986-1991, Zhuhai | 5.7% (Zhuhai) |
| *Vaginulus alte* | 1988, Hekou | 1988, Hekou | 4.2% (Hekou) |
| *Vaginulus sp.* | 1980-1982, Guangzhou | 1980-1982, Guangzhou | 49.2% (Guangzhou) |
| *Vaginulus yuxjsjs* | 1988, Hekou | 1988, Hekou | 21.0% (Hekou) |
| *Zimax flavus* | 2005, Lianjiang/Nan’an | 2005, Lianjiang/Nan’an | 10.1% (Lianjiang/Nan’an) |

So far, 12 potential paratenic host species were investigated and *A. cantonensis* larvae were isolated from four of them (*Hylarana guentheri, Bufo melanostictus, Rana limnocharis* and *R. plancyi*). There is also evidence that freshwater shrimps, fish, crabs and planariae can serve as paratenic hosts of *A. cantonensis* in other countries but no infections have been found among these animals in mainland P.R. China.

**Table 1.2** Definitive hosts naturally infected with *A. cantonensis* in the mainland of P.R. China

| Definitive host | First investigation | First positive observation | Highest infection rate (%) (locality) | Highest worm burden |
|---|---|---|---|---|
| *Bandicota indica* | 1986-1987, Xuwen | 1986-1987, Xuwen | 13.2% (Xuwen) | - |
| *Mus musculus* | 1986-1987, Xuwen | 2003-2004, Fuzhou | 2.4% (Fuzhou) | - |
| *Rattus bowersii* | 1986-1987, Xuwen | 1986-1987, Xuwen | 25.0% (Xuwen) | - |
| *Rattus confucianus* | 1986-1987, Xuwen | 1986-1987, Xuwen | 20.6% (Xuwen ) | - |
| *Rattus flavipectus* | 1983, Haikou | 1986-1987, Xuwen | 17.0% (Cangnan) | 9 (Putian) |
| *Rattus losea* | 1975, Hainan | 1975, Hainan | 16.0% (Cangnan) | - |
| *Rattus nitidus* | 1986-1987, Xuwen | 1986-1987, Xuwen | 20.0% (Xuwen) | - |
| *Rattus norvegicus* | 1933, Guangzhou | 1933, Guangzhou | 67.0% (Cangnan) | 91 (Guangzhou) |
| *Rattus rattus* | 1933, Guangzhou | 1933, Guangzhou | - | - |
| *Suncus murinus* | 1983, Haikou | 2003-2004, Fuzhou | 4.1% (Fuzhou) | - |
| *Talpa micrura hainanus* | 1975, Hainan | 1975, Hainan | - | - |

### *1.4.3. Risk factors associated with culture and socioeconomics*

Freshwater snails are traditionally consumed in P.R. China. The native freshwater snail species (e.g., *Cipangopaludina* spp. and *Bellamya* spp.) are common aquatic foodstuff on local markets in P.R. China. Recently, *P. canaliculata* (or closely related species) originating from the South Americas become the alternative of *Cipangopaludina* spp. owning to their much higher growth rate and reproductivity. Therefore, initial introduction around 1980 was successful. However, the snail species became wild after escaping from farms. Experiments showed the snail are highly susceptible to *A. cantonensis* and field surveys indeed demonstrated the high infection rate. However, the native snail species are rarely found naturally infected with *A. cantonensis*, although the considerable infection rate and worm burden were observed under laboratory conditions. *B. aeruginosa* was only recently found to be naturally infected and several infections could be linked to the consumption of snails belonging to this species (Lin et al., 2005). *C. chinensis* has been suggested to play an important role in the epidemiology of human angiostrongyliasis (Hwang and Chen, 1991), but now never found naturally infected in the mainland of P.R. China. Ecological characteristics might explain this observation. Indeed, the native snail species live at the bottom of water bodies, and *P. canaliculata* however is usually active at water surface around the banks. Furthermore, the exotic snails can also live in small ditches and ponds surrounding human settings where domestic rats, *R. norvegicus* and *R. rattus*, inhabit.

Consumption of terrestrial snails is much less common in P.R China. However, one snail species, the Africa land snail (*A. fulica*), is a considerable exception. Africa land snail was first recorded in the campus of Xiamen University in 1931. Although this snail species was indeed consumed by the Japanese soldiers and native residents on the Pacific Islands during the Second World War, there might have been other reasons to introduce this snail into P.R. China might exit. Nevertheless, the giant land snail was involved in the consumption, and hence resulted in angiostrongyliasis in the mainland of P.R. China.

In addition to the consumption of freshwater and recently Africa land snail, terrestrial slugs are, still today, used in traditional medicine in P.R. China as well as other countries in Southeast Asia (Kliks and Palumbo, 1992). Several cases of angiostrongyliasis in the mainland of P.R. China were linked to the consumption of raw slugs (Huang et al., 2003; Li et al., 2006; Liu et al., 2006; Ruan et al., 2008).

The rapid socio-economic development of P.R. China over the past three decades also impacted on the food trade and enabled the countrywide marketing of aquaculture products from the southern provinces. Especially snails are not always raised in specialized farms but rather collected from natural habitats. An example is the recent angiostrongyliasis outbreak in Beijing. *P. canaliculata* imported from Guangxi province in southwest P.R. China were traced as the source of infection. The economic development also resulted in the spread of new or regionally limited consumption habits. The residents of the southeastern coastal area have a long tradition of raw food consumption, including snails and seafood. In recent years, these special foods became popular among the middle and upper class population of urban areas throughout the country.

Angiostrongyliasis did not attract wide attention in P.R. China until the 2006 outbreak in Beijing, which was widely reported in the media. Health professionals outside known endemic areas usually have limited knowledge about the disease and often neglect it when confronted with patients from non-endemic areas. Most consumers in both endemic and non-endemic areas are not aware of the disease, its route of transmission and the most common signs and symptoms of infection.

### *1.4.4. Research and control priorities*

Although considerable efforts have been made in the last two decades to deepen the understanding of *A. cantonensis* and eosinophilic meningitis due to this parasite, there are still a number of unresolved issues that need to be addressed. First, the distribution of the parasite throughout the country has to be determined and the leading hosts should be identified. Today, still very little is known about the extent of the endemic area in P.R. China (Prociv et al., 2000). The majority of previous surveys were carried out following the detection of human infections in an area and the main motive was to identify the place where implicated food items came from. Furthermore, socioeconomic development and environmental change probably govern the distribution dynamics of *A. cantonensis* and their hosts. The currently available data pertaining to the endemic areas were mainly produced in the 1980s, which perhaps could not reflect the current status in P.R. China. Therefore, it is necessary to carry out systemic and large-scale surveys in all potential endemic areas of the country.

Second, standard protocols are needed for the diagnosis of angiostrongliasis due to *A. cantonensis*. A definite diagnosis for angiostrongyliasis was based on the discovery of larvae of *A. cantonensis* in cerebrospinal fluid or ocular chamber, or adult worms in pulmonary arteries. However, the probability was very low because most human cases are found with slight or mild infection (Lv et al., 2008). Elevated eosinophil count characterizes meningitis due to *A. cantonensis*, but can vary during the course of an infection. Seroimmunological assays hold promise in the diagnosis of angiostrongyliasis. However, the assessment of these assays is limited because the serum from parasitologically confirmed cases is very rare. The epidemiological history of cases is crucial to make final diagnosis, but is difficult in many sporadic cases because various paratenic hosts can be involved. Therefore, categorized criteria should be proposed in order to lead clinical doctors to establish new evidence to enhace the accuracy of diagnosis.

Third, animal ill-health due to *A. cantonensis* warrants special attention. There is growing evidence from wildlife and domestic animals that indicate that *A. cantonensis* can threat a range of vertebrate species, including non-human primates, horse, dog, bird, marsupial and flying fox (Mason, 1987; Higgins et al., 1997; Barrett et al., 2002; Kim et al., 2002; Rajkovic-Janje et al., 2002; Duffy et al., 2004; Monks et al., 2005; Gelis et al., 2011). One experiment was performed to observe the pathogenicity of *A. cantonensis* in non-human primate, dog and cats. Only cats were successfully infected by this parasite and manifested with symptoms related to eosinophilic meningitis (Yang et al., 2001). Duck can be also infected by this parasite and suffered from eosinophilic meningitis (Zhang et al.; personal communication). Notably, many domestic livestock, ducks and chicken, prey on or are bred with *P. canaliculata* and *A. fulica* in some areas of P.R. China. If Chinese isolates of *A. cantonensis* is infective to these animals as shown in Australia and USA, the special concern should be raised in southeast parts of P.R. China, where *A. cantonensis* is endemic.

Forth, a comparative study should be conduct among different isolates in the presumptive home range of *A. cantonensis*. It is assumed that *A. cantonensis* has spread beyond Southeast Asia probably from southeast part of P.R. China and the offshore islands (Kliks and Palumbo, 1992). This hypothesis, however, has not yet been confirmed. Characterizing the isolates by molecular markers in this area is the basis to address this issue. Unfortunately, the data like that are absent. Another issue also calls for this kind of investigation. As early as 1993 there has been an assumptiion that *A. malaysiensis* occurs in Yunnan province (Li et al., 1993). Although it was not confirmed, the morphological difference from typical *A. cantonensis* indicated potential intraspecific differentiation or introduction of exotic species. The molecular epidemiology of *A. cantonensis* in the home range, hence facilitates a deeper understanding of the biological invasion and their role of *P. canaliculata* and *A. fulica*.

Fifth, hospital-based surveillance system in endemic areas should be established. Angiostrongyliasis is one of the rare parasitic infections leading to outbreaks (Odermatt et al., 2010). Nevertheless, the real number of snail consumers who are attacked by angiostrongyliasis is difficult to estimate. There are two major explanations: (i) the relatively long incubation period, and (ii) mild infection. The average incubation period is 2 weeks but with remarked variation, primarily depending on infection intensity. Notably, many cases might not be linked 2 weeks after exposure only when the patients were familiar with each other (e.g. relatives, friends and colleagues). Furthermore, the patients can be spatially scattered within a period of 2 week, which makes it more difficult to reveal an outbreak. A hospital-based surveillance can be useful when the index patients provide the detailed information pertaining to consumption of suspected food items.

Finally, the most important preventive measure would probably be targeted at health education. The basic message is that the most important way of infection is the consumption of raw or undercooked snails. This message can be easily communicated and taken up by the public. For example, after the first angiostrongyliasis outbreak in Wenzhou in 1997, a health education campaign about safe consumption habits and the most prominent disease symptoms was launched in Zhejiang province. Few cases were reported since that campaign. However, not only the public has to be informed about the disease, but knowledge among the medical community has to grow as it is critically important that infections are detected in an early stage, so that, appropriate medical actions can be taken.

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# 2. Goal and objectives

## 2.1. Goal

**To update the epidemiology of *Angiostrongylus cantonensis* and eosinophilic meningitis in the mainland of People's Republic of China by a national survey pertaining to *A. cantonensis* and studying outbreaks**

## 2.2. Objectives

- To identify the control priorities in eosinophilic meningitis by observing and analyzing outbreaks
- To reveal the geographical distribution of *A. cantonensis* and to identify the major intermediate hosts
- To characterize the mitochondrial (mt) genome of *A. cantonensis* and its close relative *A. costaricensis* in order to identify genetic marker that might give rise to novel diagnostic assays and population genetic studies
- To evaluate the intraspecific differentiation of *A. cantonensis* and explore the potential dynamic scenarios in P.R. China
- To assess the genetic diversity of the invasive snail species *Pomacea* spp. and explore potential spread scenarios
- To assess the interplay between invasive snails, climate change and transmission dynamics

# 3. Human angiostrongyliasis outbreak in Dali, China

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### 3.1. Abstract

**Background** Several angiostrongyliasis outbreaks have been reported in recent years but the disease continues to be neglected in public health circles. We describe an outbreak in Dali, southwest China in order to highlight some key problems for the control of this helminth infection.

**Methodology/Principal Findings** All available medical records of suspected angiostrongyliasis patients visiting hospitals in Dali in the period 1 October 2007 - 31 March 2008 were reviewed, and tentative diagnoses of varying strength were reached according to given sets of criteria. Snails collected from local markets, restaurants and natural habitats were also screened for the presence of *Angiostrongylus cantonensis*. A total of 33 patients met criteria for infection, and 11 among them were classified as clinically confirmed. An additional 8 patients were identified through a surveillance system put in operation in response to the outbreak. The epidemic lasted for 8 months with its peak in February 2008. Of the 33 patients, 97.0% complained of severe headache. 84.8% patients had high eosinophil cell counts either in the peripheral blood or in cerebrospinal fluid (CSF). Three quarters of the patients were treated with a combination of albendazole and corticosteroids, resulting in significantly improved overall conditions. Twenty-two patients reported the consumption of raw or undercooked snails prior to the onset of the symptoms, and approximately 1.0% of the *Pomacea canaliculata* snails on sale were found to be infected with *A. cantonensis*. The snails were also found in certain habitats around Dali but no parasites were detected in these populations.

**Conclusions/Significance** The import and sale of infected *P. canaliculata* is the likely trigger for this angiostrongyliasis outbreak. Awareness for angiostrongyliasis must be raised, and standardized diagnosis and treatment are needed in order to provide clinicians with a guide to address this disease. Health education campaigns could limit the risk, and a hospital-based surveillance system should be established in order to detect future outbreaks.

**Keywords**: *Angiostrongylus cantonensis*, angiostrongyliasis, *Pomacea canaliculata*, eosinophilic meningitis, Dali, China

### 3.2. Introduction

Angiostrongyliasis caused by *Angiostrongylus cantonensis* is a potentially fatal parasitic disease. Humans are infected through several ways as its life cycle involves different edible intermediate host mollusks and a range of paratenic hosts which also pose threats (Murrell and Fried, 2007). In China and Southeast Asia, consumption of raw or undercooked snails, mainly *Pomacea canaliculata*, *Achatina fulica* and *Pila* spp., is the primary route of infection (Lv et al., 2008; Punyagupta et al., 1970; Yii et al., 1975). In the Caribbean, contaminated vegetables and condiments have been implicated, e.g. in an outbreak in Jamaica (Slom et al., 2002). Monitor lizard is considered as the main source of infection in India and Sri Lanka (Hidelaratchi et al., 2005; Parameswaran, 2006) while freshwater shrimps, fish and crabs are a suspected way of infection in the Pacific Islands (Malvy et al., 2008; Rosen et al., 1967).

Angiostrongyliasis has been widely spread in the wake of biological invasion. *Rattus norvegicus* and *A. fulica* are two top invasive species and *A. cantonensis* is thought to have trailed the two animals in establishing itself throughout the tropics (Kliks and Palumbo, 1992). Indeed, many outbreaks and sporadic cases were attributed to *A. fulica* (Hwang and Chen, 1986; Kliks et al., 1982; Yii, 1976; Yii et al., 1975) or happened only after the invasion of the two species (Prociv et al., 2000). Recently, the snail rapidly spread into Brazil (Thiengo et al., 2007), and only subsequently, *A. cantonensis* had been found to naturally infect local mollusks (Caldeira et al., 2007). In addition to *A. fulica*, another invasive snail species, i.e., *P. canaliculata*, has facilitated the emergence of angiostrongyliasis in Asia in the past decade and has since become the primary vector in this region (Lv et al., 2008; Tsai et al., 2001).

Over 2,800 cases have already been reported from more than 30 countries (Wang et al., 2008) but this figure might well be a small fraction of the real number. There are sporadic cases which are likely to go undetected due to low awareness among the medical community and a lack of diagnostic symptoms and readily available tests. Some patients also only experience transient or mild manifestations (Kliks and Palumbo, 1992; Punyagupta et al., 1975). Angiostrongyliasis is not common and is usually clustered in certain population segments. Recently, cases in returning travelers from non-endemic regions have been reported (Ali et al., 2008; Bartschi et al., 2004; Graeff-Teixeira et al., 2009; Kirsch et al., 2008; Leone et al., 2007; Ryan et al., 2002). Others include local people with special dietary habits or using traditional medicine (Cross, 1987).

Here, we describe an outbreak in Dali in Yunnan province, southwest China in order to highlight this neglected disease and the challenges involved in its diagnosis, surveillance and control.

## 3.3. Materials and methods

### *3.3.1. Ethics statement*

The investigation had been approved by the Academic Board (ethics committee) of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai (ref. no. 2006111201). All participants were informed about the study procedures and gave their written informed consent or, in the case of illiterates, oral approval.

### *3.3.2. Retrospective survey and establishment of surveillance system*

In response to an unusually high number of suspicious clinical manifestations, all medical records of suspected angiostrongyliasis patients visiting three major hospitals in Dali, namely People’s Hospital of Dali Prefecture, Dali First People’s Hospital, Affiliated Hospital of Dali Medicine College, and a specialized local medical center (Institute of Research and Control of Schistosomiasis in Dali Prefecture) between 1 October 2007 and 31 March 2008 were reviewed. The extracted data included demographic information, onset of illness, hospitalization date, clinical manifestations, examination and laboratory test results, disease progression, and treatment regimens and outcomes. The patients, including discharged patients, were then traced and interviewed regarding potential exposure (e.g. consumption of raw or undercooked snails, fish, shrimps and crabs within the past one month), the place and date of eating these food items, and the number of persons who shared the same food.

In order to follow the progress of the epidemic, a temporary hospital-based surveillance system was established to collect information on new cases from 1 April 2008 onwards. Through this system, clinicians reported suspected patients according to a given set of diagnostic criteria.

### *3.3.3. Diagnostic criteria*

Patients were stratified into three groups according to the tentative diagnostic criteria of angiostrongyliasis published by the Ministry of Health in 2006. The three classes were suspected, clinically diagnosed and parasitologically diagnosed cases. The diagnostic criteria are based on available evidence from clinical practice:

(1) Eating history: recently (within one month) ate raw or undercooked snails or other potentially infective food items such as slugs as medicine, raw or undercooked freshwater fish, shrimps, crabs, frogs and snakes.

(2) Clinical manifestations: presenting with at least one of the following symptoms: severe headache, nausea and vomiting, visual disturbances, photophobia, nuchal rigidity, hyperesthesia, and paresthesia.

(3) An elevated count of eosinophils (>500 cells/µL) in peripheral blood.

(4) An elevated count of eosinophils (>10 cells/µL) in cerebrospinal fluid.

(5) Sero-positive for specific *A. cantonensis* antigens or the corresponding antibody.

(6) Presence of *A. cantonensis* larvae in cerebrospinal fluid, anterior chamber, vitreous cavity, or subretinal space; or presence of larvae in sections of the brain and spinal cord, or worms in pulmonary arteries or the heart.

Suspected cases were those meeting criteria 1 and 2, or 1 and 5, or 2 and 3, or 2 and 4. Clinical diagnosis required meeting the criteria 1, 2, 3 and 4. Parasitologically diagnosed cases were those clinical cases where *A. cantonensis* was discovered as stipulated by criterion 6.

### *3.3.4. Additional investigations*

In order to understand the local prevalence of *A. cantonensis* in snails marketed for human consumption, *P. canaliculata* and *Cipangopaludina chinensis* snails were collected in markets and restaurants mentioned by study participants. A week-long surveillance of the snails on sale was conducted from 26 - 31 March 2008. *A. cantonensis* infections in *P. canaliculata* were diagnosed using the "lung examination" method described by Liu and co-workers (Liu et al., 2007). Negative snails were examined in batches of five using the artificial digestion method (Lv et al., 2006c). *C. chinensis* were examined by artificial digestion only. In addition, the sources of snails available on markets and in restaurants were investigated. Snails including *P. canaliculata* and *C. chinensis* collected from natural habitats were also examined using the same techniques.

### *3.3.5. Data analyses*

All data were entered in a central database using EpiInfo version 3.5 (Centers for Disease Control and Prevention, Atlanta, USA). The epidemic trend of angiostrongyliasis in Dali between 1 October 2007 and 31 March 2008 was described using bar graphs with a two-week interval. Both the onset of manifestations and the date of hospital admission for each patient are shown. Demographic information, i.e. sex, age and ethnic group, were investigated, and the proportion of suspected sources of infection (e.g. snails, fish and shrimps, unknown) calculated and clinical manifestations summarized.

The dynamic relationship between eosinophil counts in peripheral blood and CSF was analyzed using available paired data of all patients collected before treatment and over the course of the intervention to describe their co-dynamic pattern. Increased eosinophil cell counts in peripheral blood were defined as those with more than 500 cells per μL, and increased eosinophil counts in CSF were those with more than 10 cells per μL. Since the date of collection of blood and CSF samples might differ, only data originating from samples collected within an interval of less than 3 days were considered.

The treatment efficacy of the standard combination therapy involving albendazole and dexamethasone was assessed by following changes in eosinophil cell counts in the peripheral blood. Data on eosinophils were collected before onset of any treatment which might affect eosinophil cell numbers and again during and after combination treatment. The effect of illness duration (the duration of chief complaints, i.e., headache for 32 patients, numbness and muscle weakness in limbs for one patient) and the period of drug treatment were also considered.

## 3.4. Results

### *3.4.1. Epidemiology*

Starting in mid-October 2007, the three major hospitals of Dali and a specialized medical center in the same city were visited by patients with eosinophilic meningitis. The last such patient was admitted to hospital in mid-May 2008. At this time, two public health interventions had been launched: information about angiostrongyliasis had been spread through mass media in mid-March, and the sale of live snails in markets had been banned since 1 April. The peak incidence was in late February and the peak of hospital admissions was in early March (Figure 3.1). None of the suspected angiostrongyliasis cases which were characterized by significantly elevated eosinophil cell counts and typical manifestations had been parasitologically confirmed.

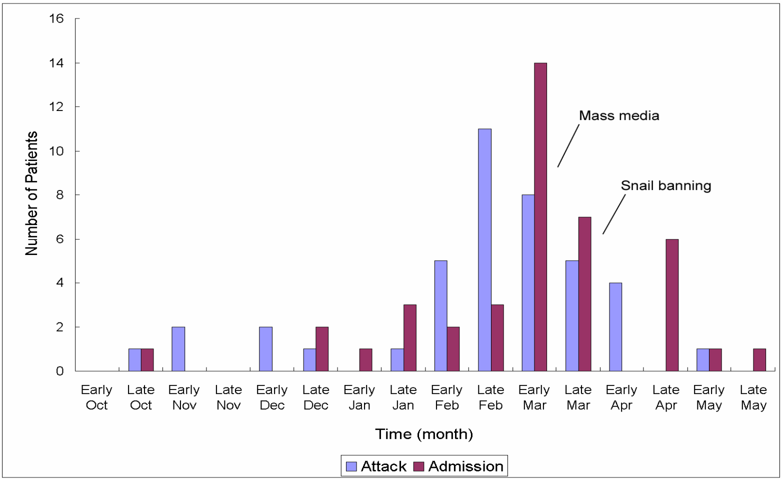

**Figure 3.1** Course of the angiostrongyliasis epidemic in Dali from October 2007 - May 2008. “Attack” and “Admission” designate the onset of symptoms and admission to hospital, respectively. “Mass media” indicates propagation of information on angiostrongyliasis through mass media and was initially performed in mid-March 2008. “Snail banning” is the prohibition of snail sales on markets since 1 April 2008.

Of 36 patients included in the retrospective survey, 3 were excluded due to lack of eating history of raw snails or other potential infective food items. Among 33 cases who met the diagnostic criteria set by the Ministry of Health, 11 could be classified as clinically diagnosed cases and 22 were suspected cases. Another 8 cases (one suspected and seven clinical cases) were picked up by the hospital-based surveillance system between 1 April and 30 September 2008.

Of the 33 patients included in this retrospective study, 18 were female (Table 3.1). They were aged 12 - 72 years and mainly belonged to the Bai ethnic minority ($n$ = 19) or were Han Chinese ($n$ = 13). All patients were locals except three who lived in adjacent counties but had consumed the potentially infectious food items in Dali city. Consumption of raw or undercooked snails in the past one month was reported by 22 study participants, and eating raw fish or unknown food items possibly related to angiostrongyliasis equally accounted for the remaining cases. The study participants mentioned a range of locations and time points where they had consumed potentially infective food: four patients ate snails obtained in local markets in private settings, six pointed out the same restaurant but had frequented it at different dates, and others had dined at several other restaurants. Most individuals had consumed the suspicious food in early February 2008 around the Spring Festival/traditional Chinese New Year.

### *3.4.2. Clinical characteristics*

The mean estimated incubation period among the 33 patients was 16 days with a range of 3-50 days. Headache was reported by 97.0% of all patients when they were admitted to hospital (Table 3.1).

One case only suffered from numbness and muscle weakness of the left limbs. Nausea and vomiting were reported by 39.4% of the cases. Fever was uncommon; most were afebrile during the entire duration of their illness. Thirty patients received lumbar punctures and 18.2% of the patients suffered from high intracranial pressure (>250 $mmH_2O$). High eosinophil counts in peripheral blood and/or CSF were noted in 84.8% of the patients. Simultaneous cytological tests in peripheral blood and CSF before treatment were done in 18 patients, and 61.1% of them had elevated eosinophil counts in both blood

**Table 3.1** Characteristics of 33 patients involved in the angiostrongyliasis epidemic in Dali in 2007-2008

| Characteristics | Patients (N=33) |
|---|---|
| Demographic information | |
| Sex (*n* [%]) | |
| Male | 15 (45.5) |
| Female | 18 (54.5) |
| Age (median [ range]) | 35 (12-72) |
| Ethnic group-no. (*n* [%]) | |
| Bai | 19 (57.6) |
| Han | 13 (39.4) |
| Other | 1 (3.0) |
| Reported eating history (*n* [%]) | |
| Snails | 22 (66.7) |
| Fish/shrimps | 6 (18.2) |
| Unknown | 5 (15.1) |
| Symptoms and signs (*n* [%]) | |
| Headache | 32 (97.0) |
| Nausea and vomiting | 13 (39.4) |
| Nuchal rigidity | 6 (18.2) |
| Muscle weakness | 5 (15.1) |
| Fever (axillary temperature > 37.8°C) | 4 (12.1) |
| Paresthesias | 3 (9.1) |
| Muscle pain | 3 (9.1) |
| Visual disturbance | 2 (6.1) |

and CSF (Figure 3.2). The three patients with increased counts of eosinophils in their CSF but normal blood values were the oldest and two youngest patients of the entire cohort. Two of the three patients with normal eosinophil counts in their CSF and increased counts in their blood had a disease history ≥33 days. Only one patient had normal eosinophil levels in both blood and CSF. Average glucose and chlorinate levels were 2.65±0.65 mmol/L and 122.59±10.00 mmol/L, respectively. Total protein varied between 189 mg/L and 3857 mg/L with a median of 753 mg/L. Differential serological testing was performed in 20 patients (schistosomiasis, trichinellosis, cysticercosis, echinococcosis) but only five patients were tested for *A. cantonensis*-specific antibody, among whom four tested positive.

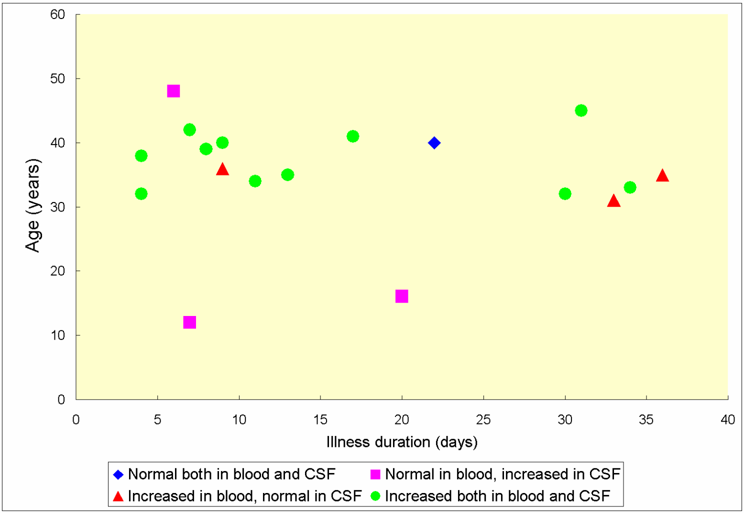

**Figure 3.2** Eosinophil counts in peripheral blood and CSF before treatment, stratified by illness duration and age. An increased level of eosinophil cell counts in blood is defined as a relative count of more than >500 cells/μL and an increased eosinophil level in CSF is defined as a count of more than >10 cells/μL. The data of the 18 patients were collected before treatment. The collection date of blood and CSF samples from the same patient may differ up to 3 days.

### *3.4.3. Treatment*

A combination of albendazole (200 - 400mg, tid) and dexamethasone (5 - 20mg/day) was employed to treat 26 study participants. In addition, patients suffering from high intracranial pressure were treated with mannitol (20%; 250ml/day). The other patients were treated with either albendazole or dexamethasone, each accompanied by mannitol, if necessary. Only one young female patient was readmitted to hospital due to relapse one month after she had been discharged. Continuous data reflecting the dynamics of key indicators, e.g. eosinophil counts, over the treatment period were only available for 7 patients. Eosinophil numbers in peripheral blood generally decreased after treatment but fluctuations were noted in two patients.

### *3.4.4. Additional investigations*

On 3 different days, *A. cantonensis* larvae were found in a total of five of the 503 *P. canaliculata* snails collected from local food markets. Samples from restaurants as well as *C. chinensis* samples were negative. Two separate *P. canaliculata* populations were discovered in the Dali area. The bigger habitat included Xihu Lake and its surroundings, upstream of Erhai Lake which is the biggest freshwater reservoir in the area. The second population was located in Heqing county in the vicinity of Dali city but none of the snails collected in these locations was found to be infected.

## **3.5. Discussion**

Dali city was considered non-endemic for angiostrongyliasis since neither human or animal cases nor infected specimens of known intermediate host mollusks were found in the area during the first national survey on angiostrongyliasis (Lv et al., 2009). However, an extended outbreak occurred in this area due to importation of contaminated snail food. In previous angiostrongyliasis outbreaks in China both within and beyond the known endemic areas, most patients involved were found to have shared the same food at a single location (Lv et al., 2008; Malvy et al., 2008; Slom et al., 2002; Tsai et al., 2001). The described epidemic in Dali differed from this picture in several important aspects: patients consumed the potentially infective food at different places and over an extended period of time, suggesting a stable supply of contaminated food. Spring festival, the most important traditional Chinese holiday, arguably facilitated the epidemic since extended meals involving special dishes are a core feature of this holiday. The native *C. chinensis* is a traditional food item in many areas across China including Dali. The snail can readily be infected with *A. cantonensis* in the laboratory (Lv et al., 2006b) and is an important source of infection in Taiwan (Chang et al., 1968; Hwang and Chen, 1991) but infected snails have rarely been detected in mainland China and no case of angiostrongyliasis in the country has ever been linked to this species. Since their population declined following environmental changes, *P. canaliculata* has increasingly been imported as a substitute, possibly also from places where *A. cantonensis* is endemic. Today, the majority of all outbreaks and sporadic cases in mainland China can be linked to this species (Deng et al., 2007; Lv et al., 2008; Lv et al., 2006a).

None of the described angiostrongyliasis cases from Dali could be parasitologically confirmed but the clinical manifestations, laboratory data, eating histories as well as the presence of *A. cantonensis* in locally sold snails suggest the suspected infection. Indeed, the probability of finding *A. cantonensis* in patients is very low (Lv et al., 2008; Punyagupta et al., 1975; Yii, 1976) and parasitological evidence is not considered mandatory for the identification of outbreaks since additional information obtainable in outbreak situations can help to reach the final decision (He et al., 2007; Slom et al., 2002; Tsai et al., 2001). This might explain the higher frequency of parasitological proof in sporadic cases than outbreak situations. We adopted the tentative diagnostic criteria of angiostrongyliasis set forth by the Chinese Ministry of Health. Based on them, our study population was categorized into 22 suspected and 11 clinically diagnosed cases. Several reasons drive this low approval rate of suspected cases. First, the criteria are strict compared to other clinic guidelines (Malvy et al., 2008; Slom et al., 2002; Yii, 1976). For example, clinical diagnosis requires elevated eosinophil numbers in both peripheral blood and CSF. However, in the present sample only 61.1% (11/18) showed consistency in eosinophil levels between peripheral blood and CSF before treatment, corroborating earlier observations (Tsai et al., 2001). Second, results from serologic tests are not considered for clinical diagnosis, leading to the classification as suspected cases of four seropositive patients. Although various immunological tests have been developed, none have been systematically evaluated and none are not widely and commercially available in China (Wang et al., 2008). Last, important indicators might have been neglected due to low levels of awareness about angiostrongyliasis among medical staff. Thus, the development, evaluation and dissemination of serologic tests and validated clinical guidelines appear imperative.

Eosinophilic meningitis is the main but not the only clinical manifestation of angiostrongyliasis cantonensis (Sawanyawisuth and Sawanyawisuth, 2008). Other symptoms include eosinophilic meningoencephalitis (Furugen et al., 2006), eosinophilic radiculomyelitis (Schmutzhard et al., 1988), ocular angiostrongyliasis (Sinawat et al., 2008), as well as nasal (Liu and An, 2000) or pulmonary infections (Li et al., 2001; Lindo et al., 2004). Further, most angiostrongyliasis infections are self-limiting and recovery without sequelae is the norm (He et al., 2007; Punyagupta et al., 1975; Yii, 1976). However, ensuing encephalitis might be fatal (Sawanyawisuth and Sawanyawisuth, 2008). All but one of the 33 patients described here developed eosinophilic meningitis and one patient suffered from eosinophilic radiculomyelitis. Severe headache and high intracranial pressure were often reported, and elevated eosinophil counts in blood and/or CSF were important characteristics (Yoshimura et al., 1994). Few studies describe the dynamics of eosinophils in peripheral blood (Punyagupta et al., 1975; Tsai et al., 2001), and no complete data are available showing the co-dynamics of counts in peripheral blood and CSF which could support diagnosis and facilitate treatment evaluation. Indeed, these changes are difficult to generalize because many factors including age, severity and duration of the infection and treatment can affect eosinophil cell numbers. In the present survey, eosinophil counts were elevated in 84.8% of the participants, either in peripheral blood and/or in CSF, but the agreement between peripheral blood and CSF when patients were not treated was only 61.1%. Although the sample was small, the available data suggested that very young and elder patients were more likely to exhibit elevated counts in the CSF rather than in peripheral blood, and that eosinophil cell counts in the CSF returned to normal more quickly than those in blood.

A combination therapy with anthelminthic drugs and corticosteroids is recommended for the treatment of eosinophilic meningitis (Chotmongkol et al., 2006; Chotmongkol et al., 2004; Tsai et al., 2001) although the role of anthelminthics in improving manifestations is still unclear (Koo et al., 1988; Sawanyawisuth and Sawanyawisuth, 2008). Presumably, anthelminthic drugs kill causative worm larvae and the corticosteroids suppress hypersensitivity reactions triggered by antigens released from the worms. Thus, anthelminthic treatment alone tends to exacerbate neurologic symptoms (Bowden, 1981; Hidelaratchi et al., 2005), and corticosteroid treatment alone improves symptoms but fails to cure patients, resulting in relapses (Chotmongkol et al., 2000; Malvy et al., 2008; Sawanyawisuth et al., 2004; Tsai et al., 2004). In the present study, 26 patients were given a combination treatment resulting in significantly improved conditions. Dramatic drops in eosinophil counts over the course of the combination treatment were noted in those seven patients for whom complete data were available. However, no reliable conclusion was drawn due to small sample size and few repeated measurements of eosinophil counts.

Unlike most food-borne viral and bacterial diseases, food-borne helminthiasis usually has a long and variable incubation period, resulting in a tempo-spatially dispersed patient population. Incubation periods ranged from 3 to 50 days with a mean of 16 days in this outbreak. Moreover, patients did not appear to have consumed contaminated food at the same time, further spreading the temporal distribution of cases and masking the onset of the epidemic. This also made it difficult to determine the population potentially exposed to *A. cantonensis* infected food. It can thus be suspected that the 41 cases detected through retrospective analysis of patient data as well as the hospital-based surveillance system might represent but a fraction of all patients and thus not accurately reflect the afflicted population and the full range of clinical presentations and disease progression. This underscored the need to pay due attention to apparently isolated cases since they might signal the onset of a protracted epidemic. Hospital-based surveillance might be an effective approach to reveal unfolding angiostrongyliasis epidemics in areas where snails are commonly eaten.

In conclusion, the reported epidemic of angiostrongyliasis in Dali highlights the risks of food-borne diseases in hitherto non-endemic areas through food trade. It appears urgent to develop reliable diagnostic tools and establish sound diagnostic criteria and treatment schedules for angiostrongyliasis. Robust health education should be initiated and hospital-based surveillance established in regions where snails, particularly *P. canaliculata* and *A. fulica*, are popular.

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# 4. Invasive snails and an emerging infectious disease: results from the first national survey on *Angiostrongylus cantonensis* in China

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## 4.1. Abstract

***Background**:* Eosinophilic meningitis (angiostrongyliasis) caused by *Angiostrongylus cantonensis* is emerging in mainland China. However, the distribution of *A. cantonensis* and its intermediate host snails, and the role of two invasive snail species in the emergence of angiostrongyliasis are not well understood.

***Methodology/Principal Findings:*** A national survey on *A. cantonensis* was carried out using a grid sampling approach (spatial resolution: 40 x 40 km). One village was randomly selected from each of a 5% random sample of grid cells located in areas where the presence of the intermediate host snail *Pomacea canaliculata* had been predicted a based on degree-day model. Potential intermediate hosts of *A. cantonensis* were collected in the field, as well as on restaurants, markets and snail farms, and examined for infection. The infection prevalence among intermediate host snails was estimated, and the prevalence of *A. cantonensis* within *P. canaliculata* was displayed on map, and predicted for non-sampled locations. It was confirmed that *P. canaliculata* and *Achatina fulica* were the predominant intermediate hosts of *A. cantonensis* in China, and the snails were found to be well established in 11 and six provinces, respectively. Infected snails of either species were found in seven provinces, closely matching the endemic area of *A. cantonensis*. Infected snails were also found on markets and restaurants. Two clusters of *A. cantonensis* in *P. canaliculata* were predicted in Fujian and Guangxi provinces.

***Conclusions/Significance:*** The first national survey revealed wide distribution of *A. cantonensis* and two invasive snail species, and a considerable number of people were still at risk of angiostrongyliasis. Health education, rigorous food inspection and surveillance are needed to prevent recurrent angiostrongyliasis outbreaks.

**Keywords**: *Angiostrongylus cantonensis*, *Pomacea canaliculata*, *Achatina fulica*, angiostrongyliasis, emerging infectious disease, invasive species, China

## 4.2. Introduction

Eosinophilic meningitis, a potentially fatal disease caused by *Angiostrongylus cantonensis*, is considered an emerging infectious disease in mainland China (Lv *et al.*, 2008; Morse, 1995). The first case of angiostrongyliasis in mainland China was reported in 1978, and a few more cases occurred until the mid-1990s. Subsequently, several outbreaks have been recorded (Lv et al., 2008). The first major angiostrongyliasis outbreak, involving 65 patients, was documented from Wenzhou in Zhejiang province in 1997 (Zheng et al., 2001). The biggest outbreak in China thus far attributed to freshwater snail, i.e., *Pomacea canaliculata*, took place in the capital Beijing in 2006 (MacDonald, 2006). Of the 160 infected individuals involved in this event, 100 were hospitalized and 99 were interviewed to shed light on the etiology of the disease (He et al., 2007). This outbreak also demonstrated that angiostrongyliasis had moved beyond its traditional endemic areas located in the southeastern coastal regions of China.

The parasite was first described by Chen based on worm specimen collected from pulmonary arteries of rats in Guangzhou (Canton) (Chen, 1935) and Dougherty gave the name *A. cantonensis* in 1946 (Murrell and Fried, 2007). Adult *A. cantonensis* live in the pulmonary arteries of its definitive hosts, i.e., rodents, especially rats, which pass infective first-stage larvae ($L_1$) in their feces. The life cycle also involves mollusks, harboring the larval stage. In humans, larvae fail to mature, and hence humans and their excreta play no role in the transmission and dissemination of the parasite. Humans become infected by ingesting third-stage larvae ($L_3$) in raw or undercooked intermediate host mollusks (e.g., snails and slugs) or paratenic hosts (e.g., freshwater prawns, crabs, frogs and fish) (Alicata, 1991; Anderson, 2000; Lai *et al.*, 2007). Lettuce and vegetable juice have also been identified as sources of infection when contaminated with intermediate or paratenic hosts (Slom *et al.*, 2002; Tsai *et al.*, 2004). Due to the low host specificity of *A. cantonensis* it is difficult to control this parasite (Lv et al., 2008). Two snail species, i.e., *Achatina fulica* and *P. canaliculata*, are believed to be closely associated with angiostrongyliasis in China. These snails were imported into mainland China in 1931 (Jarreit, 1931; Mead, 1961) and 1981 (Joshi and Sebastian, 2006; Lv *et al.*, 2008), respectively, and have rapidly extended their geographic ranges. Indeed, these two snails are now listed as invasive species by the Chinese government.

In response to the recent angiostrongyliasis outbreak in Beijing that received considerable national and international attention and mass-media coverage, the Ministry of Health (MoH) of China launched the first national survey on *A. cantonensis*. Here, we report the design and key findings of this survey. Moreover, predictions are made for the spatial distribution of *A. cantonensis* and its intermediate hosts. Finally, recommendations are offered for the prevention of angiostrongyliasis.

## 4.3. Methods

### *4.3.1. Ethics statement*

The project entitled "The first national survey on *Angiostrongylus cantonensis* in China" has been approved by the institutional ethics committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai (ref. no. 2006030101; a copy of the ethical clearance is attached with the submission). Animal experiments were conducted by adhering to institutional guidelines for animal husbandry.

### *4.3.2. Design of the national survey on* A. cantonensis

The first national survey pertaining to *A. cantonensis* and its definitive and intermediate hosts in mainland China was implemented in two phases over a 1-year period, i.e., between September and November 2006, and between March and October 2007. Considering that the distribution of *A. cantonensis* was affected by several environmental and ecological factors, the potential distribution of the parasite was first determined. Temperature was selected as the main factor to predict the potential distribution of the parasite and two invasive snail species in China. Because revealing distribution of parasite and the two snail species is an aim in the survey, the widest potential distribution (of *P. canaliculata*) was considered as the survey region. The potential range of *P. canaliculata* in China was predicted using a degree-day model based on temperature data obtained from 149 meteorologic stations across China (Zhou et al., 2003). A grid with a spatial resolution of 40 x 40 km was laid over the designated area, and ~5% of the grid cells were randomly selected. In each survey grid cell, one village was randomly selected for subsequent field work. The geographic coordinates of the survey villages were recorded using a hand-held global positioning system (GPS) device (GPSmap 70; Kansas, USA).

### *4.3.3. Field survey of* A. cantonensis *and its hosts*

Rodents are the definitive hosts of *A. cantonensis*. Some insectivores also serve as suitable definitive hosts (He *et al.*, 1985; Luo *et al.*, 2005). Therefore, rats (e.g., *Rattus norvegicus*) and insectivores (Soricidae, e.g., *Suncus murinus*) were trapped in fields and in residents' houses. All captured animals were euthanized and dissected to estimate the presence or absence of adult *A. cantonensis* in their hearts and lung arteries.

Freshwater snails (e.g., *P. canaliculata* and *Bellamya aeruginosa*), terrestrial snails (e.g., *A. fulica*) and certain terrestrial slugs were collected from the surroundings of the villages, restaurants and markets in the capital town of the counties, and snail farms across the study area and examined for the presence of *A. cantonensis* larvae. Up to 100 specimens of each species were collected at each study site. The intermediate hosts were artificially digested using routine procedures (incubation in a solution containing 0.2% pepsin and 0.7% hydrochloric acid at 37°C for 2 h) (Lv *et al.*, 2006a). Additionally, for the examination of *P. canaliculata*, a recently developed method relying on specific lung tissue features of this species was employed (Liu *et al.*, 2007; Lv *et al.*, 2006a). In brief, the lungs were separated from the snail body and opened. The nodules containing *A. cantonensis* larvae were then directly observed under a microscope. Paratenic hosts were also collected from markets and restaurants and examined for $L_3$ using an artificial digestion method.

*A. cantonensis* larvae were identified based on distinct morphological criteria described elsewhere (Lv, 2006). For quality control purpose, larvae identified as *A. cantonensis* from ~10% of the foci where *A. cantonensis* is endemic were intragastrically injected into Sprague-Dawley (SD) rats, and maintained in the laboratory to confirm the identity of adult worms.

### *4.3.4. Analysis*

An area was considered *A. cantonensis* endemic if the parasite was detected in any kind of animals captured in the field. The geographic locations of these sampling sites were linked to an existing geographic information system (GIS), using the software ArcGIS version 9.1 (ESRI, USA). Subsequently, ordinary kriging, a statistical technique for spatial prediction (Waller and Gotway, 2004), was performed, and a smoothed risk map of the *A. cantonensis* infection prevalence in *P. canaliculata* was produced.

## 4.4. Results

### *4.4.1.* A. cantonensis *hosts and their infection status*

The first national survey pertaining to *A. cantonensis* in China was implemented in 164 counties belonging to 19 provinces. A detailed list of the surveyed locations is available from the corresponding author upon request. Various mollusks were collected, belonging to one of the three following groups: (i) freshwater snail, (ii) terrestrial snail, and (iii) terrestrial slug. All collected specimens were deposited in the "Preservation Center for Parasite Specimens in China" (http://www.psic.cn), and further details are available from this center upon request. Overall, 11,709 *P. canaliculata* were screened, 6.8% of which harbored $L_3$ of *A. cantonensis*. The prevalence among the other freshwater snails (a total of 7,287 specimens were examined) was only 0.05%. Of 3,549 *A. fulica* examined, 13.4% were infected. The infection prevalence among the 1,421 other terrestrial snail specimens was only 0.3%. Finally, 5,370 terrestrial slugs were dissected, revealing an infection prevalence of 6.5%. Hence, the endemicity of *A. cantonensis* in mainland China is primarily attributable to *P. canaliculata*, *A. fulica* and terrestrial slugs (Figure 4.1).

Of the 711 rodents trapped during the field surveys, 32 were found to be infected with *A. cantonensis* (31 *R. norvegicus* and one *R. flavipectus*; overall prevalence: 4.2%). None of the 46 insectivores (*Suncus murinus*) were infected. The 652 paratenic hosts collected during the survey included frogs, shrimps, crabs, toads and fish. No *A. cantonensis* was identified in any of these animals.

The prediction prevalence of *A. cantonensis* in *P. canaliculata* in China, using an ordinary kriging approach with a spherical model, highlighted two potential clusters with prevalences of 19-28% in Guangxi province and 28-40% in Fujian province, respectively (Figure 4.2).

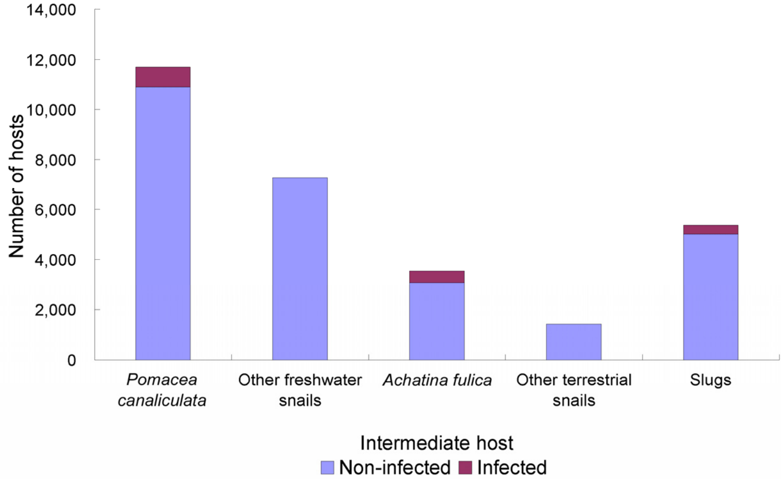

**Figure 4.1** Number and infection status of potential intermediate hosts of *Angiostrongylus cantonensis* examined during the first national survey on *A. cantonensis* in mainland China, 2006/2007.

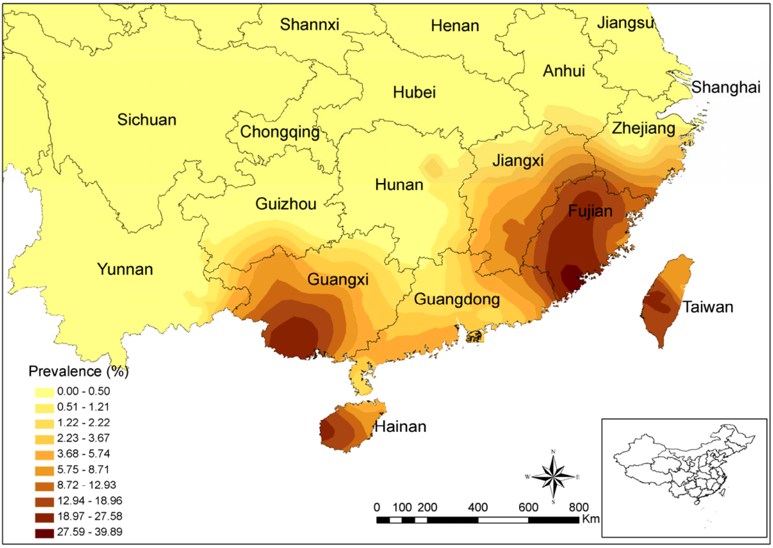

**Figure 4.2** Predicted *Angiostrongylus cantonensis* prevalence in *Pomacea canaliculata* in mainland China, 2006/2007. The map is made based on the current data of the prevalence of *A. cantonensis* in *P. canaliculata* using ordinary kriging and the predicted prevalence is classified into 10 categories by smart quantile.

### *4.4.2. Geographic distribution of* A. cantonensis *and its main hosts*

Figure 4.3 shows the current distribution of *A. cantonensis* at county level in China. The parasite was identified in 59 of the 164 surveyed counties (36.0%). Most of the *A. cantonensis*-endemic areas were linked to infections in *P. canaliculata* and/or *A. fulica* snails. Only in three counties infected rats were found, but the presence of the parasite in intermediate hosts could not be detected. Seven provinces in southeastern China (i.e., Hainan, Guangdong, Guangxi, Fujian, Jiangxi, Hunan, and Zhejiang) were identified as *A. cantonensis* endemic.

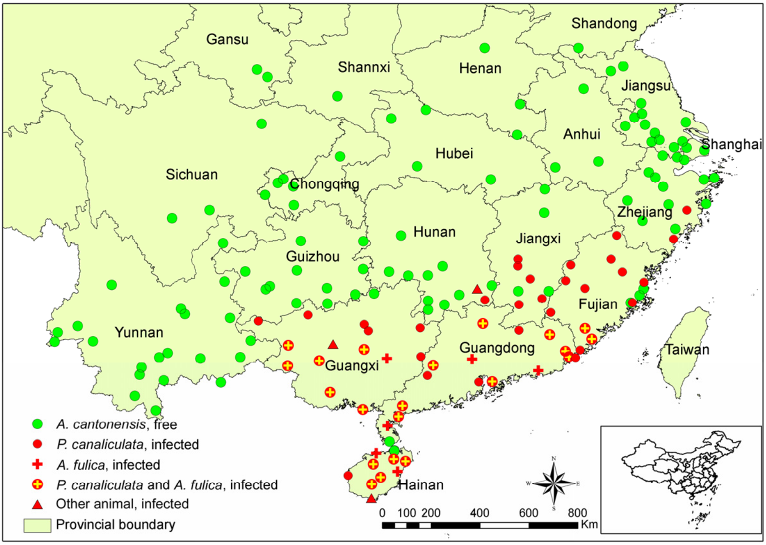

**Figure 4.3** Occurrence of *Angiostrongylus cantonensis* according to infections in hosts in mainland China, 2006/2007. Each point represents a county where the national survey was conducted. All the field sites are located in the region limited to north latitude 18°13'-34°50' and east longitude 97°50'- 122°07'. The two invasive species, i.e., *Pomacea canaliculata* and *Achatina fulica*, are highlighted in this map, and the infection status of other animals was omitted when infected invasive snails were found in the same places.

*P. canaliculata* was introduced in Zhongshan city, Guangdong province in 1981 (Joshi and Sebastian, 2006). As shown in Figure 4.4, *P. canaliculata* is now well established in southern China in a band spanning northeast-southwest. A separate endemic area is located in the Sichuan basin. The snail now colonizes almost the entire Pearl River valley, the Southern River system and the Southeast River system. The snail has also been observed in mountainous areas at high elevations in Yunnan province. Moreover, *P. canaliculata* snails have crossed from the Pearl River valley to the Yangtze River valley, and currently also inhabit the southeast section of the latter.

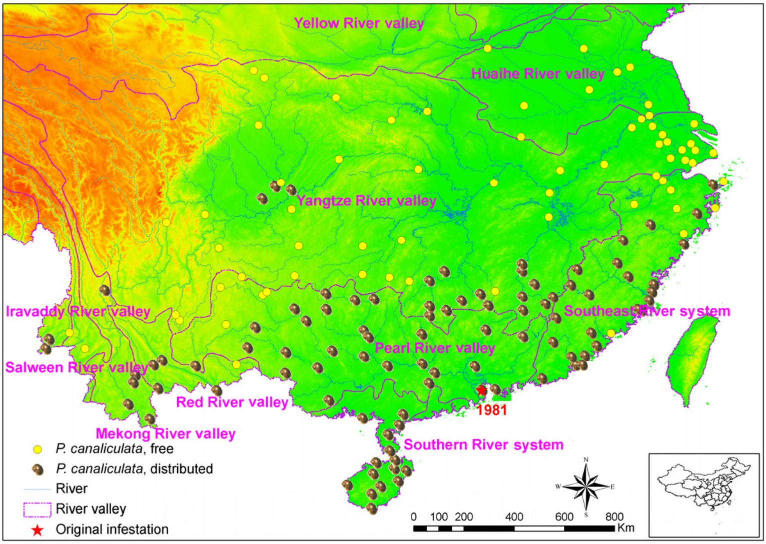

**Figure 4.4** Distribution of *Pomacea canaliculata* in mainland China, 2006/2007. All the field sites are located in the region limited to north latitude 18°13'- 34°50' and east longitude 97°50'- 122°07'. Also shown are the terrain and the waterways along with the point of introduction of the aquatic snail (Zhongshan city; red star in this map). The terrain type is shown in color spectrum the green indicates lowest elevation or plains and the red indicates highest elevation or mountains.

Figure 4.5 shows that *A. fulica* has a more focal distribution than *P. canaliculata*, although the former species had been introduced into China half a century earlier than the latter. At present, *A. fulica* is known to occur in the provinces of Guangdong, Hainan, and Guangxi, in the southern areas of Yunnan and Fujian provinces, and in one county of Guizhou province. Unlike *P. canaliculata*, *A. fulica* occurs only south of 25° N latitude, and does not appear to be associated with major river networks.

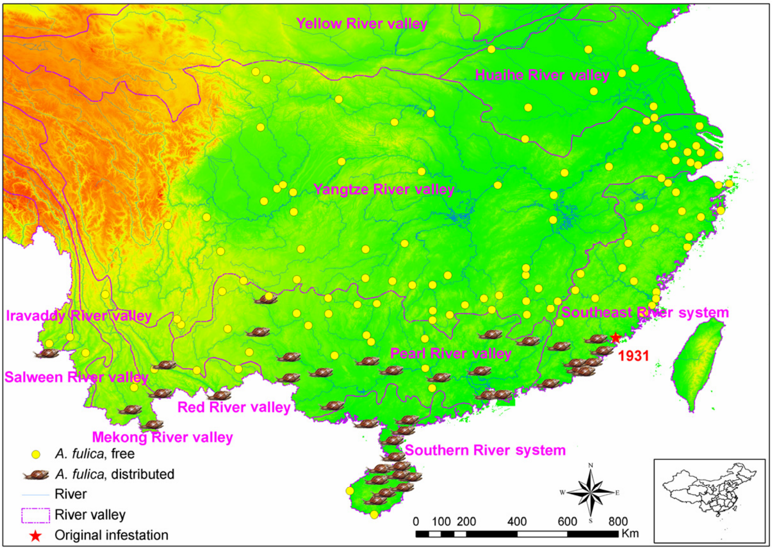

**Figure 4.5** Distribution of *Achatina fulica* in mainland China, 2006/2007. All the field sites are located in the region limited to north latitude 18°13’- 34°50’ and east longitude 97°50’- 122°07’. The terrain and the waterways along with the point of introduction of the terrestrial *A. fulica* (Xiamen; red star indicated) are presented in this map. The terrain type is shown in color spectrum the green indicates lowest elevation or plains and the red indicates highest elevation or mountains.

### *4.4.3. Intermediate host snails on markets and restaurants*

*P. canaliculata* snails were found on markets and/or restaurants in 21 counties, whereas *A. fulica* snails were detected in three counties only. Infected *P. canaliculata* and *A. fulica* were found in nine and two counties, respectively. Additionally, two native freshwater snail species, i.e., *B. aeruginosa* and *Cipangopaludina chinensis*, were commonly found to be on sale on many markets and restaurants. *C. chinensis* is one of the key intermediate hosts of *A. cantonensis* in Taiwan and, in the current survey, infected specimens were detected in a restaurant in Fujian province. To our knowledge, this is the first report of *A. cantonensis*-infected *C. chinensis* from mainland China. On two markets in Guangdong and Guangxi provinces, infected *B. aeruginosa* snails were detected.

#### *4.4.4. Commercial snail farming*

Only two commercial snail farms for *P. canaliculata* (located in Jiangsu and Jiangxi provinces) and one for *A. fulica* (located in Zhejiang province) were identified during this survey. None of the snails collected in these farms contained *A. cantonensis*.

### 4.5. Discussion

Eosinophilic meningitis caused by *A. cantonensis* is endemic in Southeast Asia, Australia, the Pacific Islands and the Caribbean. To date, more than 2800 human cases have been reported (Wang et al., 2008). It had been speculated that the parasite dispersed from East Asia to other regions in two important hosts, i.e., rats and *A. fulica*, especially during the second world war (Kliks and Palumbo, 1992). However, the parasite is still expanding its range and the associated disease is emerging in some regions, including China (Caldeira *et al.*, 2007; Lv *et al.*, 2008; Prociv and Carlisle, 2001; Prociv *et al.*, 2000; Senanayake *et al.*, 2003).

The results of the first national survey on the distribution of *A. cantonensis* and its hosts in China reported here indicate that there is a need for strengthening food safety inspections and food-borne disease surveillance. Long-distance trade, biological invasion and animal migration are contributing to the emergence of new diseases and the re-emergence of diseases that have previously been controlled (Enserink, 2007; Kilpatrick *et al.*, 2006; McMichael and Beaglehole, 2000; Rappole *et al.*, 2000). Angiostrongyliasis in mainland China is an example of an emerging food-borne disease. Its spread can be linked to the introduction, farming and consumption of certain snail species. The incidence of angiostrongyliasis is likely to further increase in China, although the 2006 outbreak in Beijing triggered considerable attention and a change in attitude toward this parasitic infection not only in the medical and research community, but also the general public.

The results of the national survey can be summarized as follows. First, the *A. cantonensis*-endemic area is very wide, covering seven southern provinces. Not a single snail or rat infected with *A. cantonensis* was found in Yunnan province. This observation is surprising, since the parasite was first documented in Yunnan some 20 years ago (Li et al., 1989), and several outbreaks have occurred subsequently (Han *et al.*, 2005; Wei *et al.*, 2005), most recently in Dali (early 2008). Hence, Yunnan must clearly be considered a potentially endemic province.

Second, several freshwater and terrestrial snail species were found on local markets and restaurants, and *A. cantonensis*-infected *P. canaliculata* and *A. fulica* clearly destined for human consumption were recorded. This observation suggests that the transmission of *A. cantonensis* to humans is ongoing, and that the health education and awareness raising campaigns initiated after the 2006 outbreak in Beijing – targeting consumers, health personnel and officials alike – must be improved since they appeared to have failed yet to stop the sale and consumption of infected snails. It follows that the impact of the previous health education campaigns through mass media to change human behavior has probably been overestimated, because angiostrongyliasis outbreaks repeated in Guangdong province in 2007 (Deng et al., 2007) and Yunnan province in early-2008, involving six and 41 patients, respectively.

Third, culturally-routed dietary habits of certain ethnic groups increase the risk of *A. cantonensis* infection. For example, the consumption of raw or undercooked freshwater snails is held responsible for the early-2008 angiostrongyliasis outbreak in Dali. As a direct consequence of the booming inland tourism in China, the interest in minority dishes is growing, and ethnic dining has become popular among tourists and wealthy urban residents alike. Travelers to endemic regions with a tradition of preparing snails for human consumption should be better informed about the risks associated with certain dishes, and food inspection and hygiene regulations need to be enforced.

Fourth, among the different factors facilitating the spread and transmission of *A. cantonensis* in China, the two invasive mollusk species *P. canaliculata* and *A. fulica* play a central role. A range of mollusks which can serve as intermediate hosts of *A. cantonensis* were examined during the national survey. The prevalence of *A. cantonensis* infection among *P. canaliculata*, *A. fulica* and terrestrial slugs was shown to govern the endemicity in China. However, terrestrial slugs were rarely found to be associated with human angiostrongyliasis. The only exception is their occasional use in local traditional medicine (Cross, 1987; Li *et al.*, 2006; Liu *et al.*, 2006). Thus, *P. canaliculata* and *A. fulica* are responsible for most angiostrongyliasis cases. Both snails not only expand their range, but also frequently go on the table for human consumption.

Interestingly, *P. canaliculata* and *A. fulica* have facilitated the spread of *A. cantonensis* rather than the introduction of a new pathogen. Man-made ecological transformations and climate change are important drivers of the spread of exotic species and their establishment in new areas (Bardsley and Edwards-Jones, 2007; McMichael and Beaglehole, 2000; Stachowicz *et al.*, 2002). The emergence of several infectious diseases has been attributed to the invasion of efficient vectors or hosts involved in their life cycle (Juliano and Lounibos, 2005). The two invasive snail species impact on the endemicity and transmission of *A. cantonensis* in at least two ways. First, the invasion of these snails facilitates the establishment of life cycle of the parasite and thereby increases the exposure opportunity of native mollusks to *A. cantonensis* in existing endemic areas. Previous experiment indeed documented a significantly higher susceptibility to *A. cantonensis* than that of native snails (Lv *et al.*, 2006b). Second, these invasive snails accelerate the spread of *A. cantonensis* since they expand their range, resulting in the local establishment of the snail and – sometimes – the parasite life cycle in previously non-endemic areas.

*A. fulica* was recorded for the first time in mainland China in 1931 (Jarreit, 1931). It has been suggested that eggs of *A. fulica* were accidentally imported from Singapore with shipments of plants, and that an initial snail population established itself in Xiamen (Amoy) (Mead, 1961). These terrestrial snails are nocturnal and become active under high-humidity conditions (Barker, 2002). The snails feed on plants and deposit their eggs in the soil nearby. This behavior facilitates their dispersal through long-distance transportation of pot plants (Mead, 1961). Since their unintentional introduction, *A. fulica* spreads across southern China, probably facilitated by the rapid expansion of long-distance trade and an increasing demand for farmed plants going hand-in-hand with China's ongoing economic development. It has also been speculated that *A. fulica* invaded China more than once. For example, the snail populations in Yunnan province might derive from trade with Indochina (Mekong basin) rather than eastern China (Chen et al., 1996). The public health significance of *A. fulica* in mainland China was only noted when a parasitologically-confirmed case of angiostrongyliasis found in 1984 could be linked to this snail (He et al., 1984). However, the consumption of *A. fulica* snails is less popular than that of *P. canaliculata* in mainland China.

The freshwater snail *P. canaliculata* was deliberately introduced into China for human consumption. The invasion process can be stratified into three stages, i.e., (i) introduction, (ii) establishment, and (iii) spread (Jeschke and Strayer, 2005). It was first imported into Zhongshan city in Guangdong province ~30 years ago (Lv et al., 2008). Subsequently, the snail was farmed in most southern provinces with commercial aims (Joshi and Sebastian, 2006). However, within a few years, the snail also became established outside due to abandoning of farms and deliberate release (Joshi and Sebastian, 2006). Now the snails have reached at north latitude of 30° and highest elevation of 1960 m in Yunan province. It is conceivable that the dense river networks in eastern and southern China contributed to the dispersal of this snail. The isolated snail population in the Sichuan basin has expanded freely in this area for about 20 years. The easternmost natural colonies were observed in Zhoushan in Zhejiang province, suggesting a line from Zhoushan to the Sichuan basin south of which climate conditions are suitable for the snails to thrive. This line might move further northward as a consequence of global warming (Yang et al., 2006). The public health significance of *P. canaliculata* was emphasized by the first major angiostrongyliasis outbreak in Wenzhou in 1997 (Zheng et al., 2001). The results of the national survey presented here suggest a close relationship between the endemicity of *A. cantonensis* and the area where *P. canaliculata* breed two or even three generations per year (Zhou et al., 2003), suggesting that *A. cantonensis* largely depends on this freshwater snail. Although *P. canaliculata* in the whole endemic area of *A. cantonensis* can be infected, point prevalences of infection are heterogeneous: two heavily endemic areas were identified in the provinces of Fujian and Guangxi. The snail is responsible for many sporadic cases recorded in Fujian province. However, it remains to be investigated why no angiostrongyliasis cases have been observed in Guangxi province.

Although both *A. fulica* and *P. canaliculata* appear to have contributed to the emergence of angiostrongyliasis in China, several characteristics of *P. canaliculata* suggest that this species is more important. This claim is justified as follows. First, the aquatic *P. canaliculata* probably spread along waterways which can be accelerated through flooding events. This might partly explain why *P. canaliculata* more rapidly expanded its range than *A. fulica*, which depends on human-facilitated transport. Second, the area colonized by *P. canaliculata* also expands far beyond that of *A. fulica* despite a considerably longer presence of the latter in China. Third, the consumption of *P. canaliculata* is more popular than that of *A. fulica*. During the national survey, for example, *P. canaliculata* was on sale in 21 counties, while *A. fulica* was only noted in three counties.

The national survey shed light on different important aspects regarding the distribution of *A. cantonensis* and its hosts in China. The results indicate a need for more pointed attention to this emerging threat through awareness-raising campaigns among the medical community, the establishment of a hospital-based sentinel surveillance system, improved community-based health education and strengthening of food safety inspection. A number of pressing research questions were also identified. For example, the model for predicting the prevalence of *A. cantonensis* in *P. canaliculata* identified two high-prevalence clusters. However, the accuracy of this prediction has not been assessed since no ground truthing of the predictions have been made thus far. The small-scale distribution, the range of hosts and the actual points of *A. cantonensis* transmission between its natural hosts should also be investigated since in several locations, infections could not be demonstrated in each member of the transmission cycle.

In conclusion, the first national survey revealed the distribution of *A. cantonensis* and two invasive snail species, i.e., *P. canaliculata* and *A. fulica*, and the pivotal role of these invasive snails in transmission of this parasite. The results of the survey also suggest that people are still at risk of angiostongyliasis because the snails and even infected were found on many markets and restaurants. Continued health education, rigorous food inspection and hospital-based surveillance are needed to prevent recurrent angiostrongyliasis outbreaks in China.

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# 5. The complete mitochondrial genome of the rodent intra-arterial nematodes *Angiostrongylus cantonensis* and *A. costaricensis*

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### 5.1. Abstract

**Background**: The two rodent intra-arterial nematodes, *Angiostrongylus cantonensis* and *A. costaricensis*, can cause human ill-health. A mitochondrial (mt) genome-wide analysis between different species of *Angiostrongylus* might aid in the identification of markers for population genetic studies and new targets for diagnosis, drugs and vaccines, as well as clarify their phylogenetic relationship and the position in the phylum of Nematoda.

**Results:** The complete mt genomes of *A. cantonensis* and *A. costaricensis* are 13,497 bp and 13,585 bp in length, respectively. Hence, they are the smallest in the class of Chromadorea characterized thus far. Nucleotide identity of the two mt genomes is 81.6%, ranging from 77.7% to 87.1% in individual gene pairs. Similar codon usage pattern was found; UUU, UUG, UUA, GUU, UAU, and AUU identifed as dominant codons. Our mt genome-wide analysis identified three major gene arrangement patterns (II-1, 2, and 3) from 48 nematode mt genomes. Pattern II-1 includes all nematode species in the order Strongylida, some Rhabditida, and a single member of the Diplogasterida. Pattern II-2 covered Ascaridida and only differed from II-1 in the location of adenine and thymine (AT)-rich region. Both patterns II-1 and II-2 are distinct from pattern II-3, which covers the Spirurida, supporting a closer relationship between Ascaridida and Strongylida rather than Spirurida. Thymine was highly concentrated on coding strands in Chromadorea, but balanced between the two strands in Enoplea, probably due to the gene arrangement pattern.

**Conclusion:** The complete mt genome of *A. cantonensis* and *A. costaricensis* show considerable variation in both the full sequences and specific genes. Our findings are important for population genetic studies and target identification. Gene arrangement pattern of mt genomes as well as phylogenetic analysis based on concatenated amino acid indicated a closer relationship between the order Ascaridida and Rhabditida rather than Spirurida as indicated in previous study, which raised a consideration pertaining to biomarker in phylogenetic studies of nematode.

**Keywords**: mitochondrial genome, *Angiostrongylus cantonensis*, *Angiostrongylus costaricensis*, nematode, phylogenetic analysis

## 5.2. Introduction

Rodent *Angiostrongylus* belong to the superfamily Metastrongyloidea of the phylum of Nematoda. They parasitize rodents and are located in the bronchioles (e.g., *Angiostrongylus andersoni*), pulmonary arteries (e.g., *A. cantonensis*, *A. mackerrasae*, and *A. malaysiensis*), or mesenteric arteries (e.g., *A. costaricensis* and *A. siamensis*) (Anderson, 2000). As an exception in the bursate group, *Angiostrongylus* spp. require intermediate hosts to complete their life cycle (Anderson, 2000). Terrestrial mollusks, such as snails and slugs, normally play this role, but some freshwater snails have been found to be particularly important for the transmission of angiostrongyliasis (Lv et al., 2009; Lv et al., 2008; Morley, 2006). Most of them are highly specific with regard to their definitive rodent host species and even intermediate mollusk hosts, yet cross infections have been observed (Lv et al., 2008). Hence, the distribution of angiostrongyliasis is dependent on the distribution of definitive and intermediate host species. With regard to *A. cantonensis*, it is commonly believed that this species originated from Southeast Asia from where it subsequently spread over tropical regions following biological invasion of their suitable definitive hosts *Rattus norvegicus* and *R. rattus* (Kliks and Palumbo, 1992; Prociv et al., 2000).

Among the many species of rodent intra-arterial nematode described thus far, only *A. cantonensis* and *A. costaricensis* are known to cause human ill-health. The former species is implicated in eosinophilic meningitis, whereas the latter can cause granulomatous inflammation of intestinal wall (Kramer et al., 1998; Lv et al., 2010; Wang et al., 2008). An infection in human is acquired primarily via consumption of undercooked snails or foodstuff contaminated with infective mollusks (Lv et al., 2010). Several studies reported that *A. cantonensis* not only infects humans but also wildlife, such as primates, flying foxes, and birds. Hence, *A. cantonensis* is a potential threat to wildlife (Barrett et al., 2002; Duffy et al., 2004; Gelis et al., 2011; Kim et al., 2002; Monks et al., 2005). Regarding *A. costaricensis*, this species can also parasitize primates (Miller et al., 2006). Interestingly, *A. mackerrasae* (endemic in Australia) and *A. malaysiensis* (endemic in Malaysia) virtually have the same life cycles like *A. cantonensis*, whereas *A. siamensis* in Thailand shares a very similar life cycle to that of *A. costaricensis*. However, neither *A. mackerrasae*, nor *A. malaysiensis*, nor *A. siamensis* has been reported to be involved in human and wildlife infections. At present, the diagnosis of an *Angiostrongylus* infection in humans and animals is most often based on morphological characteristics of adult worms or larvae. However, several *Angiostrongylus* species show similar morphology and migration route in the hosts, and hence differential diagnosis is a challenge. Indeed, here is a need for a more accurate diagnosis and molecular approaches might play a role to readily distinguish closely related species and different isolates.

Genetic markers derived from mitochondrial (mt) DNA hold promise for molecular diagnosis. The rapid mutation rate of mt DNA compared to nuclear DNA render the former a promising genetic marker to distinguish various clades or species at a lower taxonomic level, which might explain their frequent use in population genetic and diagnostic studies (Blouin et al., 1998; Gissi et al., 2008). An mt genome-wide analysis between different *Angiostrongylus* species is therefore needed not only for the identification of suitable genetic marker population genetics and diagnosis, but also for clarifying their phylogenetic relationship from a molecular point of view.

The aims of the current study were (i) to sequence the mt genomes of *A. cantonensis* and *A. costaricensis* and (ii) to compare these mt genomes with other species of the phylum of Nematoda. A deeper understanding of the phylogenetic relationship of nematodes provides potential genetic markers for subsequent population genetics studies and the identification of new targets for diagnosis, drugs and vaccines.

## 5.3. Materials and methods

### *5.3.1. Parasites and DNA extraction*

*A. cantonensis* was obtained from Lijiang, Fujian province in People's Republic of China (P.R. China). The nematode was maintained in the laboratory of the National Institute of Parasitic Diseases (Shanghai, P.R. China). Adult worms were recovered from the pulmonary arteries of an infected Sprague-Dawley (SD) rat. *A. costaricensis*, obtained from Santa Rosa, Rio Grande do Sul in Brazil, was maintained in the laboratory cultured in the laboratory of the Instituto de Pesquisas Biomédicas da PUCRS (Porto Alegre, Brazil). Adult worms were recovered from mesenteric arteries of rodents and kept in pure alcohol.

From both species, a single female worm was used. Specimens were washed several times with physiological saline. Total genomic DNA was extracted from nematodes using sodium dodecyl-sulphate/proteinase K treatment (Gasser et al., 1993). The individual worm was put into a 2.5 ml tube with 500 µl extraction buffer and homogenized with a polytron. The tubes were incubated at 37 °C overnight. The suspension was then centrifuged for 60 sec at 10,000 ×*g* and the supernatant transferred to a clean tube and extracted with phenol/chloroform/isoamyl alcohol (v:v:v = 25:24:1). The aqueous phase was precipitated with a double volume of absolute ethanol and centrifuged for 3 min at 10,000 ×*g*. The DNA pellet was suspended in 50 µl $H_2O$ and kept at -20 °C.

### *5.3.2. PCR amplification and sequencing*

The primers were designed according to the conversed sequences of currently available mt genomes, i.e., those of *Ancylostoma duodenale* [NC_003415], *N. americanus* [NC_003416], and *Cooperia oncophora* [NC_004806], which are close relatives of *Angiostrongylus* spp. according to conventional classification. Some of the primers used for *A. costaricensis* were designed based on sequenced mt genome of *A. cantonensis*. All adjacent fragments overlapped. PCR cycling conditions used were 94 °C for 10 min, and then 35 cycles with 94 °C for 60 sec, around 48 °C for 90 sec, and 72 °C for 90 sec, followed by 72 °C for 10 min for the final extension. Each PCR reaction yielded a single band detected in a 1% agarose gel upon ethidium-bromide staining. PCR products were recovered from the gel using mini-spin columns (Axygen). Purified PCR products were ligated into pGEM®-T Easy vectors with the LigaFast ligation system (Promega). The plasmid vector with the target fragment was transformed into JM109 or DH5α *Escherichia coli*, following the manufacturer's instructions. The positive clones were sequenced with the dideoxynucleotide termination method.

### *5.3.3. Sequence analyses*

The sequences were assembled and edited using the Vector NTI package (version 9.1, Invitrogen). The proteins encoding genes were identified using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) set for the invertebrate mt genetic codes. The initiation and termination codons were determined by comparison with the corresponding sequences of *A. duodenale*, *N. americanus*, and *C. oncophora*. Two ribosomal RNA (*rrn*) genes were identified by comparison to other nematode mt genomes. Transfer RNA (*trn*) genes in the mt genome of *A. cantonensis* were identified using the tRNA scan program (http://www.queensu.ca/micr/faculty/ kropinski/online.html) (Lowe and Eddy, 1997), whereas two *trn*S genes were recognized by their potential to be folded into *trn*-like secondary structures and by their anticodon sequences. Secondary structures of tRNAs and rRNAs were edited using RNAviz (De Rijk and De Wachter, 1997). The stem-loop structures of non-coding mt regions were inferred using the web Mfold program (http://bioweb2.pasteur.fr/nucleic/intro-en.html) (Zuker, 2003). The AT-rich region was determined using the "Tandem Repeats Finder" program (http://tandem.bu.edu/trf/trf.html) (Benson, 1999).

The arrangement of genes in the nematode mt genome was compared among all nematodes whose mt genome sequences have been determined and made publicly available. A+T content were compared among all nematodes whose mt genomes are available.

### *5.3.4. Phylogenetic analysis*

For the phylogenetic analysis, 46 nematode mt genomes available from GenBank were used, in addition to the complete mtDNA sequences of *A. cantonensis* and *A. costaricensis* determined in this study. These mtDNA sequences were: *Agamermis* spp. BH-2006, *A. caninum*, *A. duodenale*, *Anisakis simplex*, *A. suum*, *Brugia malayi*, *Bunostomum phlebotomum*, *C. briggsae*, *C. elegans*, *Chabertia ovina*, *C. quiscali*, *Contracaecum rudolphii*, *C. oncophora*, *Cylicocyclus insignis*, *Dirofilaria immitis*, *E. vermicularis*, *H. contortus*, *H. bacteriophora*, *Hexamermis agrotis*, *M. digitatus*, *M. pudendotectus*, *M. salmi*, *N. americanus*, *Oesophagostomum dentatum*, *O. quadrispinulatum*, *O. volvulus*, *P. pacificus*, *R. similis*, *Romanomermis culicivorax*, *R. iyengari*, *R. nielseni*, *Setaria digitata*, *S. carpocapsae*, *Strelkovimermis spiculatus*, *Strongylus vulgaris*, *S. stercoralis*, *Syngamus trachea*, *Teladorsagia circumcincta*, *T. cosgrovei*, *T. canis*, *T. cati*, *T. malaysiensis*, *T. spiralis*, *Trichostrongylus axei*, *T. vitrinus*, and *X. americanum*.

The amino acid sequences encoded by 12 protein coding genes from each species were individually concatenated and subjected to alignment using ClustalX. Conversed blocks were selected for phylogenetic analysis using the G-blocks website service (Castresana, 2000) (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The molecular phylogeny was reconstructed based on Bayesian inference using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The posterior probabilities were calculated using Metropolis-coupled Markov chain Monte Carlo simulations. The consensus tree was drawn in TreeView version 1.6.6.

## 5.4. Results and discussion

### *5.4.1. Mitochondrial genome of* A. cantonensis *and* A. costaricensis

The complete mt genome of *A. cantonensis* (GQ398121) and *A. costaricensis* (GQ398122) were sequenced based on genetic material isolated from single female worms. Both mt genomes are circular with 13,497 bp and 13,585 bp, respectively. These two mt genomes are the smallest thus far characterized in the class of Chromadorea. Indeed, the average size of the mt genome of the 39 Chromadorea nematode species for which sequence data are currently available is 14.16±0.55 kb. The respective size is 18.80±3.23 kb for the nine Enoplea nematodes. Unlike *Xiphinema americanum*, which lacks several tRNA genes (He et al., 2005), *A. cantonensis* and *A. costaricensis* possess the same mt gene content as other nematodes, except for *Trichinella spiralis*, which has a unique *atp8* gene (Lavrov and Brown, 2001). Specifically, the mt genomes of the two *Angiostrongylus* species sequenced here contain 12 protein-coding genes (*atp6*, *cox1-3*, *cytb*, *nad1-6*, and *nad4L*), two ribosomal RNA genes (*rrnS* and *rrnL*), and 22 tRNA genes. Moreover, the mt genomes have the same gene arrangement pattern and all genes are transcribed in the same direction (Figure 5.1). There are two major non-coding regions (NCR) in both mt genomes. The shorter NCR is located between *nad4* and *cox1*, while the longer NCR (adenine plus thymine (A+T)-rich region or putative control region) is located between tRNA-Ala and tRNA-Pro genes.

The overall identity between the mt genomes of *A. cantonensis* and *A. costaricensis* was 81.6%, with a considerable range (from 77.7% for *nad6* to 87.1% for *rrnS*) observed for protein-coding and rRNA genes (Table 5.1). Consistent with previous studies (Hu et al., 2002; Jex et al., 2009; Li et al., 2008), our findings showed a similar identity pattern in protein-coding and rRNA genes. For example, the genes *cox1-3* and *rrnS* showed higher identity among family members, whereas *nad2*, *nad4*, and *nad6* showed higher variation. Hence, the genes *nad2*, *nad4*, and *nad6* could be used as markers in population genetic studies of *A. cantonensis*. However, it should be note that the comparison of the mt genome of different isolates of the hookworm species *Nector americanus* indicated a different pattern at intraspecific level: *nad1* and *nad3* showed higher difference than other protein-coding genes (Hu et al., 2003a). It follows that the final candidate marker for population genetics of *A. cantonensis* should ideally be determined based on the comparison of mt genome of different strains or isolates. Nonetheless, the analysis of the two close *Angiostrongylus* relatives in our view provides helpful information for the identification of candidate biomarkers.

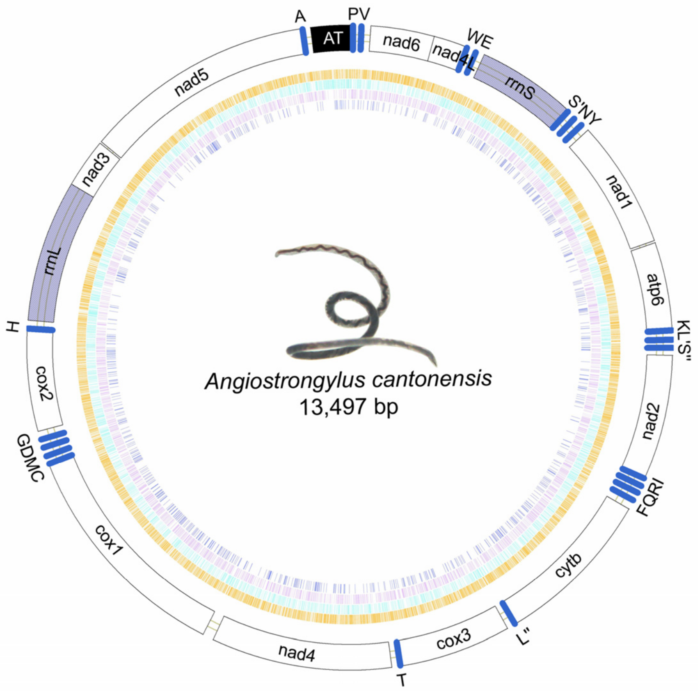


**Figure 5.1** The consensus structure of the mitochondrial (mt) genome based on that of *Angiostrongylus cantonensis*. Each tRNA gene was indicated by the amino acid code according to international union of pure and applied chemistry (IUPAC). The two leucine genes were annotated by L'(UAA) and L"(UAG), and the two serine genes by S'(UGA) and S"(UCU). "AT" with black background denotes A+T-rich region. All the genes are located on the same strand and transcribed in the same direction (clockwise).

**Table 5.1** Comparison of protein-coding genes and ribosomal RNA genes of *Angiostrongylus cantonensis* and *A. costaricensis*

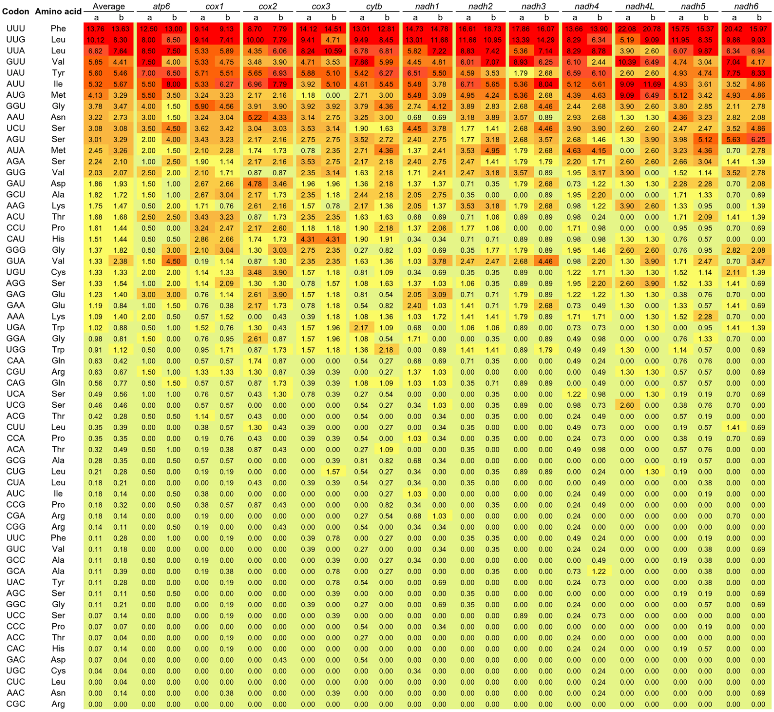
| Gene | *A. cantonensis* | | *A. costaricensis* | | Identity |
|---|---|---|---|---|---|
| | Ini/Ter* | Length | Ini/Ter* | Length | |
| *atp6* | ATT/TAG | 600 | ATT/TAG | 600 | 0.80 |
| *cox1* | ATT/TAG | 1578 | ATT/TAA | 1578 | 0.86 |
| *cox2* | TTG/TAG | 693 | TTG/TAA | 693 | 0.86 |
| *cox3* | TTG/T | 766 | TTG/T | 766 | 0.84 |
| *cytb* | TTG/TAA | 1110 | ATG/TAG | 1101 | 0.81 |
| *nad1* | TTG/TAG | 876 | TTG/TAG | 873 | 0.82 |
| *nad2* | TTG/TAG | 849 | TTG/TAA | 849 | 0.79 |
| *nad3* | TTG/TAG | 336 | TTG/TAG | 336 | 0.80 |
| *nad4* | TTG/TAG | 1230 | TTG/TAA | 1230 | 0.79 |
| *nad4L* | ATT/T | 232 | ATT/T | 232 | 0.84 |
| *nad5* | ATA/T | 1582 | ATA/T | 1582 | 0.81 |
| *nad6* | ATG/TAG | 426 | ATG/TAG | 432 | 0.78 |
| *rrnL* | | 961 | | 966 | 0.82 |
| *rrnS* | | 696 | | 695 | 0.87 |

* Ini and Ter denote initiation and termination codon, respectively.

The length of each mt gene pair of *A. cantonensis* and *A. costaricensis* was similar; a small difference could be identified in *cytb*, *nad1*, *nad6*, as well as the two rRNA genes. Initiation codons were the same for each gene pair except for *cytb*, for which the initiation codon was TTG for *A. cantonensis* but ATG for *A. costaricensis*. However, termination codon usage showed considerable variation; a difference was found in *cox1*, *cox2*, *nad2*, *nad4*, and *cytb*. For the first four genes, *A. cantonensis* employed TAG as termination codon, while *A. costaricensis* utilized TAA. A reversed termination codon usage occurred in the *cytb* gene. As has been observed for other nematodes (Hu et al., 2003a; Li et al., 2008), *A. cantonensis* and *A. costaricensis* utilized truncated termination codons, like those observed in *cox3*, *nad4L*, and *nad5*.

A considerable bias of codon usage (frequency and relative synonymous codon usage) in protein-coding genes in the mt genome of *A. cantonensis* and *A. costaricensis* was identified (Figure 5.2). Overall, UUU (Phe), UUG (Leu), UUA (Leu), GUU (Val), UAU

(Tyr) and AUU (Ile) are dominant codons, which is similar to other nematode species (Hu et al., 2002, , 2003b; Kang et al., 2009; Lavrov and Brown, 2001; Montiel et al., 2006). However, the proportion of these codons showed a marked variation in different genes. For example, UUU (Phe) exceeded 20% in *nad4L* in both mt genomes, but was below 10% in *cox1* and *cox2*. Pairwise comparison of specific genes between these two genomes showed a similar codon usage, whereas distinctive differences were observed in some gene pairs. For instance, *A. cantonensis* uses the codon GUU to encode valine, whereas *A. costaricensis* uses codons GUU and GUA, at the same proportion to encode valine.



| Codon | Amino acid | Average |  | *atp6* |  | *cox1* |  | *cox2* |  | *cox3* |  | *cytb* |  | *nadh1* |  | *nadh2* |  | *nadh3* |  | *nadh4* |  | *nadh4L* |  | *nadh5* |  | *nadh6* |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b |
| UUU | Phe | 13.76 | 13.63 | 12.50 | 13.00 | 9.14 | 9.13 | 8.70 | 7.79 | 14.12 | 14.51 | 13.01 | 12.81 | 14.73 | 14.78 | 16.61 | 18.73 | 17.86 | 16.07 | 13.66 | 13.90 | 22.08 | 20.78 | 15.75 | 15.37 | 20.42 | 15.97 |
| UUG | Leu | 10.12 | 8.30 | 8.00 | 6.50 | 9.14 | 7.41 | 10.00 | 7.79 | 9.41 | 4.71 | 9.49 | 8.45 | 13.01 | 11.68 | 11.66 | 10.95 | 13.39 | 14.29 | 8.29 | 6.34 | 5.19 | 9.09 | 11.95 | 8.35 | 9.86 | 9.03 |
| UUA | Leu | 6.62 | 7.64 | 8.50 | 7.50 | 5.33 | 5.89 | 4.35 | 6.06 | 8.24 | 10.59 | 6.78 | 6.81 | 5.82 | 7.22 | 8.83 | 7.42 | 5.36 | 7.14 | 8.29 | 8.78 | 3.90 | 2.60 | 6.07 | 9.87 | 6.34 | 6.94 |
| GUU | Val | 5.85 | 4.41 | 7.50 | 4.00 | 5.33 | 4.75 | 3.48 | 3.90 | 4.71 | 3.53 | 7.86 | 5.99 | 4.45 | 4.81 | 6.01 | 7.07 | 8.93 | 6.25 | 6.10 | 2.44 | 10.39 | 6.49 | 4.74 | 3.04 | 7.04 | 4.17 |
| UAU | Tyr | 5.60 | 5.46 | 7.00 | 6.50 | 5.71 | 5.51 | 5.65 | 6.93 | 5.88 | 5.10 | 5.42 | 6.27 | 6.51 | 5.50 | 4.59 | 3.53 | 1.79 | 2.68 | 6.59 | 6.10 | 2.60 | 2.60 | 4.93 | 4.74 | 7.75 | 8.33 |
| AUU | Ile | 5.32 | 5.67 | 5.50 | 8.00 | 5.33 | 6.27 | 6.96 | 7.79 | 3.92 | 5.10 | 4.61 | 5.45 | 5.48 | 3.78 | 6.71 | 5.65 | 5.36 | 8.04 | 5.12 | 5.61 | 9.09 | 11.69 | 4.93 | 3.61 | 3.52 | 4.86 |
| AUG | Met | 4.13 | 3.29 | 5.50 | 3.50 | 3.24 | 3.23 | 2.17 | 2.16 | 1.18 | 0.00 | 2.71 | 3.00 | 5.48 | 3.09 | 4.95 | 4.24 | 5.36 | 2.68 | 4.39 | 4.63 | 9.09 | 6.49 | 5.12 | 3.42 | 4.93 | 4.86 |
| GGU | Gly | 3.78 | 3.47 | 4.00 | 1.50 | 5.90 | 4.56 | 3.91 | 3.90 | 3.92 | 3.92 | 3.79 | 4.36 | 2.74 | 4.12 | 3.89 | 2.83 | 2.68 | 4.46 | 2.44 | 2.68 | 3.90 | 2.60 | 3.80 | 2.85 | 2.11 | 2.78 |
| AAU | Asn | 3.22 | 2.73 | 3.00 | 1.50 | 3.24 | 3.04 | 5.22 | 4.33 | 3.14 | 2.75 | 3.25 | 3.00 | 0.68 | 0.69 | 3.18 | 3.89 | 3.57 | 0.89 | 2.93 | 2.68 | 1.30 | 1.30 | 4.36 | 3.23 | 2.82 | 2.08 |
| UCU | Ser | 3.08 | 3.08 | 3.50 | 4.50 | 3.62 | 3.42 | 3.04 | 3.03 | 3.53 | 3.14 | 1.90 | 1.63 | 4.45 | 3.78 | 1.77 | 1.41 | 2.68 | 4.46 | 3.90 | 3.90 | 2.60 | 2.60 | 2.47 | 2.47 | 3.52 | 4.86 |
| AGU | Ser | 3.01 | 3.29 | 2.00 | 4.00 | 3.43 | 3.23 | 2.17 | 2.16 | 2.75 | 2.75 | 3.52 | 2.72 | 2.40 | 2.75 | 1.77 | 3.18 | 2.68 | 3.57 | 2.68 | 1.46 | 1.30 | 3.90 | 3.98 | 5.12 | 5.63 | 6.25 |
| AUA | Met | 2.45 | 3.26 | 2.00 | 1.50 | 2.10 | 2.28 | 1.74 | 1.73 | 0.78 | 2.35 | 2.71 | 4.36 | 1.37 | 2.41 | 3.53 | 4.95 | 1.79 | 2.68 | 4.63 | 4.15 | 0.00 | 2.60 | 3.23 | 4.36 | 0.70 | 2.78 |
| AGA | Ser | 2.24 | 2.10 | 1.00 | 2.50 | 1.90 | 1.14 | 2.17 | 2.16 | 3.53 | 2.75 | 2.17 | 2.18 | 2.40 | 2.75 | 2.47 | 1.41 | 1.79 | 1.79 | 2.20 | 1.71 | 2.60 | 2.60 | 2.66 | 3.04 | 1.41 | 1.39 |
| GUG | Val | 2.03 | 2.07 | 2.50 | 2.00 | 2.10 | 1.71 | 0.87 | 0.87 | 2.35 | 3.14 | 1.63 | 2.18 | 1.71 | 2.41 | 2.47 | 3.18 | 3.57 | 0.89 | 1.95 | 3.17 | 3.90 | 0.00 | 1.52 | 1.14 | 3.52 | 2.78 |
| GAU | Asp | 1.86 | 1.93 | 1.50 | 1.00 | 2.67 | 2.66 | 4.78 | 3.46 | 1.96 | 1.96 | 1.36 | 2.18 | 1.37 | 1.37 | 0.71 | 0.35 | 1.79 | 2.68 | 0.73 | 1.22 | 1.30 | 1.30 | 2.28 | 2.28 | 0.70 | 2.08 |
| GCU | Ala | 1.82 | 1.72 | 1.50 | 1.00 | 2.67 | 3.04 | 2.17 | 1.73 | 2.35 | 1.18 | 2.44 | 2.18 | 2.05 | 2.75 | 0.35 | 0.00 | 0.00 | 0.89 | 1.95 | 2.20 | 0.00 | 0.00 | 1.71 | 1.33 | 0.70 | 0.69 |
| AAG | Lys | 1.75 | 1.47 | 0.50 | 2.00 | 1.71 | 0.76 | 2.61 | 2.16 | 1.57 | 0.78 | 2.17 | 1.36 | 2.05 | 1.37 | 3.53 | 3.18 | 1.79 | 2.68 | 0.98 | 1.22 | 3.90 | 2.60 | 1.33 | 0.95 | 0.00 | 1.39 |
| ACU | Thr | 1.68 | 1.68 | 2.50 | 2.50 | 3.43 | 3.23 | 0.87 | 1.73 | 2.35 | 2.35 | 1.63 | 1.63 | 0.68 | 0.69 | 0.71 | 1.06 | 0.89 | 0.89 | 0.98 | 0.24 | 0.00 | 0.00 | 1.71 | 2.09 | 1.41 | 1.39 |
| CCU | Pro | 1.61 | 1.44 | 0.50 | 0.00 | 3.24 | 2.47 | 2.17 | 2.60 | 1.18 | 1.18 | 1.90 | 2.18 | 1.37 | 2.06 | 1.77 | 1.06 | 0.00 | 0.00 | 1.71 | 0.98 | 0.00 | 0.00 | 0.95 | 0.95 | 0.70 | 0.69 |
| CAU | His | 1.51 | 1.44 | 0.50 | 0.50 | 2.86 | 2.66 | 1.74 | 1.73 | 4.31 | 4.31 | 1.90 | 1.91 | 0.34 | 0.34 | 0.71 | 0.71 | 0.89 | 0.89 | 0.98 | 0.98 | 1.30 | 1.30 | 0.76 | 0.57 | 0.00 | 0.00 |
| GGG | Gly | 1.37 | 1.82 | 0.50 | 3.00 | 2.10 | 3.04 | 1.30 | 3.03 | 2.75 | 2.35 | 0.27 | 0.82 | 1.03 | 0.69 | 0.35 | 1.77 | 1.79 | 0.89 | 1.95 | 1.46 | 2.60 | 2.60 | 0.76 | 0.95 | 2.82 | 2.08 |
| GUA | Val | 1.33 | 2.38 | 1.50 | 4.50 | 0.19 | 1.14 | 0.87 | 1.30 | 2.35 | 2.35 | 1.63 | 1.36 | 1.03 | 3.78 | 2.47 | 2.47 | 2.68 | 4.46 | 0.98 | 2.20 | 1.30 | 3.90 | 1.71 | 2.47 | 0.70 | 3.47 |
| UGU | Cys | 1.33 | 1.33 | 2.00 | 2.00 | 1.14 | 1.33 | 3.48 | 3.90 | 1.57 | 1.18 | 0.81 | 1.09 | 0.34 | 0.69 | 0.35 | 0.35 | 0.89 | 0.00 | 1.22 | 1.71 | 1.30 | 1.30 | 1.52 | 1.14 | 2.11 | 1.39 |
| AGG | Ser | 1.33 | 1.54 | 1.00 | 2.00 | 1.14 | 2.09 | 1.30 | 1.30 | 0.78 | 1.57 | 1.08 | 1.63 | 1.37 | 1.03 | 1.06 | 0.35 | 0.89 | 0.89 | 1.95 | 2.20 | 2.60 | 3.90 | 1.52 | 1.33 | 1.41 | 0.69 |
| GAG | Glu | 1.23 | 1.40 | 3.00 | 3.00 | 0.76 | 1.14 | 2.61 | 3.90 | 1.57 | 1.18 | 0.81 | 0.54 | 2.05 | 3.09 | 0.71 | 0.71 | 1.79 | 0.89 | 1.22 | 1.22 | 1.30 | 1.30 | 0.38 | 0.76 | 0.70 | 0.00 |
| GAA | Glu | 1.19 | 0.84 | 1.00 | 1.50 | 0.76 | 0.38 | 2.17 | 1.73 | 0.78 | 1.18 | 0.54 | 0.82 | 2.40 | 1.03 | 1.41 | 0.71 | 1.79 | 2.68 | 0.73 | 0.49 | 1.30 | 0.00 | 1.33 | 0.57 | 1.41 | 0.69 |
| AAA | Lys | 1.09 | 1.40 | 2.00 | 0.50 | 0.57 | 1.52 | 0.00 | 0.43 | 0.39 | 1.18 | 1.08 | 1.36 | 1.03 | 1.72 | 1.41 | 1.41 | 1.79 | 0.89 | 1.71 | 1.71 | 0.00 | 1.30 | 1.52 | 2.28 | 0.70 | 0.00 |
| UGA | Trp | 1.02 | 0.88 | 0.50 | 1.00 | 1.52 | 0.76 | 1.30 | 0.43 | 1.57 | 1.96 | 2.17 | 1.09 | 0.68 | 0.00 | 1.06 | 1.06 | 0.89 | 0.00 | 0.73 | 0.73 | 0.00 | 1.30 | 0.00 | 0.95 | 1.41 | 1.39 |
| GGA | Gly | 0.98 | 0.81 | 1.50 | 0.00 | 0.76 | 0.95 | 2.61 | 0.87 | 1.57 | 1.96 | 1.08 | 0.54 | 1.71 | 0.00 | 0.00 | 0.35 | 0.00 | 0.89 | 0.49 | 0.98 | 0.00 | 0.00 | 0.76 | 1.33 | 0.70 | 0.00 |
| UGG | Trp | 0.91 | 1.12 | 0.50 | 0.00 | 0.95 | 1.71 | 0.87 | 1.73 | 1.57 | 1.18 | 1.36 | 2.18 | 0.00 | 0.69 | 1.41 | 1.41 | 0.89 | 1.79 | 0.49 | 0.49 | 1.30 | 0.00 | 1.14 | 0.57 | 0.00 | 0.69 |
| CAA | Gln | 0.63 | 0.42 | 1.00 | 0.00 | 0.57 | 0.57 | 1.74 | 0.87 | 0.00 | 0.00 | 0.54 | 0.27 | 0.68 | 0.69 | 0.71 | 0.35 | 0.00 | 0.00 | 0.49 | 0.24 | 0.00 | 0.00 | 0.76 | 0.76 | 0.00 | 0.00 |
| CGU | Arg | 0.63 | 0.67 | 1.50 | 1.00 | 1.33 | 1.33 | 1.30 | 0.87 | 0.39 | 0.39 | 0.00 | 0.27 | 1.37 | 1.03 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.49 | 1.30 | 1.30 | 0.57 | 0.57 | 0.00 | 0.69 |
| CAG | Gln | 0.56 | 0.77 | 0.50 | 1.50 | 0.57 | 0.57 | 0.87 | 1.73 | 0.39 | 0.39 | 1.08 | 1.09 | 1.03 | 1.03 | 0.35 | 0.71 | 0.89 | 0.89 | 0.00 | 0.49 | 0.00 | 0.00 | 0.57 | 0.57 | 0.00 | 0.00 |
| UCA | Ser | 0.49 | 0.56 | 1.00 | 1.00 | 0.76 | 0.57 | 0.43 | 1.30 | 0.78 | 0.39 | 0.27 | 0.54 | 0.00 | 0.00 | 0.00 | 0.35 | 0.00 | 0.00 | 1.22 | 0.98 | 0.00 | 1.30 | 0.19 | 0.19 | 0.70 | 0.69 |
| UCG | Ser | 0.46 | 0.46 | 0.00 | 0.00 | 0.57 | 0.57 | 0.00 | 0.00 | 0.00 | 0.00 | 0.54 | 0.27 | 0.34 | 1.03 | 0.00 | 0.35 | 0.89 | 0.00 | 0.98 | 0.73 | 2.60 | 0.00 | 0.38 | 0.76 | 0.70 | 0.00 |
| ACG | Thr | 0.42 | 0.28 | 0.50 | 0.50 | 1.14 | 0.57 | 0.43 | 0.00 | 0.00 | 0.00 | 0.54 | 0.00 | 0.00 | 0.34 | 0.00 | 0.35 | 0.00 | 0.00 | 0.24 | 0.49 | 0.00 | 0.00 | 0.57 | 0.19 | 0.00 | 0.00 |
| CUU | Leu | 0.35 | 0.39 | 0.00 | 0.00 | 0.38 | 0.57 | 1.30 | 0.43 | 0.39 | 0.00 | 0.00 | 0.27 | 0.00 | 0.34 | 0.35 | 0.00 | 0.00 | 0.00 | 0.49 | 0.73 | 0.00 | 0.00 | 0.19 | 0.57 | 1.41 | 0.69 |
| CCA | Pro | 0.35 | 0.35 | 0.00 | 0.00 | 0.19 | 0.76 | 0.43 | 0.00 | 0.39 | 0.39 | 0.54 | 0.00 | 1.03 | 0.34 | 0.00 | 0.35 | 0.00 | 0.00 | 0.24 | 0.73 | 0.00 | 0.00 | 0.38 | 0.19 | 0.70 | 0.69 |
| ACA | Thr | 0.32 | 0.49 | 0.50 | 1.00 | 0.19 | 0.38 | 0.87 | 0.43 | 0.00 | 0.00 | 0.27 | 1.09 | 0.00 | 0.00 | 0.35 | 0.00 | 0.00 | 0.00 | 0.49 | 0.98 | 0.00 | 0.00 | 0.57 | 0.76 | 0.00 | 0.00 |
| GCG | Ala | 0.28 | 0.35 | 0.00 | 0.50 | 0.57 | 0.57 | 0.00 | 0.00 | 0.00 | 0.39 | 0.81 | 0.82 | 0.68 | 0.34 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.19 | 0.57 | 0.00 | 0.00 |
| CUG | Leu | 0.21 | 0.28 | 0.50 | 0.00 | 0.19 | 0.19 | 0.00 | 0.00 | 0.00 | 1.57 | 0.54 | 0.27 | 0.34 | 0.00 | 0.00 | 0.35 | 0.89 | 0.89 | 0.00 | 0.24 | 0.00 | 1.30 | 0.19 | 0.00 | 0.00 | 0.00 |
| CUA | Leu | 0.18 | 0.21 | 0.00 | 0.00 | 0.00 | 0.19 | 0.43 | 0.00 | 0.39 | 0.39 | 0.54 | 0.27 | 0.34 | 0.00 | 0.35 | 0.00 | 0.00 | 0.00 | 0.00 | 0.49 | 0.00 | 0.00 | 0.00 | 0.38 | 0.00 | 0.00 |
| AUC | Ile | 0.18 | 0.14 | 0.00 | 0.50 | 0.38 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.27 | 1.03 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.49 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 |
| CCG | Pro | 0.18 | 0.32 | 0.00 | 0.50 | 0.38 | 0.57 | 0.87 | 0.43 | 0.00 | 0.00 | 0.00 | 0.82 | 0.34 | 0.00 | 0.00 | 0.35 | 0.00 | 0.00 | 0.24 | 0.49 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| CGA | Arg | 0.18 | 0.14 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.27 | 0.54 | 0.68 | 1.03 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.70 | 0.00 |
| CGG | Arg | 0.14 | 0.11 | 0.00 | 0.50 | 0.19 | 0.00 | 0.00 | 0.43 | 0.00 | 0.00 | 0.54 | 0.00 | 0.34 | 0.34 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| UUC | Phe | 0.11 | 0.28 | 0.00 | 1.00 | 0.00 | 0.19 | 0.00 | 0.00 | 0.39 | 0.78 | 0.00 | 0.27 | 0.00 | 0.00 | 0.35 | 0.35 | 0.00 | 0.00 | 0.49 | 0.24 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 |
| GUC | Val | 0.11 | 0.18 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.39 | 0.00 | 0.27 | 0.00 | 0.00 | 0.34 | 0.00 | 0.35 | 0.00 | 0.00 | 0.24 | 0.24 | 0.00 | 0.00 | 0.00 | 0.38 | 0.00 | 0.69 |
| GCC | Ala | 0.11 | 0.18 | 0.50 | 0.00 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.39 | 0.00 | 0.27 | 0.34 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.49 | 0.00 | 0.00 | 0.19 | 0.38 | 0.00 | 0.00 |
| GCA | Ala | 0.11 | 0.39 | 0.00 | 0.00 | 0.19 | 0.38 | 0.00 | 0.00 | 0.00 | 0.78 | 0.00 | 0.27 | 0.00 | 0.00 | 0.00 | 0.35 | 0.00 | 0.00 | 0.73 | 1.22 | 0.00 | 0.00 | 0.00 | 0.38 | 0.00 | 0.00 |
| UAC | Tyr | 0.11 | 0.28 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 | 0.00 | 0.78 | 0.54 | 0.00 | 0.00 | 0.69 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.73 | 0.00 | 0.00 | 0.00 | 0.38 | 0.00 | 0.00 |
| AGC | Ser | 0.11 | 0.11 | 0.50 | 0.50 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.39 | 0.00 | 0.00 | 0.00 | 0.00 | 0.35 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.19 | 0.19 | 0.00 | 0.69 |
| GGC | Gly | 0.11 | 0.21 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 | 0.39 | 0.00 | 0.27 | 0.00 | 0.00 | 0.69 | 0.35 | 0.00 | 0.00 | 0.00 | 0.24 | 0.00 | 0.00 | 0.00 | 0.00 | 0.57 | 0.00 | 0.69 |
| UCC | Ser | 0.07 | 0.14 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 | 0.00 | 0.39 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.89 | 0.00 | 0.24 | 0.73 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| CCC | Pro | 0.07 | 0.07 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.54 | 0.00 | 0.00 | 0.34 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 |
| ACC | Thr | 0.07 | 0.04 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.27 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| CAC | His | 0.07 | 0.14 | 0.00 | 0.00 | 0.00 | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.24 | 0.00 | 0.00 | 0.19 | 0.57 | 0.00 | 0.00 |
| GAC | Asp | 0.07 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.43 | 0.00 | 0.00 | 0.54 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| UGC | Cys | 0.04 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.34 | 0.00 | 0.34 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| CUC | Leu | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| AAC | Asn | 0.00 | 0.14 | 0.00 | 0.00 | 0.00 | 0.38 | 0.00 | 0.00 | 0.00 | 0.39 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.24 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.69 |
| CGC | Arg | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

**Figure 5.2** The frequency of codons in protein-coding genes of mitochondrial (mt) genomes of *Angiostrongylus cantonensis* and *A. costaricensis*. "a" and "b" in the title row denote *A. cantonensis* and *A. costaricensis*, respectively. The color from light green (low frequency) to red (high frequency) indicates different frequencies of codon usage.

Twenty and 18 tRNA genes were identified by the scan program in the mt genome of *A. cantonensis* and *A. costaricensis*, respectively (Table 5.2). The tRNA-Arg genes as well as two tRNA-Ser genes in both mt genomes failed to be identified. For *A. costaricensis* one more tRNA gene (tRNA-Val) was identified by eyes. Remarkably, one additional tRNA-Ile gene was found in the AT-rich region in the mt genome of *A. cantonensis* by the tRNA scan program. However, the tRNA lacks a typical secondary structure, the anticodon loop, i.e., one more base located in the loop. Furthermore, it possesses a high proportion of A+T (57/60). This pseudogene was similar to the tRNA-Ile identified in *Metastrongylus pudendotectus* (Jex et al., 2010).

**Table 5.2** tRNA anticodon and length of *Angiostrongylus cantonensis* and *A. costaricensis*

| tRNA | *A. cantonensis* | | *A. costaricensis* | |
|---|---|---|---|---|
| | Anticodon | Length | Anticodon | Length |
| Pro | UGG | 55 | UGG | 54 |
| Val | UAC | 54 | UAC* | 54 |
| Trp | UCA | 57 | UCA | 59 |
| Glu | UUC | 55 | UUC | 59 |
| Ser | UGA* | 53 | UGA* | 52 |
| Asn | GUU | 56 | GUU | 60 |
| Tyr | GUA | 60 | GUA | 55 |
| Lys | UUU | 61 | UUU | 60 |
| Leu | UAA | 56 | UAA | 56 |
| Ser | UCU* | 50 | UCU* | 52 |
| Ile | GAU | 57 | GAU | 54 |
| Arg | ACG* | 54 | ACG* | 51 |
| Gln | UUG | 56 | UUG | 55 |
| Phe | GAA | 56 | GAA | 57 |
| Leu | UAG | 56 | UAG | 56 |
| Thr | UGU | 58 | UGU | 58 |
| Cys | GCA | 57 | GCA | 56 |
| Met | CAU | 57 | CAU | 59 |
| Asp | GUC | 53 | GUC | 54 |
| Gly | UCC | 57 | UCC | 58 |
| His | GUG | 55 | GUG | 56 |
| Ala | UGC | 55 | UGC | 55 |
| Ile | GAU† | 60 | - | - |

* tRNAs failed to be identified by tRNAscan-SE program; instead was complied according to other complete mt genomes

† tRNA identified by tRNAscan-SE program, but lacked typical structure of anticodon loop

### *5.4.2. Comparison with other nematode mt genomes*

Analysis of a suite of 48 nematode mt genomes revealed a consistently high A+T content, yet variation between species was found to be considerable (Figure 5.3). *Radopholus similes*, commonly known as banana-root nematode, possesses the highest A+T content (85.4%). On the other band of the spectrum is *X. americanum* (American dagger nematode), which shows the lowest A+T content (66.5%). The variation in A+T content was observed across orders; it ranges from 73.2% (*A. costaricensis*) to 79.7% (*Mecistocirrus digitatus*) in the order Strongylida, from 75.6% (*Caenorhabditis briggsae*) to 76.7% (*Strongyloides stercoralis*) in the order Rhabditida, from 68.6% (*Toxocara canis*) to 72.0% (*Ascaris suum*) in the order Ascaridida, from 73.3% (*Onchocerca volvulus*) to 77.7% (*Chandlerella quiscali*) in the order Spirurida, and from 71.4% (*Thaumamermis cosgrovei*) to 80.5% (*Agamermis* spp.) in the order Mermithida. The single member *Enterobius vermicularis* (pinworm) in the order Oxyurida and *T. spiralis* in the order Trichocephalida have relatively smaller A+T contents, i.e., 71.2% and 67.0%, respectively. Indeed, a strong mutational bias toward A and T has been observed in nematode mt genes (Blouin et al., 1998).

In contrast to the positive skewing in A+T content in each nematode order, heterogeneity was observed in the ratio of A to T. Nematodes in the class Chromadorea, including the orders Strongylida, Diplogasterida, Ascaridida, Rhabditida, Oxyurida, Spirurida, and Tylenchida, without exception, had higher T than A. The percentage (T/(A+T)) ranges from 57.2% (*Haemonchus contortus*) to 74.2% (*Setaria digitata*) with a median of 64.7%. The highest percentages were found in the orders Ascaridida and Spirurida. Indeed, previous studies showed that substitution tended to be thymine in nematode mt genomes (Blouin et al., 1998; Nadler and Hudspeth, 2000). However, in the class Enoplea, consisting of the orders Dorylaimida, Trichocephalida, and Mermithida, the percentage approaches 50% and ranges between 39.6% (*T. spiralis*) and 55.1% (*T. cosgrovei*) with a median of 50.6%. This phenomenon could be explained by the transverse translocation of genes between two DNA strands in Enoplea, which might balance the proportion of A to T on both strands.

The 39 nematode species belonging to the Chromadorea possess a compact mt genome containing 36 genes without repeats. The genes were located on a single strand with the same transcriptional direction. With a few exceptions, e.g. *Heterorhabditis bacteriophora* and *R. similes*, Chromadorea nematodes rarely have long repeated or NCRs except the AT-rich region. In contrast to the constant gene content, the arrangement of genes showed a variation across orders. Three distinct patterns of gene arrangement (including major non-coding locality) were identified in these nematode mt genomes (Figure 5.3; II-1, 2, and 3). All nematodes in the order Strongylida fell into group II-1 and shared the same gene arrangement with the exception of *M. pudendotectus* in which the tRNA-Ile gene moved close to the AT-rich region. Interestingly, in our study one more tRNA-Ile gene was identified in the AT-rich region by the tRNA scan program. It is similar to the tRNA-Ile of *M. pudendotectus*. However, it can be excluded from the tRNA due to its inconsistency with the typical structure of the anticodon loop. Nevertheless, this finding indicates a high similarity between gene arrangement patterns among these members of the superfamily Metastrongyloidea and should be assigned to group II-1.

Additionally, two species (*Caenorhabditis elegans* and *C. briggsae*) in the order Rhabditida and the single species (*Pristionchus pacificus*) in the order Diplogasterida also share the pattern II-1. In contrast, three other members in the order Rhabditida, i.e., *Steinernema carpocapsae*, *H. bacteriophora*, and *S. stercoralis*, show distinct arrangement patterns. The gene arrangement of *S. carpocapsae* was a mediate between II-1 and II-2; namely, a location change of the AT-rich region and a tRNA-Asn gene, which might result in either II-1 or II-2. A few short linkages of genes in pattern II-1, e.g., fragments between *cox2* and *nad3*, between tRNA-Gln and *nad4*, between tRNA-Val and tRNA-Arg, could be detected in the mt genome of *H. bacteriophora*, while similar gene linkages have not been observed in *S. stercoralis*. In the order Ascaridida, all mt genomes showed the same arrangement pattern (II-2). The only difference between II-2 and II-1 were in the location of the AT-rich region. Unlike pattern II-2, II-3 was distinctively different from pattern II-1; only a few short gene linkages (2-5 genes) could be detected in both II-1 and II-3. Within the group II-3 the gene arrangement of *O. volvulus* and *C. quiscali* was slightly different from the other three species in the location of the five adjacent tRNA genes.

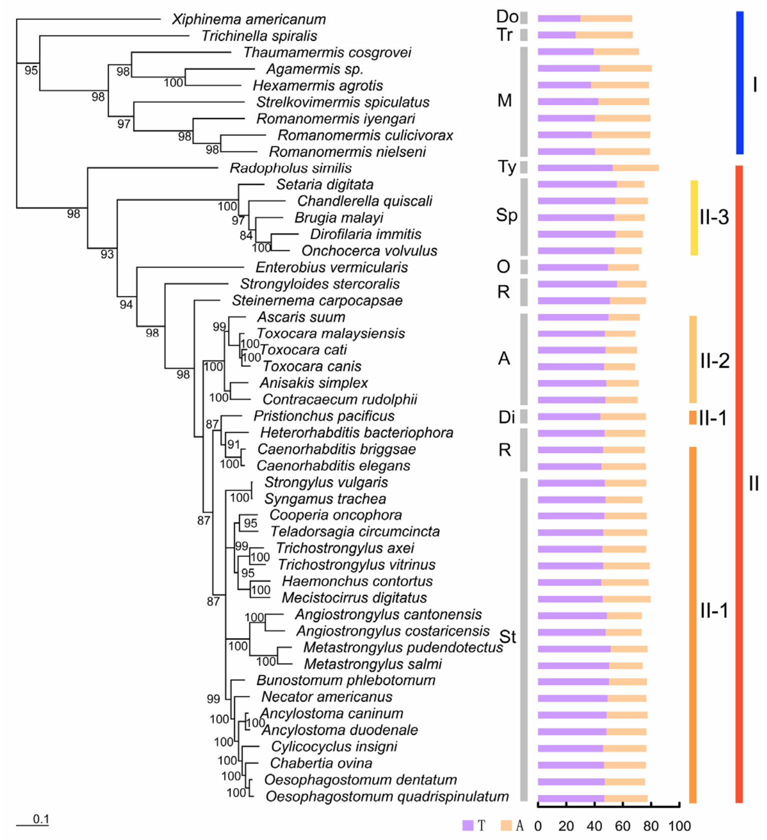


**Figure 5.3** Phylogenetic tree of nematode mitochondrial (mt) amino acid sequence based on Bayesian inference. The gray bars with capital letter indicate different order of nematode (Do, Dorylaimida; Tr, Trichocephalida; M, Mermithida; Ty, Tylenchida; Sp, Spirurida; O, Oxyurida; R, Rhabditida; A, Ascaridida; Di, Diplogasterida; St, Strongylida). The transverse bars denote the proportion of adenine (A) and thymine (T) in each nematode mt genome. The vertical color bars indicate the arrangement of mt genes (I, gene located on both light and heavy strands; II, gene located on heavy strands; II-1, 2, 3 indicate the group in which the members share a similar gene arrangement, respectively). The posterior probability (as a percentage) is indicated on branch lines.

In contrast, the genes of the mt genomes belonging to the class of Enoplea were allocated to both strands. Among the seven members of Mermithida duplication or repeats of genes were common. Interestingly, no duplication or repeats were observed in the other two orders (Dorylaimida and Trichocephalida). Instead, *T. spiralis* (Trichocephalida) showed a unique *atp8* gene (Lavrov and Brown, 2001), whereas *X. americanum* (Dorylaimida) lacked the tRNA-Asn, tRNA-Cyr, and one of two tRNA-Ser genes when compared to the other nematode species (He et al., 2005). Unlike species belonging to Chromadorea, all Enoplea nematodes, even within family, had distinct gene arrangement patterns and lacked detectable similarity.

### *5.4.3. Phylogenetic analysis*

Figure 5.3 shows a phylogenetic tree, which was constructed based on the concatenated amino acid sequence consisting of 2266 amino acids according to G-block, which effectively distinguished the orders from each other with the exception of *S. carpocapsae* and *S. stercoralis* that conventionally have been classified as belonging to the order Rhabditida, but were far away from other members. Indeed, the phylogeny of Rhabditida is the most complex in the phylum Nematoda. It might be paraphyletic, as indicated by previous studies (Blaxter et al., 1998). In addition to the phylogenetic analysis based on the amino acid sequence, gene arrangement pattern further support this hypothesis. *H. bacteriophora* was placed in the same clade with *C. elegans* and *C. briggsae*, but has a different gene arrangement pattern. In constrast, *P. pacificus*, which is a member of the order Diplogasterida, showed the same pattern as *C. elegans* and *C. briggsae*, although there was a suppressor tRNA located in the D-loop (Molnar et al., 2011(in press)). Indeed, our phylogenetic analysis indicated that *P. pacificus* is genetically close to the order Rhabditida. The unconsistent findings from the phylogenetic analysis and gene arrangement patterns highlight that there is a need for further studies pertaining to *H. bacteriophora*. The trophic niche (*H. bacteriophora* is entomopathogen and *Caenorhabditis* spp. is bacteriovore) should be considered when further pursuing this scientific inquiry.

Previous studies based on the nuclear small subunit ribosomal DNA (SSU) sequence indicated a close relationship between Ascaridida and Spirurida (Blaxter et al., 1998; Meldal et al., 2007). However, findings from the present study along with results from several recent investigations (Jex et al., 2009; Kang et al., 2009; Kim et al., 2006) pertaining to nematode mt genome analysis indicate that Ascaridida had a closer relationship with Rhabditida instead. Furthermore, gene arrangement patterns were more similar between Ascaridida and Rhabditida rather than Spirurida. We also employed the method of maximum parsimony used in previous studies to restructure the phylogeny (data not shown) but failed to significantly change the topology based on Bayesian inference. We also note that some studies indeed implied a potential conflict in phylogeny based on nuclear and mitochondrial DNA (Shaw, 2002), although most studies had shown a similar phylogenetic relationship. Nevertheless, few conflicts were noted at higher taxonomic level. Therefore, the position of the order Ascaridida in phylogenetic tree should be reappraised.

### 5.5. Conclusion

The complete mt genomes of the two rodent intra-arterial nematodes that can cause human (and wildlife) ill-health, *A. cantonensis* and *A. costaricensis* represent the smallest mt genomes characterized thus far in the class of Chromadorea. The gene content of these two mt genomes, however, is consistent with other species in this class. A mt-genome-wide comparison revealed that the mt genomes of *Angiostrongylus* showed considerable variation in different genes, which might provide a basis for identifying markers for population genetic studies and targets for development of novel diagnostic arrays, drugs and vaccines. The comparison between 48 mt genomes of nematode species showed different A+T content and gene arrangement, which along with phylogenetic analysis using concatenated amino acid, support a closer relationship between Ascaridida and Rhabditida rather than Spirurida, as suggested by previous studies using nuclear genes. This apparent inconsistence calls for a reappraisal pertaining to the position of the order Ascaridida in phylogenetic relationship.

### Authors' contributions

SL had the main responsibility for this investigation, including designing the study, collecting specimen from field sites, laboratory work, data analysis, and drafting the manuscript. YZ, HXL, and LH collected the specimen of *A. cantonensis* in P.R. China and maintained the life cycle in the laboratory. LZ, QL, and FRW provided laboratory support. GGT provided the specimen of *A. costaricensis* from Brazil. SL, PS, CGT, XNZ, and JU drafted and revised the manuscript.

## 5.6. Acknowledgement

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# 6. Genetic diversity of *Angiostrongylus cantonensis* in its presumptive home range, and potential scenarios for its spread

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## 6.1. Abstract

The rat lungworm *Angiostrongylus cantonensis*, a major cause of human eosinophilic meningitis, was first discovered in southern People's Republic of China (P.R. China) in the early 1930s. To date, the genetic diversity and geographic distribution of *A. cantonensis* in its presumptive "home range" are still poorly understood. We analyzed the mitochondrial *nad1* gene sequences of *A. cantonensis* from 32 collection sites in known endemic areas in southern P.R. China and one isolate from Thailand. A total of 73 haplotypes were identified from 143 sequences that can be grouped into 7 distinct clades. In contrast to the obvious divergence between clades, the differentiation within clade was marginal. A biased substitution pattern between clades was observed on coding strand; 62.6% of substitutions occurred between adenine (A) and guanine (G). The potential invasion routes of clades were inferred based on network analysis. Our findings argue an Asian origin of *A. cantonensis* from a genetics point of view and provide a genetic basis for investigation of the global spread pattern of *A. cantonensis*.

**Keywords**: genetic diversity, *Angiostrongylus cantonensis*, mitochondrial DNA, *nad1* gene, mutation pattern, network analysis, spread

## 6.2. Introduction

The rat lungworm *Angiostrongylus cantonensis* is the major cause of human eosinophilic meningitis, particularly in tropical and subtropical regions *(Graeff-Teixeira et al., 2009; Lv et al.*, 2010). This parasite was first discovered in the pulmonary arteries of rats collected in Guangzhou (formerly known as Canton) in the People's Republic of China (P.R. China) in 1933, and hence was named *Pulmonema cantonensis* (Chen, 1935). In the second half of the 1930s, the same species was also described in Taiwan and was named *Haemostrongylus ratti* (Matsumoto, 1937; Yokogawa, 1937). Therefore, the south of P.R. China is considered the presumptive home range of *A. cantonensis* (Kliks and Palumbo, 1992).

Over the past decades, *A. cantonensis* spread beyond Southeast Asia, probably facilitated by long-distance dispersal of infected rats (*Rattus norvegicus* and *R. rattus*) and African land snails (*Achatina fulica*) (Kliks and Palumbo, 1992). The Pacific War and other human activities are considered the main drivers for the dispersal of *A. cantonensis*. Indeed, since the early 1960s, many countries in the Pacific Ocean basin and the Caribbean region have become invaded. Current studies suggest that the geographic range of *A. cantonensis* is still expanding (Archer *et al.*, 2011; Chikweto *et al.*, 2009; Foronda *et al.*, 2010; Maldonado *et al.*, 2010; Pincay *et al.*, 2009). Several hypotheses were forwarded to explain the global spread of *A. cantonensis* (Kliks and Palumbo, 1992), yet the exact dispersal routes remain to be elucidated. Phylogeographical studies can help to shed new light on distribution patterns, and hence potential dispersal routes. However, only few studies have been conducted thus far and all relied on a limited number of isolates (Eamsobhana *et al.*, 2010a, b; Liu *et al.*, 2011b). Importantly, detailed investigations regarding the population genetic structure of *A. cantonensis* in its presumptive home range have not yet been performed.

Moreover, genetic studies can reveal the intraspecific differentiation and thus are helpful to determine feasibility of developing sensitive tools to diagnose *Angiostrongylus* species and isolates. Two other rat lungworm species, i.e., *A. mackerrasae* in Australia and *A. malaysiensis* in Malaysia, virtually sharing the same life cycle with *A. cantonensis*, including migration route in definitive hosts, are not yet confirmed to be implicated in human and wildlife eosinophilic meningitis. These two rat lungworms showed higher specificity to definitive hosts than *A. cantonensis* (Lv *et al.*, 2008; Prociv *et al.*, 2000) and are normally found in native rat species (Stokes *et al.*, 2007). Indeed, *A. cantonensis* is more frequently discovered in the global invasive species *R. norvegicus* and *R. rattus*. However, most current diagnoses are based on morphological characteristics. The similarity in morphology of three closely related *Angiostrongylus* species probably hindered making an accurate diagnosis, and thus the importance of *A. mackerrasae* and *A. malaysiensis* might be underestimated. Recently, molecular techniques were developed to detect the larvae of *A. cantonensis* from mollusks (Caldeira *et al.*, 2003; Liu *et al.*, 2011a; Qvarnstrom *et al.*, 2010; Qvarnstrom *et al.*, 2007), but failed to take into account the differentiation among these three species.

Here, we studied the genetic diversity of *A. cantonensis* using a part of the mitochondrial (mt) gene *nad1* (i) to assess whether there is intraspecific differentiation in the presumptive home range; (ii) if any, to determine the potential pattern of differentiation; and (iii) to determine the geographic distribution of haplotypes. Finally, we speculate on the likely transmission dynamics of *A. cantonensis* using network analysis.

## 6.3. Methods

### *6.3.1.* A. cantonensis *specimen and total DNA preparation*

*A. cantonensis* samples were obtained through the first national survey pertaining to angiostrongyliasis in the mainland of P.R. China which had been performed in 2006 and 2007 (Lv *et al.*, 2009). Some of adult *A. cantonensis* specimens were directly collected from the pulmonary arteries and heart of wild rats. The others were recovered from Sprague-Dawley rats maintained in the laboratory. These rats had been experimentally infected with *A. cantonensis* $L_3$ larvae, isolated from mollusks collected in the field. *A. cantonensis* specimens from Thailand were kindly provided by Dr. Praphathip Eamsobhana from Mahidol University, using the Flinders Technology Associates (FTA) card (Eamsobhana *et al.*, 2010a). Adult worms were primarily identified by morphology and kept in 100% ethanol after recovery from pulmonary arteries of laboratory rats.

*A. cantonensis* specimens were individually washed three times using phosphate buffered saline (PBS) and placed into clean 1.5 ml tubes. Worms were cut into small pieces and incubated with sodium dodecyl-sulphate/proteinase K at 56°C for 4-6 hours (Gasser *et al.*, 1993). The suspension was centrifuged and the supernatant transferred into another tube and extracted with phenol/chloroform/isoamyl alcohol (v:v:v = 25:24:1). The DNA pellet was suspended in 30-50 µl $H_2O$ and kept at -20°C pending analysis.

### *6.3.2. PCR and sequencing*

The primers targeting the mt gene *nad1* were designed according to the complete mt genome of *A. cantonensis* (GQ398121). The forward primer was 5'-GATTTAGTTATTCTTGTTG-3' and the reverse primer was 5'-CCAACAAAAACACATCTAAC-3'. PCR cycling conditions were as follows: 94°C for 5 min, 35 cycles at 94°C for 60 sec, around 48°C for 60 sec, and 72°C for 60 sec, followed by 72°C for 10 min for the final extension. PCR products were recovered from the gel over mini-spin columns (Axygen). The purified PCR products were ligated into pGEM$^{®}$-T Easy vectors with the LigaFast ligation system (Promega). The plasmid vector with the target fragment was transformed into JM109 or DH5α *Escherichia coli*, according to the manufacturer’s instructions. Positive clones were then subjected to sequencing using the dideoxynucleotide termination method. All fragments were determined by double-strand sequencing. All unique mutations were carefully checked and ambiguous bases due to alignment of two sequencings were confirmed by cycling sequencing.

### *6.3.3. Network and phylogenetic analysis*

All sequences were aligned using ClustalX and trimmed using BioEdit version 7. The truncated sequences were then presented to DanSP version 5 to collapse into unique haplotypes, which were then be subjected to a network analysis using Network version 4.6 to produce the shortest tree without consideration of aligned gaps. The representative sequences from each clade according to this network analysis were used to construct a phylogenetic tree by Bayesian inference and, independently, Neighbor-Joining. Bayesian inference was conducted in MrBayes version 3.1 with sampling every 10 generations among a total of 100,000 generations. Neighbor-Joining estimate was performed in Mega version 3.1 with bootstrap testing (1000 replicates). The posterior probability and bootstrap values were recorded to assess relative reliability of clade divergence.

### *6.3.4. Mutation analysis*

Polymorphic sites and DNA polymorphism were analyzed by DnaSP version 5.0. The haplotype diversity (Hd), and nucleotide diversity (Pi) were estimated. A hierarchical analysis of the *nad1* mutation pattern was conducted at intra-clade, intraspecific, and interspecific levels. In the intra-clade analysis, all variable sites of the sequences of one clade, including insert/delete sites, were considered. The mutation direction was defined from dominant bases to rare ones. For intraspecific analysis, only parsimony informative sites were considered to avoid inference from intra-clade variation. Additionally, mutation patterns were also studied between different nematode species of Metastrongyloidea, compared to the full *nad1* gene of *A. cantonensis* (GQ398121). The following species were included in this analysis: *Angiostrongylus costaricensis* (NC_013067), *Metastrongylus pudendotectus* (NC_013813), *Metastrongylus salmi* (NC_013815). We also employed *Ancylostoma duodenale* (NC_003415) as outgroup. The corresponding *nad1* genes were obtained from complete mt genomes.

In order to explore the relationship between the clades and the possibility of recent evolution, we estimated the divergence time of clades using the clade-related parsimony informative substitutions. We made the following assumption. The overall substitution rate in *C. elegans* has been estimated as 8.9 per site per million year (My) (Denver *et al.*, 2000). However, the observed substitution rate might be 10-fold lower than the true mutation rate due to natural selection (Montooth and Rand, 2008). Hence, we set the range from 0.89 to 8.9 per site per My. The divergence time ($T_d$) was defined as the ratio of the total numbers of observed mutations ($M_o$) and expected mutations ($M_e$). $M_e$ was the product of the substitution rate per site per year (0.89 per site per My for the lower limit, $M_{el}$; and 8.9 for the upper limit, $M_{eu}$) and the length of observed DNA fragment (843 bp).

$$T_d = \frac{M_o}{M_e}\,;\ M_{el} = 0.89E^{-6} \times 843\,,\ M_{eu} = 8.9E^{-6} \times 843$$

### *6.3.5. Analysis of geographic distribution patterns*

The potential distribution patterns of *A. cantonensis* were determined according to the correlation between genetic and geographic distance. The geographic distance between any two populations (i.e. all individuals from the same collection site) was calculated by the tool PointDistance in ArcMap version 9.0. The genetic distances between each sequences was determined on a pairwise level using Mega version 3.1. Correlation between genetic (Fst) and geographic distance were tested by the programme Isolation by Distance (IBD) (Bohonak, 2002; Jensen *et al.*, 2005). Additionally, association between the genetic and geographic distance was also assessed within clades. Clades for which the sequences occurred in only one or two study sites were excluded.

The haplotypes were mapped using ArcMap version 9.0. The potential spreading routes of these haplotypes were inferred based on the results of the network analysis. The potential origin of a clade was defined as the place where some haplotypes were located on a branch basis in a network or where a common ancient haplotype was found. Connections between different collection sites were determined based on the evolutionary direction from ancient haplotypes to the end of network branches.

**Table 6.1** Haplotype (Hd) and nucleotide (Pi) diversity of mitochondrial gene *nad1* of *Angiostrongylus cantonensis* by clades

| Clade | Sequence number | Haplotype number | Haplotype diversity (Hd±SD) | Nucleotide diversity (Pi±SD) | Nucleotide differences (k) |
|---|---|---|---|---|---|
| Mainland | 80 | 32 | 0.628± 0.066 | 0.001±0.000 | 1.099 |
| Hainan | 23 | 13 | 0.818± 0.082 | 0.002±0.000 | 1.747 |
| TianE | 19 | 13 | 0.906± 0.060 | 0.005±0.001 | 4.374 |
| Sanya | 11 | 9 | 0.945± 0.066 | 0.003±0.000 | 2.218 |
| Nanao | 4 | 3 | 0.833± 0.222 | 0.002±0.001 | 2.000 |
| Zixing | 3 | 2 | 0.667± 0.314 | 0.001±0.000 | 0.667 |
| Thailand* | 3 | 1 | 1.000 | 0 | 0 |
| Total | 143 | 73 | 0.877±0.026 | 0.026±0.002 | 21.913 |

* Samples were collected from laboratory and the origin of *A. cantonensis* was Khon Kaen, Thailand; SD, standard deviation

## 6.4. Results

### *6.4.1. Diversity of* A. cantonensis *and haplotype network*

Overall, 157 *nad1* gene sequences of *A. cantonensis* from 33 study sites were determined. Among them, 14 repeated sequences were excluded for subsequent analyses. All sequences were trimmed to 843 bp and collapsed into 73 unique haplotypes without consideration of eight aligned gaps (Table 6.1). The overall Hd was 0.877. A total of 158 variable sites were identified, including 66 singleton variable sites and 92 parsimony informative sites. The Pi was 0.026. With regard to the parsimony informative sites, 88 were strongly associated with clades and the other four were located in only a few sequences distributed in different clades. Most variable sites had two variants. In four sites, however, three variants were observed.

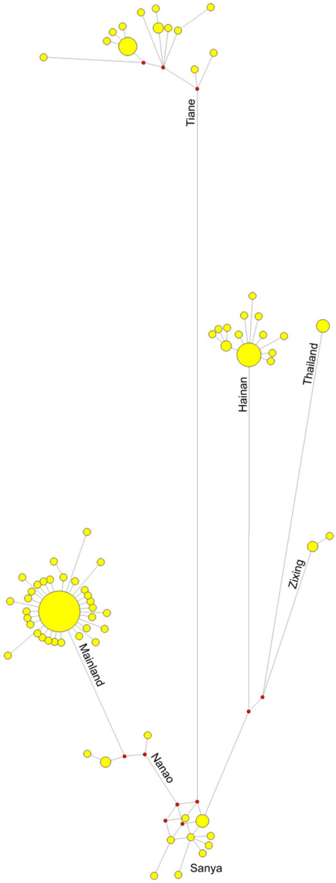

**Figure 6.1** Network of *nad1* haplotypes of *Angiostrongylus cantonensis*. The length of branches is proportional to observed mutations; the shortest branch represents one mutation. The node size is related to haplotype frequency. The red full circle denote the inferred transient haplotypes which were not observed in this study.

The shortest tree, including 204 mutations, was inferred by network analysis, and seven distinctive clades were identified (the clades were named after a typical collection site) (Figure 6.1). The shortest connect occurred between the Sanya and Nanao clades. It consisted of an average 8.091 mutations with a divergence of 0.010 (Table 6.2). The longest connect was found between the Hainan and Tiane clades: it consisted of 52.382 mutations with an overall divergence of 0.062. In contrast to considerable divergence between clades, much smaller Pi within clades was found in spite of remarkable Hd. This observation was confirmed by the fact that there were only a few (0-4.374) substitutions within each clade, in contrast to 21.913 substitutions overall.

**Table 6.2** Average divergence (below diagonal in average number of nucleotide substitution per site) and estimated divergence timing (above diagonal in thousand years) between clades

| | Mainland | Hainan | TianE | Sanya | Nanao | Zixing | Thailand |
|---|---|---|---|---|---|---|---|
| Mainland | - | 4.4-44.0 | 6.1-61.3 | 1.5-14.7 | 1.2-12.0 | 2.8-28.0 | 3.6-36.0 |
| Hainan | 0.040 | - | 6.7-66.6 | 3.5-34.7 | 4.0-40.0 | 3.7-37.3 | 3.5-34.7 |
| Tiane | 0.058 | 0.062 | - | 5.2-52.0 | 5.6-56.0 | 5.7-57.3 | 6.1-61.3 |
| Sanya | 0.015 | 0.032 | 0.050 | - | 1.1-10.6 | 2.1-21.3 | 3.5-34.7 |
| Nanao | 0.012 | 0.036 | 0.054 | 0.010 | - | 2.4-24.0 | 3.5-34.7 |
| Zixing | 0.026 | 0.034 | 0.055 | 0.020 | 0.022 | - | 3.5-34.7 |
| Thailand | 0.034 | 0.030 | 0.057 | 0.033 | 0.033 | 0.033 | - |

Note: the estimated divergence timing was based on the difference in 88 clade-related parsimony informative substitutions. We use the dominant base (occurring in the same site among more than half sequences) to make comparison between any two clades; the difference (two or more variants in the same site) within clade was considered as the intra-clade variation.

Our network analysis revealed that the clades Mainland and Hainan utilized the dominant haplotypes as the basis of the branches. Exceptions to these observations were the clades Tiane, Sanya, and Nanao. Furthermore, transient or common ancient haplotypes were clearly absent from the clades Tiane and Nanao.

Both Bayesian inference and Neighbor-Joining approaches produced similar topologies of *A. cantonensis* clades (Figure 6.2). The phylogenetic tree supported a closer relationship between the clades Mainland, Nanao, and Sanya. The clade Hainan was more likely to be related to the *A. cantonensis* sequences from Thailand. Our network analysis failed to produce conclusive evidence in support of the clade Tiane showing a close relationship to any other clade.

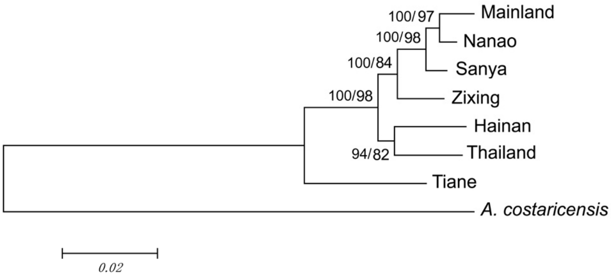

**Figure 6.2** Phylogenetic tree of *Angiostrongylus cantonensis* with *A. costaricensis* as outgroup. Neighbor-Joining and Bayesian inference produced similar trees. The posterior probability as percentage (before) of Bayesian inference and bootstrap value (after) of Neighbor-Joining are placed on the branches. For clade codes, referred to Table 6.1.

### *6.4.2. Mutation analysis*

Mutation analysis at the intra-clade level showed an overwhelming transition mutation (i.e. A↔G or C↔T) in each clade, except for Zixing and Thailand due to smaller sequence numbers. However, the proportion of different mutation types varied considerably across clades (Table 6.3). The predominant substitution in the clades Mainland and Hainan was T→C. However, this mutation type was reduced in the clades Tiane, Sanya, and Nanao. Transversion was rarely detected, although a considerable proportion due to a small account of mutations had been observed in clades Nanao and Zixing. Indel (insert or delete) mutations, particularly deletions of T, were commonly found across clades and this mutation was most likely to occur at the sites with T:A homopolymers (usually more than seven Ts in the coding strand). Nonsynonymous substitutions were predominant in the clades Mainland, Hainan, and Sanya. For the clades Tiane and Nanao the nonsynonymous and synonymous substitutions occurred almost equally. The only substitution T↔G in the clade Zixing was nonsynonymous.

Similar to mutation patterns at intra-clade level, the analysis of intraspecific mutation patterns also showed that transition was the dominant substitution. However, the transition

A↔G was more common and accounted for 62.0%, which was approximately 2.7-fold higher than that between T and C (22.8%). The transversion G↔T and A↔T occurred at a frequency of 9.8% and 3.2%, respectively. The remaining share was a mixture of A, T, and G (Table 6.4). In order to understand the bias in mutations, the substitution direction was defined as that from the predominant base (in six of seven clades) to a rare base (in the other one clade) in the same site. Reciprocal substitution A↔G was balanced, but a bias was found in substitutions C↔T and G↔T. For the former, a tendency of T→C was found. The latter showed a mutation bias to T. Only three substitutions A↔T were identified; two of them showed a mutation direction from A to T, whereas the other one was reversed.

**Table 6.3** Mutation type (percentage) and direction of *nad1* gene within clade

| Type | Direction* | Mainland | Hainan | Tiane | Sanya | Nanao | Zixing | Thailand |
|---|---|---|---|---|---|---|---|---|
| Transition | | | | | | | | |
| | T→C | 38.3 | 45.0 | 20.7 | 33.3 | 14.3 | 0 | 0 |
| | C→T | 6.4 | 5.0 | 10.3 | 8.3 | 0 | 0 | 0 |
| | A→G | 23.4 | 25.0 | 17.2 | 33.3 | 14.3 | 0 | 0 |
| | G→A | 14.9 | 5.0 | 27.6 | 8.3 | 14.3 | 0 | 0 |
| Transversion | | | | | | | | |
| | T→G | 4.3 | 0 | 6.9 | 0 | 0 | 33.3 | 0 |
| | G→T | 0 | 5.0 | 3.5 | 0 | 0 | 0 | 0 |
| | T→A | 0 | 0 | 0 | 0 | 14.3 | 0 | 0 |
| | A→T | 4.3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | C→A | 0 | 0 | 3.5 | 0 | 0 | 0 | 0 |
| Indel | | | | | | | | |
| | T→. | 6.4 | 15.0 | 10.3 | 16.7 | 42.9 | 66.7 | 100.0 |
| | G→. | 2.1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nonsyn/Syn** | | 30:13 | 12:5 | 13:13 | 6:4 | 2:2 | 1:0 | 0:0 |

* Defined as from dominant base to rare one in the same site; "." indicates delete;
** Ratio of nonsynonymous and synonymous substitution (excluding indel mutation);
Total number of mutations for Mainland, Hainan, Tiane, Nanao, Zixing and Thailand are 47, 20, 29, 12, 7, 3 and 2, respectively.

The 92 substitutions in parsimony informative sites were positively skewed to the third codon position, which accounted for 68 substitutions. The others occurred either at the first (n=18) or second (n=6) position. The ratio of nonsynonymous and synonymous mutations was 26:66, and hence resulted in 21 shifts of amino acids in a putative 281-amino-acid protein sequence. Ten of them were caused by a mutation at the first codon position. Four and three were caused by mutations at second and third codon position, respectively. Two amino acid changes were induced by both mutations at first and third position, and one by both mutations at second and third position. Only one change was observed by a mutation at three positions simultaneously.

**Table 6.4** Mutation type and direction in *nad1* coding strand of *Angiostrongylus cantonensis* at clade level based on 92 parsimony informative subsitutions.

| Type | No. (%) | Direction* | Outcome of substitution | | | | |
|---|---|---|---|---|---|---|---|
| | | | Ambiguous | A | G | C | T |
| AG | 57 (62.0) | A→G | | | 20 | | |
| | | G→A | | 18 | | | |
| | | A::G | 19 | | | | |
| CT | 21 (22.8) | C→T | | | | | 3 |
| | | T→C | | | | 14 | |
| | | C::T | 4 | | | | |
| AT | 3 (3.2) | A→T | | | | | 2 |
| | | T→A | | 1 | | | |
| | | A::T | 0 | | | | |
| GT | 9 (9.8) | G→T | | | | | 7 |
| | | T→G | | | 0 | | |
| | | G::T | 2 | | | | |
| ATG | 2 (2.2) | A::T::G | 2 | | | | |
| Total | 92 (100.0) | | 27 | 19 | 20 | 14 | 12 |

* Defined as from the predominant base (in six of seven clades) to a rare base (in the other one clade) in the same site

Compared to the intraspecific mutation patterns of *A. cantonensis*, the interspecific analysis showed a different pattern. The proportion of transversions generally increased with genetic distance to *A. cantonensis* (Figure 6.3).

### *6.4.3. Scenarios for spread of* A. cantonensis

The Mantel test (DNA distance method: Kimura 2-parameter) in the IBD programme showed no significant correlation between the genetic and geographic distances of *A. cantonensis* at population level (r = 0.091, p=0.191). Similarly, the test of correlation between genetic (pairwise difference using Kimura 2-parameter) and geographic distances within clade showed no significant correlation in the clades Mainland, Hainan and Sanya.

However, a significant correlation was found for the clade Tiane (Figure 6.4).

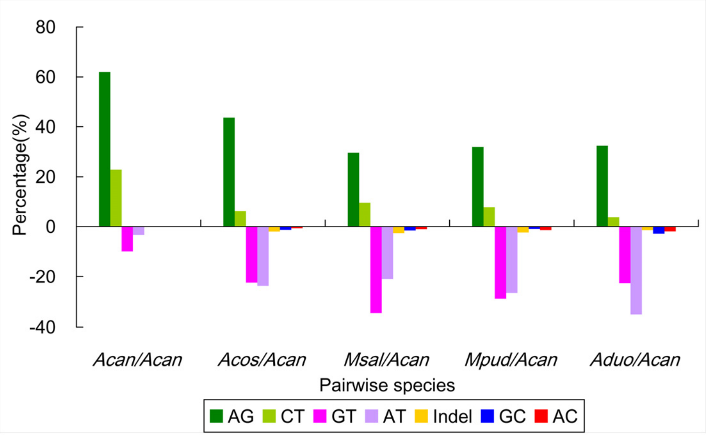

**Figure 6.3** Intraspecific and interspecific mutation patterns of *nad*1 gene compared to *Angiostrongylus cantonensis*. *Acan/Acan* denotes intraspecific mutation patterns, which was calculated according to 92 parsimony informative site. The transition substitution, i.e. A and G, T and C, are assigned above x-axis and the other below x-axis. The order of different comparison on x-axis was determined by genetic distance to *A. cantonensis*. *Acan*: *Angiostrongylus cantonensis*, *Acos*: *Angiostrongylus costaricensis*, *Mpud: Metastrongylus pudendotectus*, *Msal*: *Metastrongylus salmi*, *Aduo*: *Ancylostoma duodenale*

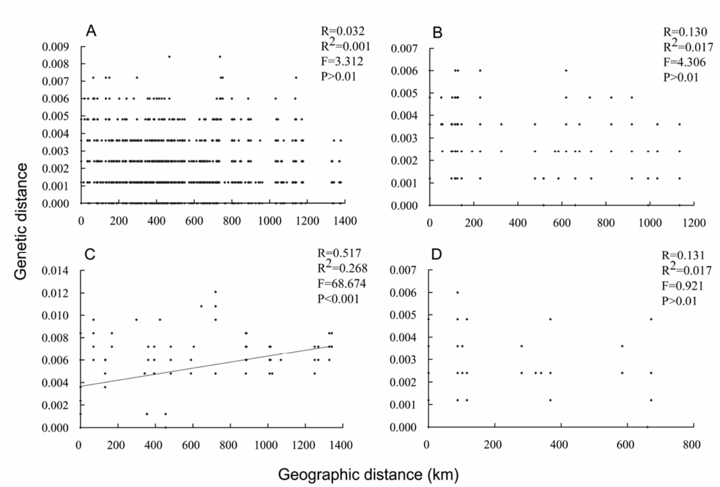

**Figure 6.4** Correlation between geographic and genetic distance by clade. The capital letters A, B, C and D represent clades Mainland, Hainan, Tiane and Sanya, respectively.

Potential routes of spread of different haplotypes of the clades Hainan, Sanya, and Tiane, inferred based on network analysis, are shown in Figure 6.5. No attempts were made to draw the spread route of Mainland haplotypes, since the place of origin could not be inferred based on the data from the present study. For the clades Nanao, Zixing and Thailand, all haplotypes were collected from single sites, and hence excluded from further analyses. Our network analysis suggests that the ancient haplotype of the clade Tiane is most likely to be close to the haplotypes from an island off Fujian province (Pingtan Island); one and two mutations were observed, respectively, in the only two haplotypes from this island. A missing common haplotype stem from this ancient haplotype was noted. There were two mutation steps between them. The original place of this missing haplotype is currently unknown. Four independent introductions to the mainland of P.R. China from this place were inferred based on network analysis. One of these introductions is in close proximity to the island mentioned above. Two separate routes were determined through which this clade was introduced into central and western parts of south P.R. China. The remaining one led to another small island (i.e. Nanao Island) off the border of Fujian and Guangdong provinces.

In three collection sites in the mainland of P.R. China, the haplotypes from the clade Hainan were intermixed with mainland clades. Two sites shared the same dominant haplotype with Dongfang county, which was inferred as the original place of this clade. Therefore, direct introduction to these two sites was inferred. The third site, located between the two aforementioned sites, harboured a haplotype that was close to that from a central county on Hainan Island. On Hainan Island, the haplotype from Dongfang county spread eastward with a few mutations.

In total, 11 sequences were identified as the clade Sanya, which was distributed in three adjacent counties of the southern parts of Hainan Island. In contrast to the clades Mainland and Hainan, the frequency of putative ancient haplotype (Figure 6.1) was very small; only one sequence located in Sanya took this role. The inferred spread routes, hence, were from Sanya to Dongfang and another neighbour county (Qiongzhong). One collection site located at the basis of the Leizhou Peninsula had yielded one sequence belonging to this clade, and hence was thought to be invaded by this clade directly from Sanya.

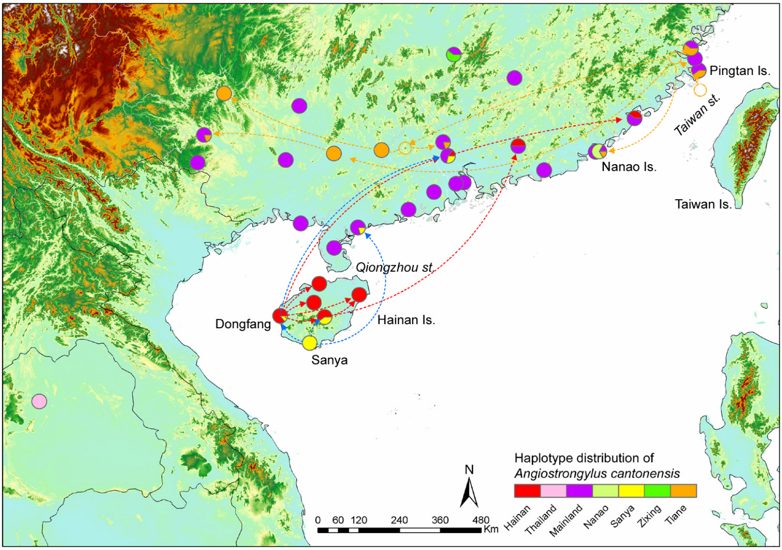

**Figure 6.5** The geographic distribution and inferred spread route of *nad1* haplotype of *Angiostrongylus cantonensis*. The different color dot line with arrow indicate the potential spread route (yellow: Tiane; red: Hainan; blue: Sanya). The yellow dash circles denote the transient haplotype of the clade Tiane and the locations are arbitrary.

## 6.5. Discussion

Three *Angiostrongylus* species, namely *A. cantonensis*, *A. malaysiensis*, and *A. mackerrase*, had been distinguished in Southeast Asia and Oceania. In principle, the latter two species could occur in our study area due to the introduction of host animals. However, this concern was lowered by the following information. First, morphology was used to primarily distinguish *A. cantonensis* from other two *Angiostrongylus* species. Second, *A. malaysiensis* could be excluded by mitochondrial genetic comparison between *A. malaysiensis* (Malaysia) and *A. cantonensis* (China, Thailand, Hawaii) (Eamsobhana *et al.*, 2010a). Third, *A. mackerrasae* was reported specific to definitive host (Stokes *et al.*, 2007; Prociv, 2000). To our knowledge, its primary definitive host, *Rattus fuscipes*, is absent or at least rare in P.R. China. Fourth, all specimens were collected within or around villages rather than forest where *A. malaysiensis* and *A. mackerrasae* usually exist. Last, it is widely acknowledged that geographic isolation is associated with intraspecific differentiation and speciation, although new theories are also proposed for speciation

(Dieckmann and Doebeli, 1999; Doebeli and Dieckmann, 2003; Ogden and Thorpe, 2002; Turelli *et al.*, 2001). If the divergence of these three *Angiostrongylus* species in Australia and Asia was caused by geographic distance or differences in biomes, it would not be surprising to discover several distinct clades of one species within a relatively small area, such as the study area investigated here. Indeed, we observed a high genetic diversity of *A. cantonensis*, which sheds new light on the mutation pattern of mitochondrial DNA (mtDNA), the origin of this parasite, and the pattern of geographic distribution.

Mutation pattern of nematode mtDNA can be directly studied in mutation accumulation (MA) lines (Denver *et al.*, 2000; Howe *et al.*, 2010; Molnar *et al.*, 2011). Unlike these model nematodes, *A. cantonensis* normally takes much longer time to accomplish life cycle (Lv *et al.*, 2010), which limits establishment and observation of MA lines. Alternatively, the well divergent clades with slight intra-clade nucleotide differences in our present study provide a unique opportunity for new insights into the mutation pattern of *A. cantonensis* at different levels. We observed 92 parsimony informative substitutions in alignment of 143 sequences and 88 out of them were clade-related, and hence referred that they played the essential role in clade divergence. A purifying selection was identified in the present study. The ratio of nonsynonymous to synonymous mutations was estimated at 26:66. An obvious “wobble” (Crick, 1966) at the third codon position was found; among 21 amino acid changes resulting from 92 parsimony informative mutations, only two changes were exclusively attributed to the mutation at the third codon position, although approximately 74% parsimony informative mutations occurred at this position.

Mutation bias or direction was variable at different level of speciation according to our study. In the observation of mutation rates of *C. elegans* mtDNA, nine reciprocal substitutions C↔T, of which eight was T→C in the coding strand, and only four A↔G were identified from 16 base substitutions; a similar situation occurred among two wild isolates (Denver *et al.*, 2000). Our results indicate the substitution T→C indeed occurred more frequently within a clade. For example, the mutation T→C might be 45.0% and 38.3% in the clades Hainan and Mainland, respectively, although the proportion decreased in clade Sanya, Tiane and Nanao. Nevertheless, the definite difference of proportion of reciprocal substitutions C↔T and A↔G were not determined across all clades in our study. Similar observations have been made in other studies on nematode species (Blouin *et al.*, 1998; Jefferies *et al.*, 2010; Peng *et al.*, 2005). However, the situation dramatically changed when interclade mutations were considered. According to our analysis, the substitution A↔G was overwhelming (62.0% in all substitutions), which is similar to the substitution pattern observed in the fruit fly *Drosophila melanogaster* (Haag-Liautard *et al.*, 2008). Notably, the content of T is predominant in our target fragment of *nad1* gene, ranging from 49.2% to 49.8% in the seven clades. Therefore, the mutation is asymmetric regardless of the initial base content. This finding indicates that the substitution A↔G tends to be accumulated, and hence forms new clades, while C↔T tends to be cleaned out in natural selection. Interestingly, the substitution A↔G is gradually mitigated by the genetic distance between nematode species. Instead, the transversion becomes the dominant mutation type. This observation implies a different mutation pattern in intraspecific differentiation and speciation. We also note that substitutions G↔T and A↔T, which are dominant between species, occurred at clade level, although to a low extent. Moreover, they are almost the only substitution types except for A↔G and C↔T. Hence, we infer that the accumulation of A↔G in coding strand may force transversion (G↔T and A↔T) which results in speciation.

Although *A. cantonensis* was recognized to be mainly endemic in Southeast Asia, including the southern part of P.R. China, a hypothesis of African origin was once proposed when the importance of *A. fulica* in the transmission of this parasite was realized, with *A. fulica* clearly originating from Africa (Alicata, 1966; Prociv *et al.*, 2000). Furthermore, *A. cantonensis* was not discovered until *A. fulica* was introduced to Southeast Asia. However, this hypothesis was challenged by subsequent investigations. For example, this parasite was established in some localities before advent of *A. fulica* (Prociv *et al.*, 2000). Furthermore, the phylogeny of *Rattus* also argued against the hypothesis of African origin (Drozdz *et al.*, 1975). Our findings support the Asian origin theory in a view of *A. cantonensis* genetics. First, high diversity was found in the present study area. We observed significant intraspecific differentiation; seven distinct clades from 143 sequences were determined, which contrast to the low diversity of introduced *A. fulica* (Fontanilla, 2010). Second, the heterogeneous geographical distribution of *A. cantonensis* observed in our study also supports an Asian origin. If the parasite species originated from Africa and spread with *A. fulica*, a more homogeneous distribution would be expected, regardless of geographic isolation. Notably, the failure to detect isolation by distance in all the samples could be attributed to the long-distance dispersal of a few haplotypes, as well as the asymmetric distribution of these clades. The factors driving the range expansion of clades may include secondary introduction of *Pomacea* spp. and *A. fulica* (Lv *et al.*, 2009), as well as dispersal of rats through human activities. Third, the inferred divergence timing (several thousand years) between each clade excludes recent evolution of this parasite. *A. fulica* was clearly recorded to invade Southeast Asia only since the early 1910s (Mead, 1961).

Conventionally, the southern part of P.R. China is considered the “home range” of *A. cantonensis* (Kliks and Palumbo, 1992; Prociv *et al.*, 2000). However, our results cannot confirm this hypothesis from a genetics point of view. First, our samples were mainly collected from southern P.R. China. Only one site outside P.R. China has been included in our study, i.e. Khon Kaen in Thailand. Furthermore, the samples from this site showed considerable genetic distance from those in P.R. China. Therefore, the diversity of *A. cantonensis* in the Indochinese Peninsula is expected. Second, the extremely low intra-clade differentiation in our study area, compared to that between clades, indicates a potential introduction of *A. cantonensis*. High diversity in a given collection site is common for native species. However, the Mainland clade, for example, widely distributed in southern P.R. China, showed slight differentiation (only one point mutation or not at all; more than 60% (49/80) of sequences were identical). In the absence of strong natural selection, loss of diversity is normally induced by genetic bottleneck or founder effect, which usually occurs in biological invasion (Dlugosch and Parker, 2008; Tsutsui *et al.*, 2000).

In contrast to the clades Mainland and Hainan, the intra-clade differentiation of the clades Tiane and Sanya is higher and the “founder” haplotype is missing or rare. The clade Tiane individually displayed genetic isolation by distance, which usually characterizes native or local populations rather than invasive species (Genton *et al.*, 2005; Leblois *et al.*, 2000; Tsutsui and Case, 2001). If the clade Tiane was exotic to our study area, multiple introductions from different geographic sources must have occurred. Among the known major intermediate hosts of *A. cantonensis*, *Pomacea* spp. originating from South America most likely took this role; these snails were widely introduced as a food resource in Southeast Asia (Joshi and Sebastian, 2006). Our network analysis indicates that this clade is likely to originate from the east, probably Taiwan. Indeed, a recent study showed the isolate from Taiwan is genetically distinct from others (Eamsobhana *et al.*, 2010b). Taiwan was the first place in this region where *Pomacea* snails were introduced in the late 1970s. Within a few years, the snails escaped or were intentionally released from snail farms and the first documented outbreak of eosinophilic meningitis due to *A. cantonensis* occurred among five children who consumed raw snails (Hwang and Chen, 1986). Subsequent surveys showed a considerable prevalence of *A. cantonensis* among *Pomacea* snails (Lv *et al.*, 2009; Nishimura *et al.*, 1986). It is therefore conceivable that *A. cantonensis* was introduced into the mainland with *Pomacea* snails from Taiwan, although the population genetics of *Pomacea* snail in P.R. China might also indicate a direct introduction from South America (unpublished data).

Our results suggest that *A. cantonensis* can be easily introduced into new areas. For example, three clades simultaneously occur on a small island (Nanao). In addition to the presumptive native clade, the clades Mainland and Tiane also appeared on this island. Similar observations have been made on another small island (Pingtan) and some collection sites in the mainland. Interestingly, on the Hainan Island, we failed to detect any haplotypes from the clades Mainland and Tiane, which are widely distributed across the mainland of P.R. China. Notably, the capital city of Hainan province, located on the south bank of Qiongzhou strait, is a major port area but we failed to find *A. cantonensis* (Lv et al., 2009). However, the haplotype from the clade Mainland could occur there since port areas are the most likely founding place of new *A. cantonensis* populations (Kliks and Palumbo, 1992).

Unlike conventional studies regarding population genetics which reveal the population structures by multiple loci, the present study focuses on the genetic diversity of *A. cantonensis* and thereby infers the scenarios of spread geographically. Therefore, a single locus (*nad*1 gene) with fast substitution rate was selected to distinguish specimens and speculate the potential spread route of haplotyeps. Actually, the real ancestral and descendant haplotypes are difficult to determine in this speculation. We observed that there was marginal differentiation between hyplotypes within each clade, which resembled the observation in experiment of mutation accumulation lines of *C. elegans* and *P. pacificus*, where only few descendants possess mutation but most of them were identical to their true ancestor (the first generations) (Denver *et al.*, 2000; Molnar *et al.*, 2011). Therefore, we assumed a common haplotype as ancestor in this study. Furthermore, we utilized network analysis to infer the topology of all haplotyes. Our network is the shortest tree, which depicts the most likely relationship between haplotypes in a view of mutations. We connected all clades instead of separating them in order to find out the relative ancient haplotype, which should be closest to the haplotypes in other clades. Thereby we defined the spread route from ancient hayplotype to descendant ones.

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# 7. Population genetics of invasive snail *Pomacea* spp. in the People's Republic of China: multiple and secondary introductions

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Diversity and Distributions

## 7.1. Abstract

The apple snail (*Pomacea* spp.) originates from South America and has become established and then spread for three decades in the People's Republic of China, damaging crops and the environment as well as human health in the process. However, the true number of invasive species and their distribution patterns are still unknown. In this study, 523 sequences of the mitochondrial *cox1* gene from samples collected at 56 sites representing most of the endemic areas were obtained. A total of 25 unique haplotypes could be identified, an overall haplotype diversity of 0.702. This is higher than the results from previous studies suggest. Globally, only five out of 98 haplotypes described in previous studies were shared between introduced and native snail populations. All haplotypes clustered in ten groups, six of which occurred in the mainland of P.R. China. Two of these six groups could be traced back to South America and two were shared with other countries in Southeast Asia. The remaining two groups have been found only in P.R. China thus far; the determination of their definite origin needs further study. The phylogenetic analysis indicated that two species, namely *P. canaliculata* and *P. insularum*, coexist in the mainland of P.R. China, although the phylogenetic position of group B is still pending. Increased diversity in collection sites indicated multiple and secondary introductions.

**Keywords**: Population genetics, *Pomacea* spp., invasive species, multiple introduction, China

## 7.2. Introduction

*Pomacea* is the largest of nine extant genera in the family Ampullariidae (apple snail). There is considerable intraspecific variation in morphology and hence it is difficult to morphologically determine the true number of species (Cazzaniga, 2002; Hayes *et al.*, 2009). Thus far, some 50 species have been described from the native range, South and Central America (Cazzaniga, 2002; Cowie *et al.*, 2006). *Pomacea canaliculata* has been classified as one of the 100 top invasive species in the world (Lowe *et al.*, 2000). Indeed, around 1980 the introduction of this snail species into Southeast Asia has been confirmed; first in Taiwan and subsequently in neighboring countries (Joshi & Sebastian, 2006). "Golden apple snail" is the colloquial term to name *Pomacea* snails from South America because of their yellow colored shell and their commercial value (Cowie *et al.*, 2006). The initial introduction of the "golden apple snail" into Southeast Asia was intentional, as food item and an important source of protein. However, several years after the introduction, damage to crops and ecosystems became apparent (Joshi & Sebastian, 2006). Hence, different control strategies have been implemented, but attempts to eliminate *Pomacea* snails again proved difficult. Among the issues hindering control, the lack of detailed knowledge of species-specific distribution patterns has been emphasized. This challenge has been addressed by a growing number of studies of the morphological and molecular characteristics of *Pomacea*. Among them, they suggest that four species, namely *Pomacea canaliculata*, *Pomacea inuslarum*, *Pomacea scalaris* and *Pomacea diffusa*, spread beyond their native range, and hence should be considered invasive species (Hayes *et al.*, 2008).

In the mainland of the People's Republic of China (P.R. China), the "golden apple snail" was introduced to Zhongshan, Guangdong province in 1981. Secondary introduction for commercial aquaculture occurred between the mid 1980s and early 1990s. At present, *Pomacea* spp. is established in at least 11 provinces (Lv *et al.*, 2009) and their range is predicted to further expand northward due to climate change and environmental transformation (Lv *et al.*, 2011). Costs and benefits for humans and the environment due to the invasive snail species are difficult to estimate. Additionally, *Pomacea* snails have been identified as a major intermediate host of *Angiostrongylus cantonensis*, the rat lungworm that also infects humans has driven the emergence of human eosinophilic meningitis in P.R. China (Lv *et al.*, 2008; Lv *et al.*, 2009). The "golden apple snail" is amphibious and shows a preference for shallow ditches and ponds surrounding human settlements as its habitat, which facilitates contact with peridomestic rats (e.g. *Rattus norvegicus* and *R. rattus*), the most common definitive hosts of *A. cantonensis*. Moreover, rats are important predators of *Pomacea* snails (Yusa *et al.*, 2006). Native snail species such as *Cipangopaludina* spp. and *Bellamya* spp. which are also commonly consumed in P.R. China are rarely found to be infected with *A. cantonensis* although infections and considerable worm burdens have been observed under experimental conditions (Lv *et al.*, 2006). Ecological characteristics (e.g. their benthonic life cycle) of these native snail species probably account for the low likelihood of *A. cantonensis* infection.

Apple snails originating from South America have now spread across P.R. China for three decades. However, the first national survey regarding their distribution was not performed until 2006 (Lv *et al.*, 2009). Knowledge gaps still remain with regard to the exact number of species and the spreading patterns. Various taxonomic names, including *Ampullaria crossean*, *Ampullaria gigas*, *P. canaliculata*, *Pomacea lineata* and *Pila gigas*, were used to refer to the exotic snail species and have caused considerable confusion (Zhou *et al.*, 2009). The external morphology (e.g. shell bands and foot color) was initially considered as characteristic for different species, but its usefulness in distinguishing between species proved unreliable (Matsukura *et al.*, 2008). Recently, molecular methods (e.g. mitochondrial DNA sequence and inter simple sequence repeat) have been employed to reveal the diversity of *Pomacea* spp. (Xu *et al.*, 2008; Song *et al.*, 2010; Dong *et al.*, 2011). These studies confirmed the suspected species diversity, identifying two species, namely *P. canaliculata* and *P. insularum*. However, their distribution patterns remained to be elucidated.

In the present study, we used snail samples collected across the *Pomacea*-endemic area of P.R. China to (i) determine the number of *Pomacea* species in the mainland of P.R. China, (ii) study the genetic diversity, and (iii) reveal the distribution pattern of invasive species using the mitochondrial gene *cox1*.

## 7.3. Methods

### *7.3.1. Apple snail specimen and total DNA extraction*

Presumed *Pomacea* spp. specimens had mainly been collected during the first national survey pertaining to *A. cantonensis* in the mainland of P.R. China which was carried out in 2006 and 2007 (Lv *et al.*, 2009). For the survey, approximately 100 *Pomacea* snails were collected from each of the 56 study sites. Next, whenever possible, 10 specimens from each site were randomly selected and conserved in pure ethanol after examination for *A. cantonensis* larvae. A total of 544 samples from 56 field sites were used for the present study.

From each specimen, approximately 10 mg foot tissue was washed several times using PBS buffer, and then immersed in the same buffer overnight in order to expel any remaining ethanol. The food tissue was cut into small pieces and incubated with sodium dodecyl-sulphate/proteinase K at 56 °C for 4-6 hours. The suspension was centrifuged for 60 sec at 10,000 ×*g* and the supernatant was transferred to a clean tube and DNA extracted with phenol/chloroform/isoamyl alcohol (v:v:v = 25:24:1). The DNA pellet was suspended in 30-50 µl $H_2O$ and kept at -20 °C pending use.

### *7.3.2. PCR and sequencing*

The primers targeting *cox1* of *Pomacea* spp. were designed according to a previous study (Folmer *et al.*, 1994). For samples that failed to be amplified in the PCR, the primers were modified based on the determined sequences. PCR cycling conditions used were 94 °C for 5 min, and then 35 cycles with 94 °C for 60 sec, around 50 °C for 45 sec, and 72 °C for 60 sec, followed by 72 °C for 10 min for final extension. The PCR products were visualized in a 1% agarose gel and recovered from the gel using mini-spin columns (Axygen). The purified products were used for sequencing by the dideoxynucleotide termination method. All unique mutations were carefully checked and ambiguous bases were confirmed by double sequencing. The sequences from the present study were primarily aligned using the Blast web service of NCBI. Species identification according to these sequences was then attempted.

### *7.3.3. Data source from GenBank*

In order to deepen our understanding of the probable origin of *Pomacea* spp. in P.R. China, the genetic data pertaining to the *cox1* gene of *P. canaliculata* and *P. insularum* deposited in GenBank were employed. In total, 228 sequences were available, with most of them contributed by six studies (Rawlings *et al.*, 2007; Hayes *et al.*, 2008; Jørgensen *et al.*, 2008; Matsukura *et al.*, 2008; Hayes *et al.*, 2009; Song *et al.*, 2010). These sequences represented snail samples collected in both native habitats and in areas relatively recently colonized. The access numbers were AB433757-EF514945, EF514961-EF515058, EU274574, EU523129, EU528481-EU528499, EU528502, EU528508-EU528510, EU528513-EU528516, EU528520-EU528531, EU528533, EU528538-EU528540, EU528544, EU528549-EU528552, EU528554, EU528557-EU528563, EU528566-EU528572, EU528575, EU528576, EU528578-EU528582, EU528584, EU528586, EU528587, EU528593, FJ710313-FJ946828, GU133205-GU133207 and GU236486-GU236491.

### *7.3.4. Data analysis*

All sequences determined in the present study were aligned using Clustalx and trimmed as necessary using BioEdit for subsequent analyses. DanSP version 5 was employed to determine unique haplotypes. The total number of haplotypes and the overall diversity of haplotypes and nucleotides were calculated, and the substitution type among these truncated sequences was determined.

Analysis of molecular variance (AMOVA) was performed in Arlequin version 3.1 in order to understand the variance component and percentage. The correlation between genetic (Fst) and geographic distances was evaluated using the programme offered by the Isolation by Distance (IBD) website service. We used a Mantel Test (DNA distance method: Kimura 2- parameter) to estimate correlations.

Data regarding *cox1* sequence of *P. canaliculata* and *P. insularum* from GenBank coupled with the haplotypes produced in the present study, were aligned and truncated. The trimmed sequences were then presented to DanSP version 5 to collapse into unique haplotypes. These haplotypes were analyzed using Network version 4.6.0.0 without considering aligned gaps. The haplotypes in native and introduced range were differentiated in the network.

The clusters suggested by the network analysis were phylogenetically analyzed. Due to the observed marked variation within clusters, three haplotypes from each cluster were selected for reconstructing an evolutionary tree. Three species, i.e., *P. dolioides* (EU528556), *P. diffusa* (EU528564) and *P. scalaris* (EU528585), were used as outgroups. Two methods, i.e. minimum evolution (ME) and Bayesian inference (BI), were employed. The ME tree was constructed in Mega version 3.0 using the Kimura 2-parameter model. The resulting tree was tested with 1000 bootstrap replicates. The BI tree was accomplished in MrBayes version 3.1. The posterior probabilities were calculated via 500,000 generations using Markov chain Monte Carlo (MCMC) simulations and the chains were sampled every 100 generation. The consensus tree was visualized by TreeView.

The global distribution of haplotypes of *P. canaliculata* and *P. insularum* was mapped using ArcMap version 9.1 as suggested by previous publications (Rawlings *et al.*, 2007; Hayes *et al.*, 2008; Jørgensen *et al.*, 2008; Matsukura *et al.*, 2008; Hayes *et al.*, 2009; Song *et al.*, 2010). The location of collection sites was determined using a hand-held global positioning system (GPS) receiver (Lv *et al.*, 2009). Collection sites in close vicinity were consolidated and all haplotypes collected from the original sites were represented at the new point on the map. All haplotypes were grouped according to the results of the network analysis. For the distribution of haplotypes in P.R. China, their respective proportion in each collection site was visualized as pie charts The combination of haplotypes found in all collection sites was shown in a network.

## 7.4. Results

In total, 523 *cox1* gene sequences of *Pomacea* snails collected from 56 sites were characterized in this study after discounting duplicate sequenced data. The aligned sequences were trimmed to 503 bp and consolidated into 25 unique haplotypes. The overall haplotype diversity was 0.702 (standard deviation (SD) = 0.016). A total of 85 variable sites were indented, of which 64 were parsimony informative sites. The nucleotide diversity was 0.043 (SD=0.001). Transition was the major type of substitution (43 between A and G, and 27 between T and C). The most common haplotypes were H8 and H21, accounting for 48.2% and 20.4% respectively (Table 7.1). Less common haplotypes included H1 (13.1%), H16 (6.5%), H23 (4.0%) and H19 (2.8%). The remaining haplotypes were determined 1-3 times. All haplotypes were either identified as *P. canaliculata* or *P. insularum*.

**Table 7.1** Haplotype and frequency of *cox1* gene of *Pomacea* spp. in P.R. China

| Haplotype | Frequency (%) | Distribution* | Cluster† |
|---|---|---|---|
| H1 | 72 (13.7) | 23 | C |
| H2 | 1 (0.2) | 1 | C |
| H3 | 2 (0.4) | 1 | C |
| H4 | 1 (0.2) | 1 | C |
| H5 | 1 (0.2) | 1 | C |
| H6 | 1 (0.2) | 1 | C |
| H7 | 1 (0.2) | 1 | C |
| H8 | 252 (48.2) | 43 | E |
| H9 | 1 (0.2) | 1 | E |
| H10 | 1 (0.2) | 1 | E |
| H11 | 1 (0.2) | 1 | E |
| H12 | 1 (0.2) | 1 | E |
| H13 | 1 (0.2) | 1 | E |
| H14 | 1 (0.2) | 1 | E |
| H15 | 3 (0.6) | 2 | D |
| H16 | 34 (6.5) | 12 | D |
| H17 | 1 (0.2) | 1 | D |
| H18 | 1 (0.2) | 1 | D |
| H19 | 15 (2.8) | 6 | D |
| H20 | 1 (0.2) | 1 | B |
| H21 | 107 (20.4) | 33 | B |
| H22 | 1 (0.2) | 1 | B |
| H23 | 21 (4.0) | 12 | B |
| H24 | 1 (0.2) | 1 | B |
| H25 | 1 (0.2) | 1 | A |

*the number of collection sites; † the category referred to Figure 7.1.

AMOVA showed that the variation within populations accounted for 56.9% of the total variation whereas the remaining 43.1% of the variation occurred between populations ($p$<0.001). The total fixation index (Fst) was 0.43. No significant correlation (r=0.041, $p$=0.235) between genetic (Fst) and geographical distance was detected.

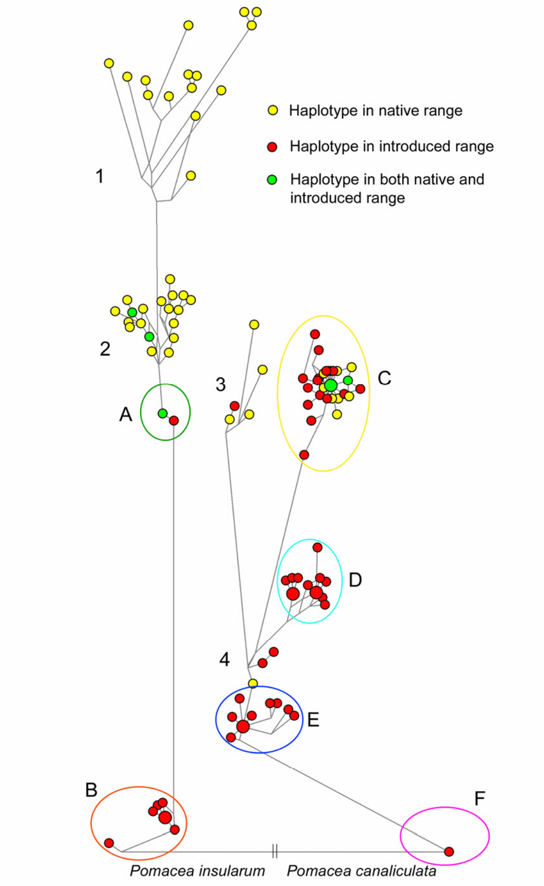


**Figure 7.1** Network of *Pomacea canaliculata* and *P. insularum* based on currently available data. The shortest distance between two haplotypes indicates one mutation in the 503-long sequence, and other distances are proportioned to the shortest distance. The larger dots indicate the haplotype which were detected independently in previous studies and the present study.

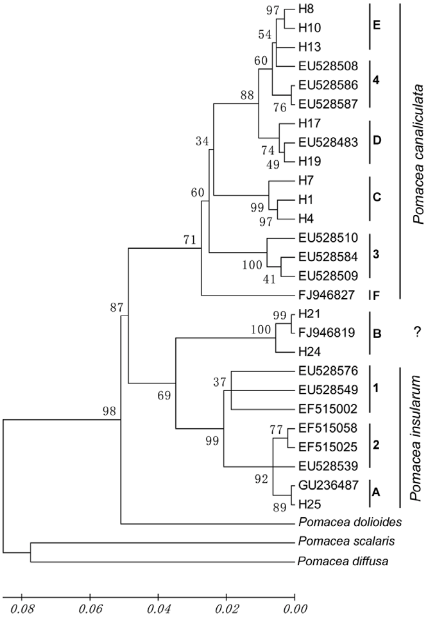
A total of 228 sequences of both *P. canaliculata* and *P. insularum* obtained from GenBank were coupled with 25 unique sequences determined in the present study and classified into 98 haplotypes overall. The shortest tree according to a network analysis identified 10 haplotype clusters (Figure 7.1). Only five haplotypes were found in both native and recently colonized areas. One haplotype, namely H1 (also referred to by the sequence EU528499), of two such shared haplotypes located in cluster C was found by this study. The other haplotype (sequence EU528489) was reported from the Philippines and Japan outside its native range (Hayes *et al.*, 2008) but was not detected in the present study. In cluster A, the common haplotype (sequence GU236487) was widely distributed across Southeast Asia but was only found in one snail sample collected from Chongqing municipality in the Sichuan Basin. Another two shared haplotypes were primarily reported from the USA and one of them also from Japan (Rawlings *et al.*, 2007; Hayes *et al.*, 2008). They have not been found in P.R. China.

Of 10 clusters in the network, six were present in P.R. China according to the results in the present study as well as the findings from two previous studies (Hayes *et al.*, 2008; Song *et al.*, 2010). The clusters B and F exclusively occurred in P.R. China. Of 25 haplotypes determined by this study one was included in cluster A, five in cluster B, seven in cluster C, five in cluster D and seven in cluster E (Table 7.1). The only haplotype in cluster F had been found in a previous study (Song *et al.*, 2010). The two most common haplotypes (i.e. H8 and H21) occurred in cluster E and B, respectively.

The phylogenetic analysis showed considerable variation at higher taxonomic level according to different methods, while haplotypes were inclusively clustered at a lower level. In the ME tree, the only sequence from cluster F was assigned to *P. canaliculata*, whereas the sequences from cluster B were clustered with the sequences of *P. insularum* (Figure 7.2). The situation changed when only one species was considered as outgroup, in that both the unique sequence from cluster F and those from cluster B were assigned to *P. insularum* (results not shown). However, the BI tree indicated that both groups F and B were likely to cluster with *P. canaliculata* (Figure 7.3).

According to the presence of particular haplotypes in P.R. China (also referred to in Figure 7.1), haplotypes were classified into six groups as well as an extraordinary group, including the haplotypes currently not yet detected in P.R. China. As shown in the network analysis, only two groups occurred both in the native range of the *Pomacea* snails and in P.R. China (Figure 7.4). One pertaining to *P. canaliculata* occurred in Argentina and the other, of *P. insularum* type, in Brazil. Two groups commonly found in Southeast Asian countries, including P.R. China, were D and E. The former has been reported from the Philippines, Japan and the southern part of Vietnam, the latter from Japan and Myanmar.

The other two groups had only been observed in P.R. China.



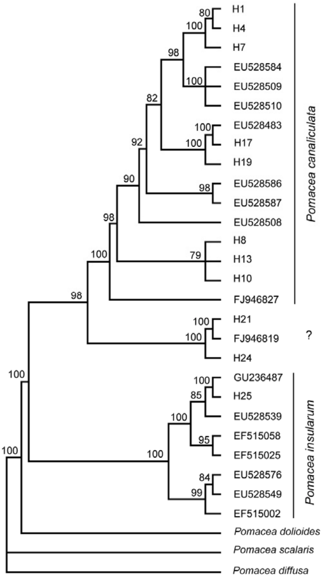


**Figure 7.2** Minimum evolution tree of apple snail haplotypes. The bootstrap values are on the branches. The clades are characterized by single letters or figures according to network analysis (see Figure 7.1). The interrogation mark denote that the position of the clade is inconsistent with that inferred from Bayesian inference tree (see Figure 7.3)

**Figure 7.3** Bayesian inference tree of apple snail haplotypes. The posterior probabilities (percentage) are placed on the branches. The interrogation mark denote that the position of the clade is inconsistent with that inferred from minimum evolution tree (see Figure 7.2).

Details of the distribution of haplotypes in P.R. China varied when compared to the global data (Figure 7.5). Groups E and B were dominant in P.R. China, while globally, groups C and A were more widely distributed. No distinctive bias of geographic distribution in P.R. China was found. The majority of the sampling sites were inhabited by two or more groups simultaneously, whereas all snails belonged to group E in 10 collection sites (Figure 7.6). These 10 collection sites were generally found outside the Pearl river basin which covers Guangdong and Guangxi provinces. The other groups occurred on their own in groups B1, D1 and D2. In contrast to group E, these groups were rare and each only occurred in one collection site. Group B2 was always found together with group B1.

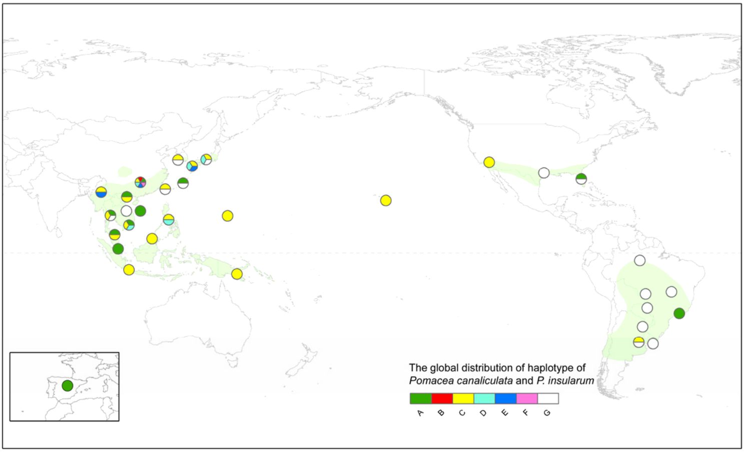


**Figure 7.4** The global distribution of haplotypes of *Pomacea canaliculata* and *P. insularum*. Six groups of haplotype identified in P.R. China were indicated using different colour and letters as see Figure 7.1. The other haplotypes which did not occur in P.R. China were allocated to group G and denoted using white pies. The locations are indicated arbitrarily according to previous studies (Rawlings et al., 2007; Hayes et al., 2008; Jørgensen et al., 2008).

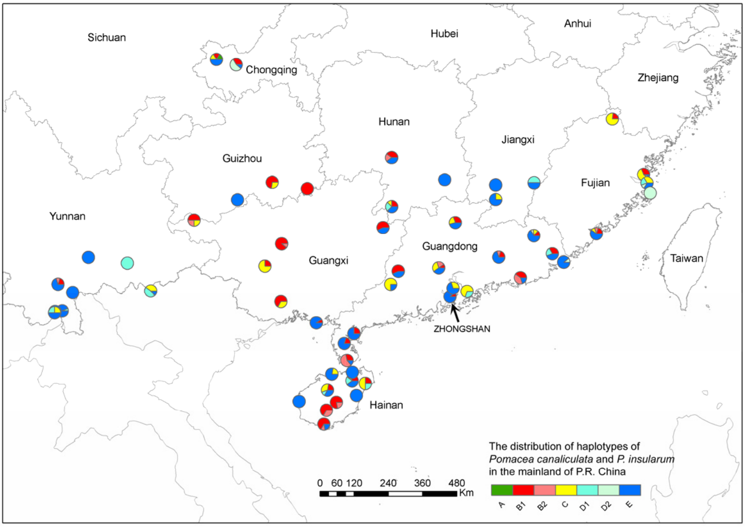


**Figure 7.5** The distribution of haplotypes of *Pomacea canaliculata* and *P. insularum* in the mainland of P.R. China. Groups B and D in Figures 1 and 2 were divided into two subgroups, respectively and denoted by B1, B2, D1 and D2. The haplotypes from previous studies in P.R. China (Hayes *et al.*, 2008; Song *et al.*, 2010) are not shown. The four sites where samples were collected from local markets were excluded.

### 7.5. Discussion

Three species of apple snails originating from South America have been confirmed to have been introduced into Southeast Asia, namely *P. canaliculata*, *P. insularum* and *P. scalaris*. The latter species is restricted to Taiwan (Hayes *et al.*, 2008), where it probably only occurs in the southern part (Wu *et al.*, 2010). The smaller hatching size and inferior growth performance compared to the other *Pomacea* species might play a role in this (Wu *et al.*, 2011). Indeed, we failed to identify this species among the more than 500 snail samples collected at 56 sites in P.R. China. In the present study we found *P. canaliculata* and *P. insularum*. Our results, along with the findings from other recent studies, suggest that these two species most likely were introduced together. Of note, the abundance of apple snails for human consumption in P.R. China and other Southeast Asian countries is such that new deliberate introduction directly from the native area is not likely. However, as any *Pomacea* snails become invasive once they have been introduced (Cazzaniga, 2002), any introduction of such snails should be formally forbidden.

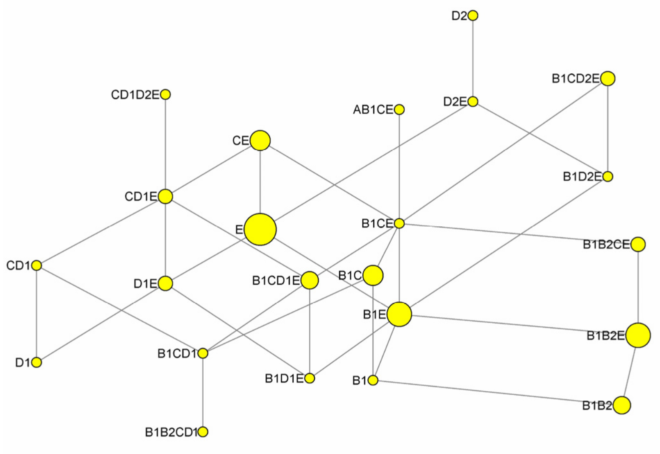


**Figure 7.6** Network of haplotype combination of apple snails in sampling sites. Two adjacent nodes were always connected by a line and differed in one haplotype. The node size is proportional to the frequency of each haplotype combination. The haplotypes are denoted as characters as shown in Figure 7.5.

The identification of *Pomacea* species on the basis of their morphology is difficult. The modern taxonomy considers both morphology and internal anatomy as well as molecular information (Cowie *et al.*, 2006). Unfortunately, to date, not all 50 *Pomacea* species have been characterized with molecular markers. In our study, we employed the closely related species *P. dolioides*, which might be placed in the "*P. canaliculata* group" (Hayes *et al.*, 2008), *P. scalaris* and *P. diffusa*, which both belong to the "*P. bridgesii* group", were considered as the outgroup to evaluate the phylogenetic relationship between *P. canaliculata* and *P. insularum*. The results of this approach show a conflict between phylogenetic trees inferred by minimum evolution and Bayesian inference; the group B could not be firmly assigned to either *P. canaliculata* or *P. insularum*. This wobble was confirmed when different outgroups were used as suggested in previous studies (Hayes *et al.*, 2009). Group B probably sits at the limit between *P. canaliculata* and *P. insularum*. Indeed, these two species share many more similarities in shell morphology and internal anatomy than differences (Cowie *et al.*, 2006). We thus suggest that the snails from this group should be considered as "*P. canaliculata* group".

The first introduction of apple snails into P.R. China has been traced back to a Brazilian of Chinese origin who brought apple snails to Zhongshan, Guangdong province to foster commercial aquaculture in 1981 (Cai & Chen, 1990). Four years later damage to crops was reported (Liang & Cai, 1993). In our study, two collection sites were located in close proximity to the initial introduction site. Except for a single sample identified as group B (tentatively *P. insularum*), all snails from the two sites fell into *P. canaliculata* groups. Moreover, group E, uncommon in other countries in Southeast Asia and even absent in South America, was predominant. Group C was found in one collection site. Additionally, in 10 of the 56 collection sites, only snails from group E were found. Hence, we speculate that the apple snails first introduced in P.R. China belonged to the group E. From P.R. China, they might have been secondarily introduced to Myanmar and Japan (Hayes *et al.*, 2008; Matsukura *et al.*, 2008). However, there is still debate regarding the exact origin. According to a recent study pertaining to the original source of Asian apple snails, *P. canaliculata* is not native to Brazil, and hence also group E must originate elsewhere (Hayes *et al.*, 2008). Indeed, the only haplotype (sequence EU528508) genetically close to group E has been reported from Argentina (Hayes *et al.*, 2008). In view of the high genetic diversity of both *P. canaliculata* and *P. insularum* in their native range, we speculate that the introduced snails might have occurred at not-yet sampled sites. It is thus worth to further expand sampling in South America with the aim of mapping the real diversity and deepen our understanding of the origin of particular strains of apple snails found in recently colonized regions.

We did not analyze the relationship between haplotypes and the niches, such as ditches, ponds and streams, where the snails had been collected. Almost all samples from a particular site had been collected from a single village or a relatively small geographical range (Lv *et al.*, 2009). Our results showed considerable genetic variation among snails, even within single niches, which contrasts previous findings, e.g. from the Philippines (Hayes *et al.*, 2008). We also noted that snails obtained from the two collection sites in close proximity to the original introduction site might have different haplotype patterns, implying a higher diversity than what we had discovered. It follows that 10 samples collected in each site might be insufficient to reveal the real haplotype diversity at a given site. It is conceivable that the original batch of apple snails introduced in P.R. China already had high genetic variation as considerable diversity has been documented in single sampling sites (Hayes *et al.*, 2008). The expected genetic bottleneck through which such a founding population should go was not apparent in our study. Instead, increased genetic variation is commonly found in single collection sites. One potential explanation for such increased genetic diversity is multiple introduction followed by secondary translocation (Kolbe *et al.*, 2004; Durka *et al.*, 2005; Genton *et al.*, 2005). Multiple introductions from different origins could pool the genetic variation in a single site. As a result, the diversity in any given location in the newly colonized area would be expected to be higher than that in the species' native range. In the present study, sites with three or four groups were not uncommon, indeed suggesting multiple and secondary introductions in the mainland of P.R. China.

Unlike some inadvertently introduced species, apple snails were introduced as food resource and hence multiple and secondary introduction over long distances are to be expected, resulting in an unpredictable distribution of apple snails and genotypes in P.R. China. For instance, the single sample that fell into group A was collected in Chongqing, but this does not mean that this group is rare, at least in the Sichuan Basin. A previous study identified four out of five snail samples from Sichuan province as haplotypes from group A (Song *et al.*, 2010). Obviously, the origin of this population is more likely Southeast Asia or the native South America rather than southeastern P.R. China.

Additionally, secondary introduction probably occurred in the same area. For example, group D2, which was relatively rare, was mainly found in coastal Fujian province, on the mainland across Taiwan, but also appeared in one site in Chongqing municipality. While intentional introductions for commercial aquaculture has probably decreased since the 1980s, the snails now tend to naturally expand their range (Yang *et al.*, 2010). Nevertheless, the spread and transportation in relation to trade are still ongoing (Lv *et al.*, 2009) and colonization of new habitats is expected.

The mosaic distribution of apple snail genotypes in the mainland of P.R. China indicates a mixture of multiple and secondary introductions. Although negative effects of the snail on crops, ecosystems and human health have been emphasized, the transportation of apple snail as foodstuff over long distances is not uncommon even at present. While invasive species often experience a loss of diversity in the long-term (Dlugosch & Parker, 2008), the increased genetic diversity resulting from multiple and secondary introductions potentially increases the success of the invasive species (Kolbe *et al.*, 2004). Therefore, the further investigations should focus on the impact of *Pomacea* introduction in P.R. China. Our study identified one clade (group B) which wobbles between two species and has not yet been found in their native range, which indicates a higher diversity and complex distribution pattern than expected. Therefore, it is worth investigating this pattern to reveal the invasion history of apple snail.

### 7.6. Acknowledgement

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# 8. The emergence of angiostrongyliasis in the People's Republic of China: the interplay of invasive snails, climate change and transmission dynamics

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### 8.1. Summary

1. Only few freshwater snail species transmit the rat lungworm *Angiostrongylus cantonensis* (Chen, 1935), which is partially explained by the low likelihood of contact between snails and infected rat faeces. The snail *Pomacea canaliculata* (Lamarck, 1822) was introduced into the People's Republic of China (P.R. China) in 1981 and has become the key intermediate host for *A. cantonensis*. Thus far, the snail has been recorded in 13 provinces of southern P.R. China.

2. We developed a biological model and assessed potential impacts of climate change on the distribution of *P. canaliculata*, and hence the transmission of *A. cantonensis*. Mean January temperature and snail generation intensity (generation number) were identified as the key factors determining *P. canaliculata* distribution. Our models predict an increase of 56.9% for the 'spread' and a decrease of 40.9% for the 'establishment' regions ('spread' and 'establishment' defined according to a national sampling survey) by the 2030s relative to the present-day.

3. Key determinants of *A. cantonensis* transmission were identified as the generation intensity in the intermediate host, the longevity of *A. cantonensis*-infected rats and the dormant period of *P. canaliculata*. Transmission of *A. cantonensis* occurs only in areas where the snail's dormant period is less than 173.2 days. The potential endemic area of *A. cantonensis* is predicted to double by the 2030s relative to the present-day.

4. The tight fit of our model predictions with data derived from a national sampling survey suggests that biological models hold promise for assessing potential impacts of climate change on infectious diseases once key determinants have been established. Geographical variation analysis may offer an approach to identify areas prone to the spread of vectors, intermediate hosts and parasites in a future warmer P.R. China and elsewhere.

*Keywords*: *Angiostrongylus cantonensis*, climate change, invasive snail, *Pomacea canaliculata*, vulnerable area

## 8.2. Introduction

*Angiostrongylus cantonensis* (Chen, 1935) (Nematoda: Metastrongylidae) is also known as 'rat lungworm', since adult worms parasitize the pulmonary arteries of rats. Molluscs, e.g. freshwater and terrestrial snails, serve as the intermediate hosts. In the human body, the worms usually fail to enter the pulmonary arterial system through the central nervous system (CNS) as they do in rats, and instead lodge in the human CNS where they induce inflammation characterized by elevated eosinophil counts in the cerebrospinal fluid (Lv et al., 2010).

Eosinophilic meningitis due to an infection with *A. cantonensis* is endemic in Southeast Asia, the Pacific Islands and the Caribbean (Wang et al., 2008). Humans acquire an infection through the consumption of undercooked snails, slugs and paratenic hosts (e.g. crabs, frogs and lizards), as well as contaminated vegetables. Only a few freshwater snail species are known to be involved in the natural transmission of angiostrongyliasis, most prominently *Pomacea canaliculata* (Lamarck, 1822) (Gastropoda: Ampullariidae), although many other snails have been demonstrated to be susceptible to *A. cantonensis* in experiments (Chang et al., 1968; Lv et al., 2006a; Yousif and Lammler, 1975). A recent review of published human angiostrongyliasis cases from the mainland of the People's Republic of China (P.R. China) suggests that three-quarters of the 334 cases reported before 2006 were directly related to the consumption of *P. canaliculata*. The first parasitologically diagnosed case report dates back to 1984 and the first recognised outbreak occurred in 1997 (Lv et al., 2008). Since then the number of cases has steadily increased. Six out of seven outbreaks involving more than five individuals could be attributed to the consumption of *P. canaliculata* (Lv et al., 2008). Two recent outbreaks in 2007 and 2008 were also attributed to this snail species (Deng et al., 2007; Lv et al., 2009a). The first national survey of *A. cantonensis* in P.R. China showed that *P. canaliculata* is widely distributed in the southern part of the country (Lv et al., 2009b). Hence, there is growing evidence that eosinophilic meningitis due to *A. cantonensis* infection is emerging in P.R. China.

The emergence of *A. cantonensis* has largely been attributed to the spread of *P. canaliculata*. Compared to other snail species, *P. canaliculata* displays a number of unusual biological features. It is amphibious, usually inhabiting slow-moving or stagnant waters such as shallow swamps, marshes, ditches, lakes and rivers, but is also able to survive outside freshwater bodies by breathing air, especially during oviposition (Joshi and Sebastian, 2006). The latter trait is significant as it enhances the chance for contacts between snails and rat faeces, and hence the risk of infection with *A. cantonensis*.

*P. canaliculata* originates from the South Americas and is the only freshwater snail listed among the global top-100 invasive species (Lowe et al., 2000). Its current invasive range includes East Asia and North America (Joshi and Sebastian, 2006). In 1979, *P. canaliculata* was introduced from Argentina by commercial snail farms into Taiwan to be cultured indoors for human consumption and, subsequently, the snail spread through eastern Asia (Joshi and Sebastian, 2006). The current distribution includes the mainland of P.R. China, Japan, the Republic of Korea, the Philippines, Indonesia, Malaysia, Singapore, Vietnam, Cambodia, Lao People's Democratic Republic (Lao PDR), Thailand and Myanmar (Joshi and Sebastian, 2006). A genetic analysis of the *P. canaliculata* complex in East Asia identified four species, namely *P. canaliculata*, *P. insularum*, *P. scalaris* and *P. diffusa*, indicating multiple independent introduction events over the past decades (Hayes et al., 2008).

Similar to other biological invasions (Jeschke and Strayer, 2005), the spread of *P. canaliculata* in eastern Asia underwent three phases, namely introduction, establishment and spread. The snail was originally intentionally introduced, but in tropical and subtropical areas, *P. canaliculata* rapidly found its way into the surroundings and spread via water networks after a short phase of establishment. A different pattern has been observed for temperate areas, e.g. in central and northern P.R. China, where the snail was commonly introduced for commercial purposes in the late 1980s and early 1990s (Wang, 1988; Wei et al., 1985; Yang and Tang, 1992; Zhao et al., 1992), but failed to establish in the wild. One of the most important factors driving the invasion of *P. canaliculata* is environmental temperature, although many other variables, such as the level of dissolved oxygen, the pH of the water and soil moisture during dormancy are implicated in the success of over-wintering (Ito, 2002). Environmental temperature not only determines the establishment of cold-blooded animals, including *P. canaliculata*, but also affects their reproductive rate and hence population dynamics.

Vector-borne diseases are generally expected to expand their range (Githeko et al.,

2000) or shift geographically (Lafferty, 2009) as a result of climate change (i.e. human-induced rise in temperature). Here, we explore the interplay of invasive *P. canaliculata*, climate change and transmission dynamics of *A. cantonensis*. In a first step, we develop a biological model of *A. cantonensis* transmission with *P. canaliculata* considered as the only intermediate host using key parameters to fit the current distribution in P.R. China. Second, we assess potential impacts of climate change on the distribution of *P. canaliculata* and consider the transmission dynamics of *A. cantonensis* in a future, warmer P.R. China.

## 8.3. Methods

### *8.3.1. Current distribution of* P. canaliculata *and* A. cantonensis

Two data sources were used to delineate the current distribution of *P. canaliculata* and *A. cantonensis* in P.R. China. The first is the national random sampling survey of *A. cantonensis* and its intermediate hosts, carried out in P.R. China in 2006 and 2007 (Lv et al., 2009b). The surveyed areas had been determined based on the potential presence of *P. canaliculata*, i.e. where more than one generation per year could survive according to a degree-day model. A grid sampling strategy was employed to identify the survey sites, with their geographical coordinates recorded using hand-held global positioning system devices (GPSmap 70; Kansas, USA). *P. canaliculata* was collected from natural habitats near human settlements (e.g. ponds, crop fields and streams). Snails were examined for the presence of *A. cantonensis* using lung-examination and artificial digestion (Lv et al., 2009b; Lv et al., 2009c). Other snails, including *Achatina fulica* (Bowdich, 1822) (Gastropoda: Achatinidae) and terrestrial slugs were also collected and examined using artificial digestion. Rats were trapped and dissected for the presence of adult *A. cantonensis* in lung arteries and the heart. The results of this survey revealed that both *P. canaliculata* and *A. cantonensis* are widely endemic in southern P.R. China (Figure 8.1).

The second source pertained to areas beyond the current distribution range of the snail identified by the national sampling survey (indicated with stars in Figure 8.1). Specifically, we collected relevant information including snail species and exact location from Chinese newspapers and Internet-based reports published between January 2008 and December 2009.

Our assumption was that the current distribution of *P. canaliculata* in P.R. China reflects the maximum extent possible under prevailing environmental conditions. Two considerations support this assumption. First, *P. canaliculata* had been repeatedly introduced into P.R. China in the 1980s, but became established only in southern provinces. Second, the isolated population in the Sichuan basin was established in the late 1980s, but failed to expand to the middle or lower Yangtze River valley. It is important to note that 'establishment' areas, which include the distribution according to local reports, usually consist of isolated populations, and hence are difficult to discover by a random sampling survey. The current distribution of *P. canaliculata* in P.R. China was therefore stratified into two regions; (i) the aforementioned 'establishment' area and, (ii) the 'spread' area, which corresponds to the habitat where *P. canaliculata* snails were readily found during the national sampling survey.

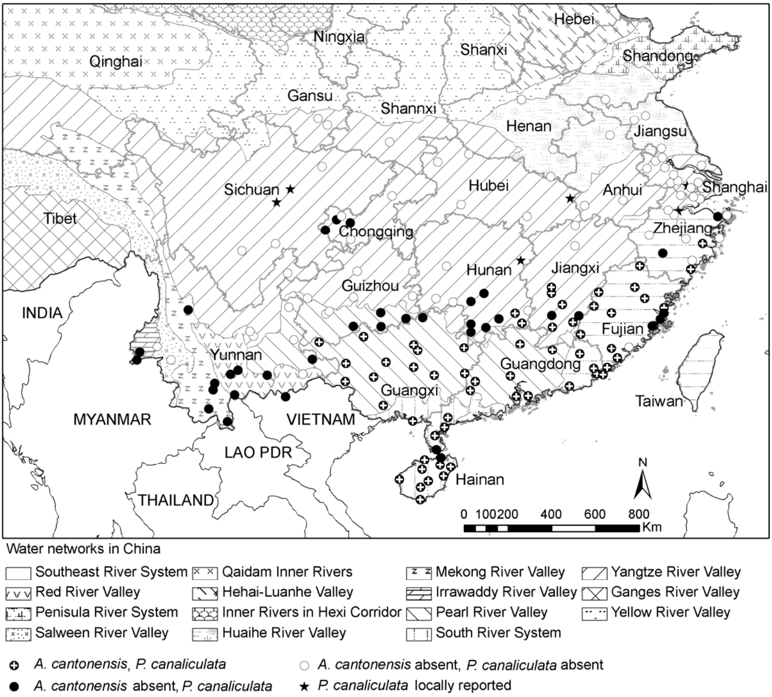

**Figure 8.1** Current distribution of *Pomacea canaliculata*, *Angiostrongylus cantonensis* and major river catchments in P.R. China.

### *8.3.2. Key transmission factors of* A. cantonensis

The transmission of *A. cantonensis* is complex and involves two distinct phases; namely, parasitism in a warm-blooded animal (i.e. rats), and in a cold-blooded animal (i.e. molluscs). Only the latter phase is markedly affected by environmental temperatures. Moreover, the transmission can involve other mollusc species whose roles in the transmission may be asymmetrical. For example, terrestrial slugs and snails may play a role in sustaining the life cycle of *A. cantonensis* in a particular location. Here, we consider only *P. canaliculata* because it is by far the most important snail species implicated in human infections in P.R. China (Lv et al., 2008). Moreover, this species can spread *A. cantonensis* over long distances via water networks and it occurs in larger habitats than, for example, *A. fulica*, another intermediate host snail found in P.R. China (Lv et al., 2009b).

We developed a biological model and considered several factors that relate to temperature, such as the population density of the snail intermediate host, the developmental period of *A. cantonensis* within *P. canaliculata* and the transmission season of *A. cantonensis* (Figure 8.2). Since snail dormancy may occur, the longevity of infected rats and the parasite and the cold tolerance of snails and parasites were also taken into account. Parameters used in our model are summarised in Table 8.1 and further detailed below.

The first parameter pertains to the generation intensity of *P. canaliculata*, which is determined by the accumulated effective thermal energy (AET) and developmental threshold temperature (DTT). These two parameters are derived from empirical theory, which assumes that a plant or a poikilotherm animal develops only once the environmental temperature is higher than a specific minimal threshold, and that the total thermal energy required for this development is constant (Higley and Haskell, 2001). We used the parameters derived from the data of Zhou *et al*. (2003) to calculate the generation intensity of *P. canaliculata* (see equation 1).

$$Gen = \frac{AET_y}{AET_t} \quad \text{..........} \quad (1)$$

*Gen* is the generation intensity (number of generations per year), $AET_y$ is the yearly AET, and $AET_t$ denotes the AET for one generation.

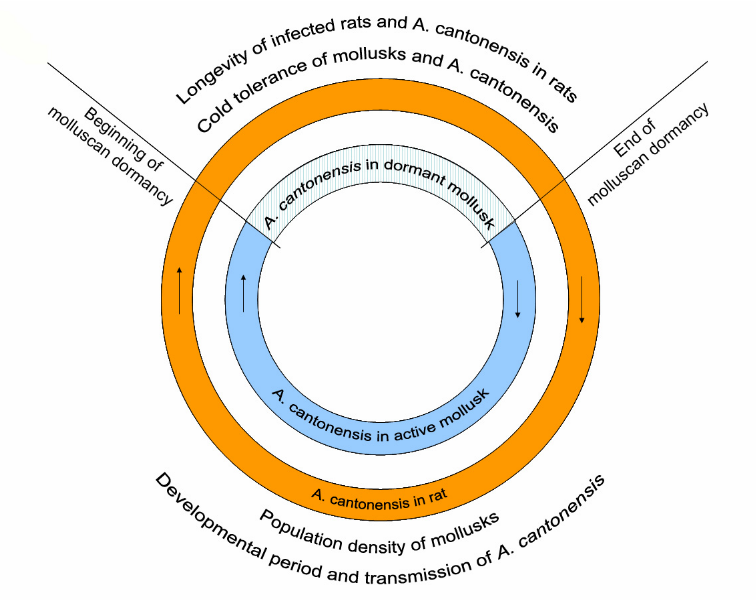

**Figure 8.2** Determinants of the transmission of *Angiostrongylus cantonensis*, with an emphasis on factors pertaining to environmental temperature. The two circles represent distinct phases in the life cycle of *A. cantonensis*.

In our study, the lower limit of the generation intensity delimiting the potential range of *P. canaliculata* was fitted using the known distribution. First, we selected the point with the lowest generation intensity as the index point on the current distribution limits of the snails. The boundary was produced using the contour function of ArcGIS (version 9.1, ESRI, USA).

The second parameter considered is the developmental period of *A. cantonensis* within *P. canaliculata*. Of the five larval development stages, the first three ($L_1$-$L_3$) take place within the snail, and hence are directly influenced by temperature. On the other hand, the fourth and fifth larval stages ($L_4$ and $L_5$), as well as adult worms are barely affected by the environmental temperature, as they take place within rats. The period between $L_3$ larvae infecting rats and the emergence of $L_1$ larvae in rat faeces is 5-6 weeks, but depends

**Table 8.1** Parameters considered for modeling and predicting the transmission of *Angiostrongylus cantonensis* in P.R. China

| Host/parasite | Determinant | Parameter | Values | Unit | References |
|---|---|---|---|---|---|
| *Pomacea canaliculata* | Generation intensity | DTT* | 11.67 | °C | Zhou *et al*. (2003) |
| | | AET* | 1404.1 | Degree-day | Zhou *et al*. (2003) |
| | Cold tolerance | Low temperature for 'spread' | 5.43 | °C | † |
| | | Low temperature for 'establishment' | 3.62 | °C | † |
| | Dormancy | Threshold temperature | 17.0 | °C | Yin *et al*. (2006) |
| Rat | Longevity of infected rats | Life span | 173.2 | Day | †† |
| *Angiostrongylus cantonensis* | Generation intensity | DTT* | 15.04 | °C | Lv *et al*. (2006b) |
| | | AET* | 262.5 | Degree-day | Lv *et al*. (2006b) |
| | Transmission season | Days above 15.04 °C | 212 | Day | † |
| | Cold tolerance | Low lethal temperature | - | °C | ‡ |
| | Longevity in rats | Life span | 414.2 | Day | Kino (1984) |

* DTT = developmental threshold temperature; AET = accumulated effective thermal energy
† Fitted according to the current distributions
†† Obtained from a fitted function based on published data
‡ No specific data available, but cold tolerance of nematode considered equal or greater than that of their host, as supported by experiments

on worm burden (Kino, 1984). The DTT and AET for this phase have been determined in the laboratory under constant temperature (Lv et al., 2006b). A linear equation based on a degree-day model was fitted in this experiment to describe the developmental period of *A. cantonensis*, with key parameters summarised in Table 8.1. The generation intensity within the intermediate host can be determined by equation 1.

The third parameter relates to the transmission season of *A. cantonensis*. Transmission is on hold during the dormant period of the snail intermediate host. However, the transmission may be interrupted even during periods when snails are active if the development of *A. cantonensis* has not been triggered. Therefore, the DTT of *A. cantonensis*, determined at 15.04 °C, is a key factor considered in our model. The number of days with mean temperature above 15.04 °C has thus been retained in the model.

The fourth parameter considered is the cold tolerance of *P. canaliculata* and *A. cantonensis*. Reliable experimental data pertaining to cold tolerance of *P. canaliculata* are not available (Howells et al., 2006). A field observation made in Japan found a linear relationship between winter (December to February) air temperature and mortality of *P. canaliculata* (Shoubu et al., 2001). The minimal threshold temperature for snail survival was estimated at 9.67 °C. However, a preliminary analysis using the winter temperature of December to February showed that the distribution of *P. canaliculata* in P.R. China stretched far beyond this 9.67 °C isotherm. Indeed, the present global distribution of the snail further indicates that populations can tolerate winter temperatures of 4-5 °C (Cazzaniga, 2006). Therefore, we used the current distribution of *P. canaliculata* in P.R. China to fit the potential range. First, we determined the mean January temperature (the lowest monthly temperature in P.R. China) of each endemic focus and selected the location with the lowest value as an indication of northernmost extent of ‘spread’ and ‘establishment’ regions, and then produce the isolines using the contour function of ArcGIS.

We are not aware of data pertaining to cold tolerance of *A. cantonensis* within *P. canaliculata*. However, the cold tolerance of *A. cantonensis* within *A. fulica* has been assessed; the larvae were found to be alive and infective after snails had been kept at 0 °C for a period of seven days (Alicata, 1967). Although the viability of the snails themselves had not been reported, many other experiments demonstrated that parasites have equal or greater cold tolerance than their hosts (Smith et al., 2008; Tyrrell et al., 1994; Woodhams et al., 2000). Hence, we assumed that the survival of *A. cantonensis* in low temperatures is equal to or higher than that of *P. canaliculata*.

The fifth parameter relates to the longevity of infected rats and *A. cantonensis* in rats. The leading definitive host is *Rattus norvegicus* (Berkenhout, 1769). Most of the trapped wild *A. cantonensis*-infected rats were infected with fewer than 30 adult worms and the average number of worms (worm burden) carried by an individual rat reported in different publications ranged from 2 to 19.9. A few rats were found to be infected with up to 90 worms (Ding et al., 1982; Ye et al., 2007). Laboratory experiments demonstrated that heavily infected rats (>30 adult worms) usually died within three months (Kino, 1984; Liang et al., 1984). We therefore concluded that the longevity of *A. cantonensis*-infected rats is associated with worm burden (Kino, 1984).

We fitted an exponential function relating longevity of *A. cantonensis*-infected rats and worm burden based on an adaptation of data derived from experiments conducted by Kino (1984). The literature on field surveys of rats infected with *A. cantonensis* in P.R. China was reviewed, and the average worm burden among wild rats determined as 11.30. The mean life span of infected rats was estimated at 173.2 days according to an exponential function and the average worm burden mentioned above. We assumed that *A. cantonensis* transmission ceased if snails cannot ingest infective rat faeces for a period equal to or longer than the mean longevity of infected rats. The mean reproductive duration of the worms was estimated to be as long as 414.2 days in rats carrying two worms (one female and one male) (Kino, 1984). Therefore, the lifespan of *A. cantonensis* in rats does not influence the life cycle in case of dormancy of *P. canaliculata*.

The sixth and final parameter considered is the dormant period of *P. canaliculata*. A decrease in activity among intermediate host snails eventually interrupts the transmission of *A. cantonensis*. We defined dormancy as a significant decrease in snail activity from the perspective of *A. cantonensis* transmission. Under natural conditions, *P. canaliculata* becomes inactive and enters dormancy if the water temperature drops below 17 °C (Ito, 2002; Yin et al., 2006). In our study, we set the water temperature equal to the air temperature, because *P. canaliculata* usually lives in shallow water and is active close to the water surface where temperatures approximate those of the air. The number of days with mean temperatures below 17 °C was designated as the dormant period.

### *8.3.3. Climate data*

Climate data, specifically daily minimum, mean and maximum temperatures at 740 observing stations across P.R. China, were obtained from the Chinese National Satellite Meteorological Center in Beijing. Stations with incomplete data were excluded from the analysis. For each station, the geographical coordinates were assigned and the temperature at locations between observing stations was estimated using ordinary kriging. This geostatistical technique is used to infer a value for non-surveyed location based on observed values in neighbouring points. Temperature data spanning one decade (i.e. 1991-2000) were employed for analyzing the distributions of *P. canaliculata* and *A. cantonensis*.

The predicted temperature data from 1991-2100 were derived from the PRECIS model (PRECIS stands for ‘Providing Regional Climates for Impacts Studies’, which downscaled the UK Hadley Centre Atmospheric Model (HadAM3), based on the ‘Special Report on Emissions Scenarios’ (SRES) A2 (Nakicenovic et al., 2000)). The spatial resolution of the dataset is 50 × 50 km, and contains more than 10,000 grid points with daily mean temperatures at the respective grid points, and covers the entire area of P.R. China. In the PRECIS model the climate data for the period 1961-1990 were considered as the baseline. The 2020s (2021-2030) and 2030s (2031-2040) were selected as the target decades for predicting the distributions of *P. canaliculata* and *A. cantonensis*.

To explore whether the predicted data were accurate, we compared the real temperature data observed between 1991 and 2000 with the predicted data for the same period. We first used the predicted average annual temperature to generate a smooth prediction map of P.R. China using a geographical information system (GIS) platform and then extracted the values at the corresponding observing stations. The difference was estimated for each station by comparing real data with those predicted by the model. The difference was employed to produce another smooth map, and the corresponding values were extracted. Finally, the adjusted daily temperature values were obtained by adding the differences to the predicted daily temperature.

### *8.3.4. Modeling and mapping in a GIS platform*

We used ordinary kriging as an interpolator to predict values at non-sampled locations, employing a GIS established in ArcMap. Data, including the generation intensity for snails and parasites and dormant periods, were transformed to achieve normal distributions using a square-root transformation. Kriging models with different orders were compared using standard tools for comparison, and the best-fitting model was selected. Surface analysis (i.e. the contour function) was employed to produce isolines of the mean January temperature, generation intensity and snail dormant period.

The potential range of *P. canaliculata* was determined by superpositioning the respective areas determined by the lower limit of the generation intensity and cold tolerance of the snail. The distribution of *A. cantonensis* was determined using a combination of several factors with details provided in Table 8.1. The at-risk areas for transmission of *A. cantonensis* were predicted for the 2020s and 2030s.

We defined 'vulnerable areas' as regions where a small change (e.g. one standard deviation (SD)) of a climatic factor (e.g. mean January temperature) or a biological factor (e.g. snail dormant period) would tip the status of the region, e.g. from 'establishment' to 'spread'. To identify the 'vulnerable areas' we assessed the annual geographical variation of the mean January temperature and dormant period of *P. canaliculata*. For each observing station or grid point, we used the average plus one SD as the upper value and the average minus one SD as the minimum extent, and then generated prediction maps, employing ordinary kriging. The same isolines were identified in all maps and the region between them was considered the vulnerable area.

## **8.4. Results**

### *8.4.1. Current distribution of* P. canaliculata *in P.R. China, including uncertainty*

A total of 98 endemic foci were identified during the national sampling survey (Figure 8.1), and the area where they were found consequently classified as 'spread' regions. Among them, Dali in the northern part of Yunnan province showed the lowest annual thermal energy for the development of *P. canaliculata*, allowing only 1.12 (indicated as square root, i.e. 1.06 in Figure 8.3a) generations per year. The 'establishment' region was determined based on six isolated populations identified from local reports. Of these, Dayi on the western edge of the Sichuan basin had the lowest annual thermal energy, allowing only 1.09 (square root 1.04 in Figure 8.3a) generations per year. Areas characterised by more than 1.12 or 1.09 generations, respectively consist of two major parts; the first is in southeast P.R. China and the second in the desert area of Xinjiang province in the northwest of the country.

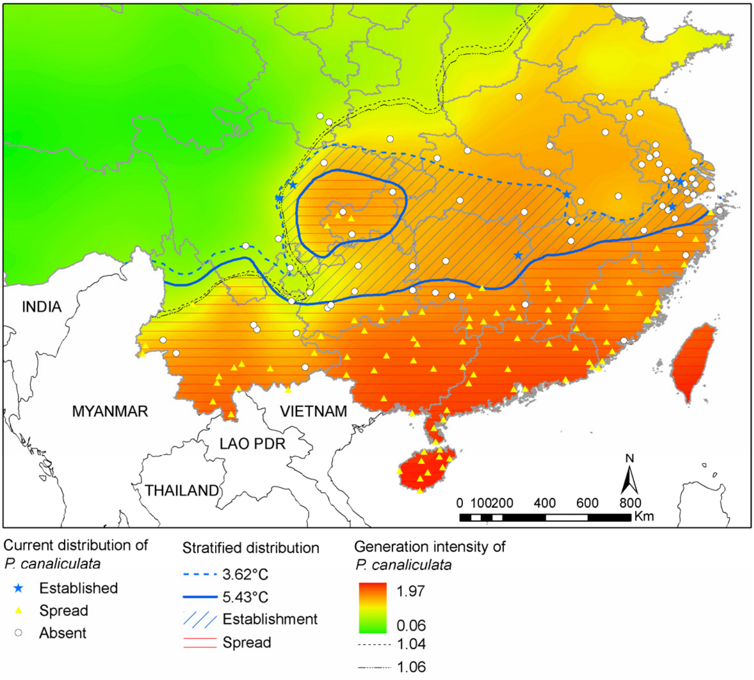

**Figure 8.3a** Current distribution of *Pomacea canaliculata* in P.R. China. The distribution is stratified into 'spread' and 'establishment' regions.

With a mean January temperature of 5.43 °C, Jiangdong in eastern Zhejiang province had the lowest temperature among all endemic foci in the 'spread' region. Yingshan in eastern Hubei province had the lowest mean January temperature (3.62 °C) among the six foci in the 'establishment' region. Considering these isotherms and generation contours, we predicted that *P. canaliculata* can only become firmly established in the southeastern part of the country (Figure 8.3a). Our data suggest that compared to the annual thermal energy required for the development of *P. canaliculata* (more than one generation per year), cold tolerance is the crucial factor determining its distribution.

The 'spread' region covers the provinces of Guangdong, Hainan and Fujian and Guangxi Zhuang autonomous region, as well as the southern parts of Yunnan, Guizhou, Hunan, Jiangxi and Zhejiang provinces. Isolated areas occur in the eastern part of Sichuan province and the western part of Chongqing municipality. The 'establishment' region mainly consists of the northern parts of Guizhou, Hunan, Jiangxi and Zhejiang provinces, as well as southern Hubei and Jiangsu provinces. Shanghai municipality and the eastern parts of Sichuan province and Chongqing municipality also form part of the 'establishment' area. The areas of the 'spread' and 'establishment' regions were 1,217,434 km$^2$ and 554,658 km$^2$, respectively (Table 8.2).

**Table 8.2** Current and predicted distribution range of *Pomacea canaliculata* and *Angiostrongylus cantonensis* in P.R. China

| Region name | At present | 2020s (2021-2030) | | 2030s (2031-2040) | |
|---|---|---|---|---|---|
| | Area (km$^2$) | Area (km$^2$) | Change (km$^2$)* | Area (km$^2$) | Change (km$^2$)* |
| 'Spread' region of *P. canaliculata* | 1,217,434 | 1,531,810 | 314,376 | 1,910,510 | 693,076 |
| 'Establishment' region of *P. canaliculata* | 554,658 | 452,908 | -101,705 | 327,806 | -226,807 |
| Endemic area of *A. cantonensis* | 881,735 | 1,494,014† | 612,279 | 2,199,694‡ | 1,317,959 |
| Overlap** | 874,690 | 1,311,943 | 437,253 | 1,697,181 | 822,491 |

* Increment compared to present situation

** Between endemic area of *A. cantonensis* and 'spread' region of *P. canaliculata*

† Including 24,188 km$^2$ in desert area in central part of Xinjiang Uyghur autonomous region

‡ Including 238,364 km$^2$ desert area in the central part of Xinjiang Uyghur autonomous region

The locations of the 5.43 °C and 3.62 °C mean January isotherms varied considerably between 1991 and 2000 (Figure 8.4). In the 'spread' region, the geographical variation was considerable between the isotherm of 5.43 °C and the line of 5.43 °C plus one SD, particularly in the central area, while only a slight parallel change was found between the average line and the line of 5.43 °C minus one SD. The main variation was observed in the eastern part of the country. A major overlap of variations was observed in both regions, indicating wide fluctuations in winter temperatures during the period 1991-2000.

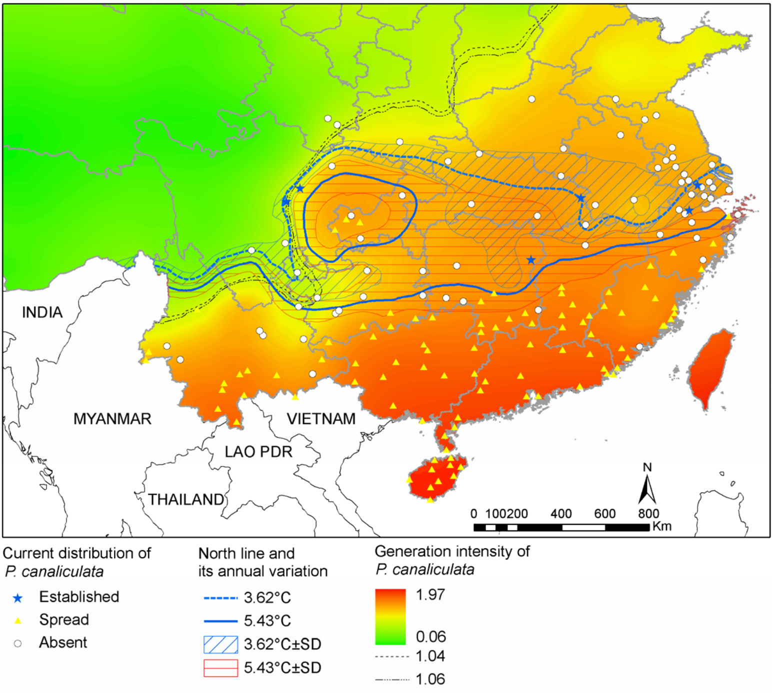

**Figure 8.4** Annual variation of the northernmost limit of the 'spread' and 'establishment' regions of *Pomacea canaliculata* in P.R. China. The thick lines describe the average extent of each region in the 1990s.

### *8.4.2. Potential change in the distribution of* P. canaliculata *in the 2020s and 2030s*

According to our predictions, the 'establishment' region will decrease and move northeastwards by the 2020s and 2030s (Figs. 8.5a and 8.6a). However, the 'spread' region will significantly expand and include almost the whole current 'establishment' region by the 2030s. Taking into consideration both generation intensity and cold tolerance of *P. canaliculata*, an estimated net increase of 314,376 km$^2$ of the 'spread' region and a decrease of 101,705 km$^2$ of the ‘establishment’ region is predicted for the 2020s. A major increase in the ‘spread’ region is predicted to occur near the boundary between Hunan, Jiangxi and Hubei provinces (Figure 8.5a). In addition to a decrease in the surface area, the ‘establishment’ region is predicted to move northeast, involving the central part of Anhui province and the southern part of Jiangsu province.

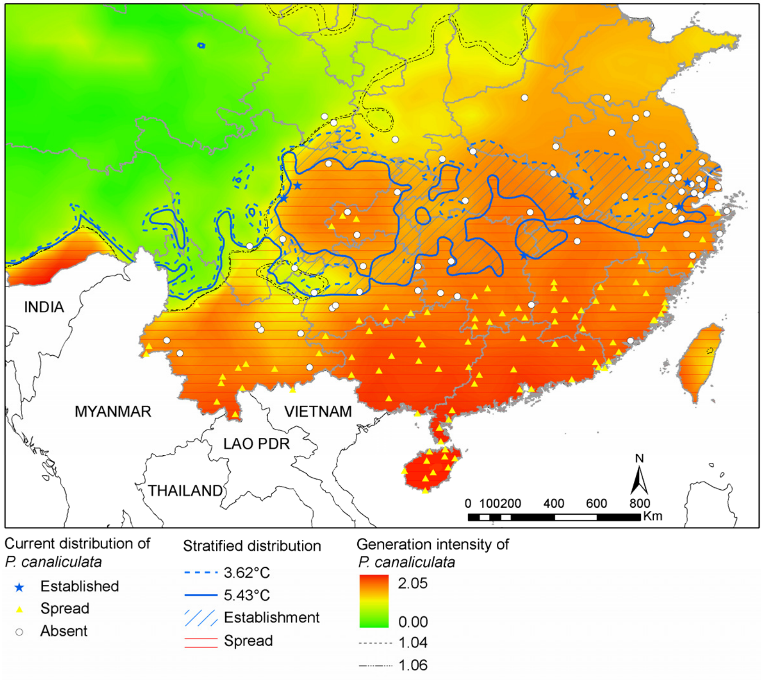

**Figure 8.5a** The potential distribution of *Pomacea canaliculata* in P.R. China in the 2020s. The distribution is stratified into ‘spread’ and ‘establishment’ regions.

Our predictions suggest that the ‘spread’ region will have further expanded northwards by the 2030s. Almost the entire Chongqing municipality as well as Hunan, Hubei, Jiangxi and Zhejiang provinces will become ‘spread’ areas (Figure 8.6a). The predicted increase in the surface area of the ‘spread’ region between the 2020s and 2030s is 378,700 km$^2$. The ‘establishment’ region will further move northeast, mainly including the Huaihe River valley in southern Henan and Anhui provinces, and central Jiangsu province. The decrease in the ‘establishment’ region between the 2020s and the 2030s is predicted at 125,102 km$^2$.

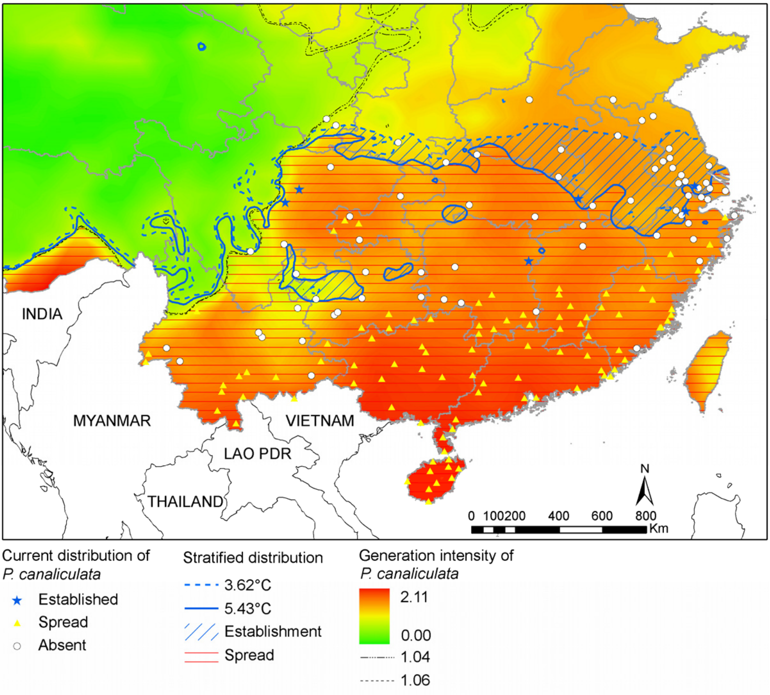

**Figure 8.6a** The potential distribution of *Pomacea canaliculata* in P.R. China in the 2030s. The distribution is stratified into 'spread' region and 'establishment' region.

### *8.4.3. The potential impact of climate change on* A. cantonensis *transmission*

The parameters considered in our biological model for assessing the distribution of *A. cantonensis* include (i) the thermal requirement for the development of the parasite in the snail intermediate host (generation intensity), (ii) the dormant period of *P. canaliculata*, (iii) the longevity of *A. cantonensis*-infected rats, (iv) the longevity of the parasite in rats, (v) the transmission season, and (vi) the cold tolerance of the parasite within the snail. In a first step, each parameter was used in our model and the predicted distribution of *A. cantonensis* was compared with the real distribution. Since the latter three parameters resulted in less meaningful predictions (i.e. the predicted ranges were far beyond the real distribution) than the former three, they were excluded from the final model.

The generation intensity of 5.29 (square root 2.30; see Figs. 3b, 5b and 6b) reflects the least stringent thermal requirements for parasite survival according to the current distribution of *A. cantonensis*, but is not the key factor to determine the range of *A. cantonensis* (Figs. 3b, 5b and 6b). Given that 17 °C is the threshold temperature for inducing winter dormancy in *P. canaliculata*, the potential *A. cantonensis* endemic area is where the dormant period is less than 173.2 days, or the active period is more than 191.8 days (square root 13.85; see Figs. 3b, 5b and 6b) according to the longevity of *A. cantonensis*-infected rats with an average worm burden of 11.3 per rat. The predicted range of *A. cantonensis* matches the one currently observed, except for Yunnan province. The isoline of 191.8 days shows considerable variation between 1991 and 2000, especially in central and eastern P.R. China, including Hunan, Hubei, Jiangxi and Zhejiang provinces.

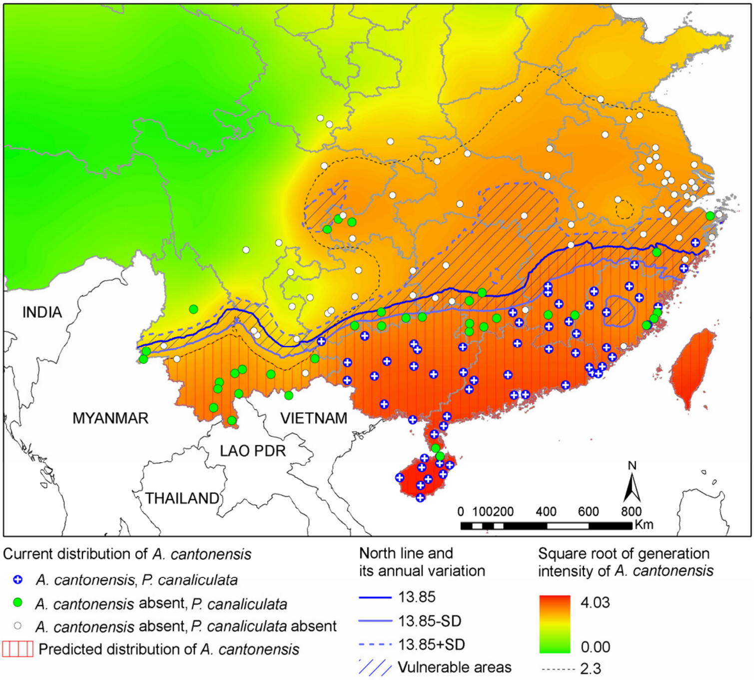

**Figure 8.3b** Current distribution of *Angiostrongylus cantonensis* in P.R. China. Coexistence of *A. cantonensis* and *Pomacea canaliculata* at each sampled site is shown in three groups. 13.85 denotes the square root of 191.8 days which is the limit active period of *P. canaliculata*.

Considering a minimal generation intensity of 5.29 for *A. cantonensis* and an active period for *P. canaliculata* of 191.8 days, the current surface area where transmission of *A. cantonensis* can occur is 881,735 km$^2$. According to our model, the potential transmission area for *A. cantonensis* will significantly expand by the 2020s (Figure 8.5b) and the 2030s (Figure 8.6b). Compared to the present situation, the increase will be 612,279 km$^2$ and 1,317,959 km$^2$, respectively. Variation analysis of the 13.85 line (square root of 191.8 days) revealed considerable spatial heterogeneity. At present, the variable area mainly lies in the central part, while the area is predicted to shift northeast in the 2020s and the 2030s.

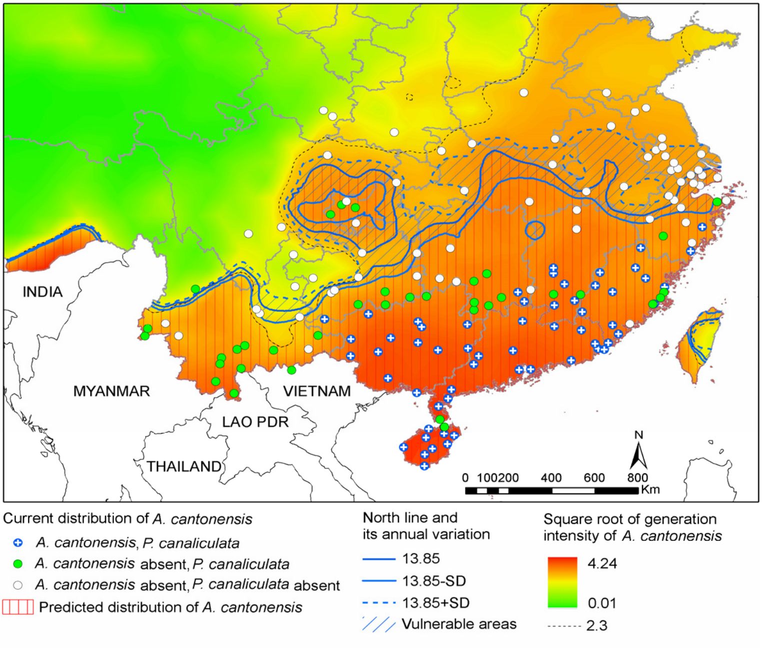

**Figure 8.5b** The potential distribution of *Angiostrongylus cantonensis* in P.R. China in the 2020s. Coexistence of *A. cantonensis* and *Pomacea canaliculata* at each sampled site is shown in three groups. 13.85 denotes the square root of 191.8 days, which is the limit active period of *P. canaliculata*.

The endemic area of *A. cantonensis* shows a parallel increase to that of *P. canaliculata*, especially in the central and eastern parts of P.R. China. An increase in the southwestern part is also observed. The overlap area between the predicted endemic area of *A. cantonensis* and the potential distribution of *P. canaliculata* is estimated to double by the 2030s compared to the present situation. Meanwhile, the fraction of this overlap accounting for *A. cantonensis*-endemic area is predicted to decrease from 99.2% at present to 87.8% in the 2020s and 77.2% in the 2030s.

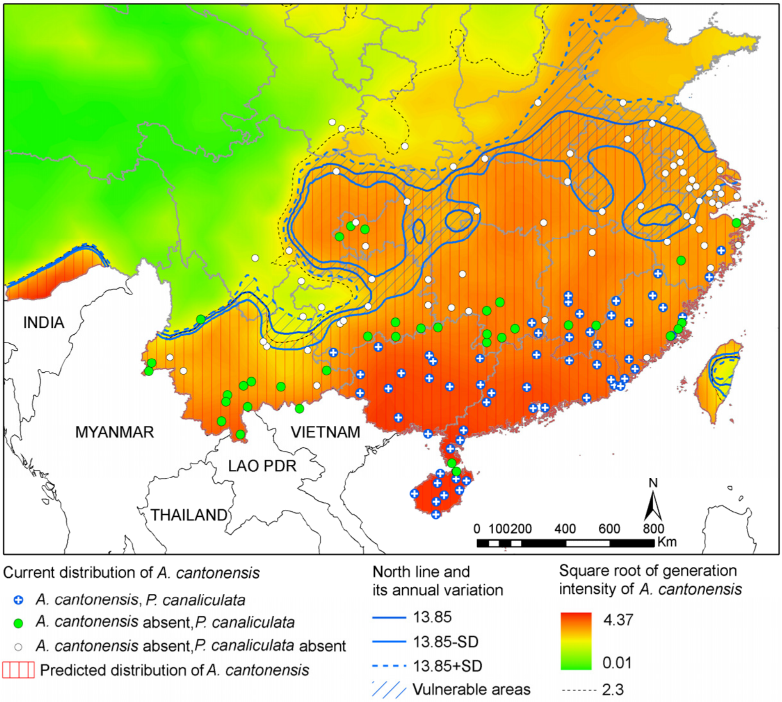

**Figure 8.6b** The potential distribution of *Angiostrongylus cantonensis* in P.R. China in the 2030s. Coexistence of *A. cantonensis* and *Pomacea canaliculata* at each sampled site is shown in three groups. 13.85 denotes the square root of 191.8 days, which is the limit active period of *P. canaliculata*.

## 8.5. Discussion

We have established a biological model to deepen our understanding of the transmission of *A. cantonensis* in P.R. China, including the role of an invasive snail species. The key features considered in the model consisted of the thermal requirement for the development of the parasite and the intermediate host snail, the dormant period of *P. canaliculata* and the longevity of *A. cantonensis*-infected rats. Importantly, our biological model facilitated appraisal of a potential impact of climate change; hence we generated predictive risk maps for the distribution of *A. cantonensis* and *P. canaliculata* in a warmer future. Our predictions suggest that the *A. cantonensis*-endemic area will expand considerably along with a further spread of *P. canaliculata*. Our results raise several issues relevant to parasite-host interactions in the context of climate change, which we offer for discussion.

### *8.5.1. Emerging infectious diseases driven by invasive species and climate change*

Many factors are involved in the emergence of infectious diseases (Morens et al., 2004; Morse, 1995). Among them, biological invasion is a central concern (Daszak et al., 2000; Enserink, 2007; Juliano and Lounibos, 2005). According to Mooney & Hobbs (Mooney and Hobbs, 2000), climate change is expected to exacerbate the invasion of exotic species, including vectors and intermediate hosts of pathogens. Indeed, an invasive freshwater snail species, i.e. *P. canaliculata*, has been identified as the major source of human angiostrongyliasis and a leading intermediate host of *A. cantonensis* in P.R. China (Lv et al., 2009b; Lv et al., 2008). Our results now suggest that the distribution of this invasive snail is likely to further expand in the face of climate change. Compared to the present situation, the predicted 'spread' area of *P. canaliculata* will increase by 25.8% in the 2020s and 56.9% in the 2030s. Most of this increase is predicted for the Yangtze River valley, the largest freshwater network in the country (Fu et al., 2003). The predicted expansion of *P. canaliculata* will potentially drive the current *A. cantonensis*-endemic area further north. Major water resources developments, particularly the South-to-North water transfer project, might further fuel the spread of *P. canaliculata*, similar to the spread of *Oncomelania hupensis*, the intermediate host snail of *Schistosoma japonicum* (Yang et al., 2005; Zhou et al., 2008).

In our study the overlap between potential distribution ranges of *P. canaliculata* and *A. cantonensis* was used to illustrate the role of this snail in the transmission dynamics of the parasite. The results show that the fraction of this overlap in the whole endemic area of *A. cantonensis* will decline, which indicates that the parasite will expand more rapidly than the intermediate host snail. In P.R. China, *A. fulica* is another snail species implicated in the transmission of *A. cantonensis* (Lv et al., 2009b), and hence the overlap region depicted in our prediction map might be an underestimation of the real future scenario. Despite this shortcoming, our model predicts that the endemic area of *A. cantonensis* will double, when comparing the present situation with that of the 2030s. Hence, the interplay of invasive snail species and climate change is likely to drive the endemicity of *A. cantonensis*, which is of considerable public health relevance.

In addition to the role of transmission, the further spread of *P. canaliculata* may facilitate the establishment of new transmission patterns. Previous field-based research suggests that the *A. cantonensis* infection intensity among terrestrial snails and slugs is significantly higher than that among freshwater snails including *P. canaliculata* (Deng et al., 2008; Hu et al., 2007; Li et al., 2006; Zhang et al., 2007). A possible explanation is that freshwater snails usually are exposed to lower concentrations of *A. cantonensis* $L_1$ larvae because rat faeces are diluted once they reach freshwater bodies. In case that *P. canaliculata* becomes the dominant intermediate host in the area where *A. cantonensis* transmission is likely to occur, it is conceivable that the infection pressure will decrease, resulting in lighter infections in wild rat populations, and hence a prolonged lifespan of infected rats compared to the current situation. A new parasite-host equilibrium might then be established when this interaction pattern between *A. cantonensis* and *P. canaliculata* shifts from the original endemic areas where terrestrial slugs and snails were abundant.

### *8.5.2. Transmission dynamics of parasites from a biological and ecological perspective*

A complex life cycle calls for a detailed biological model to describe the transmission dynamics. Unlike their vectors or intermediate hosts, parasites often are not directly exposed to environmental factors. Some parasites also have to switch their hosts to accomplish their life cycle. Different hosts do not necessarily share the same spatial distribution and ecological requirements, and hence exert asymmetrical roles in the life cycle and transmission of parasites. The transmission of *A. cantonensis* can serve as an example. While several freshwater snail species are susceptible to this nematode (Chang et al., 1968; Lv et al., 2006a; Yousif and Lammler, 1975), natural infections are rare (Lv et al., 2009b). An explanation is that most of these snails live in deep or fast-flowing water and thus contact with rat faeces is unlikely to occur. *R. norvegicus*, the most common definitive host of *A. cantonensis* (Lv et al., 2008) mainly inhabits human settlements. As a result, the mollusc infection rate and worm burden in close proximity to human habitations are significantly higher than further away (Li et al., 2006).

The longevity of vectors or intermediate hosts is an important factor to sustain the life cycle of parasites. For example, malaria parasites have to accomplish their development within female *Anopheles* mosquitoes before the vectors die (Rogers and Randolph, 2000). In our study the longevity of *P. canaliculata* is not considered since these snails usually live more than one year under suitable conditions (Cazzaniga, 2006) and are rarely influenced by infection (Lv et al., 2006a). In contrast, the life expectancy of *A. cantonensis*-infected definitive hosts (i.e. rats) is significantly affected by their worm burden (Kino, 1984). We have explicitly taken this issue into account in our model, after reviewing available data and determining the relationship between worm burden and rat longevity. This consideration brings to the fore another ecological issue, namely the dormant period of molluscs, which is closely associated with environmental temperature. Our biological model considering the two aspects indeed shows a better fit to the real distribution of *A. cantonensis* than the parasite generation intensity alone.

Asymmetrical effects of climate change on different components of parasite-host models may also influence parasite transmission dynamics. Parasites and hosts may respond to climate change in different ways. Under a scenario of rising temperatures, the development of parasites and hosts may often be accelerated, but at different rates, due to specific energetic efficiency and metabolic rates (Hellmuth & Johnson, 2010). In our study, DTT and AET differ between *A. cantonensis* and *P. canaliculata*, as manifested in different shifts in the distribution resulting from the same temperature change. Additionally, our results suggest that winter temperatures considerably affect the distribution of *P. canaliculata*, while transmission of *A. cantonensis* is driven mainly by the dormant period of molluscs and virulence in the rat definitive host. Appraisal of a potential impact of climate change on vector-borne diseases should therefore consider an ecological perspective.

### *8.5.3. Vulnerable area to climate change*

Invasive species typically go through three phases; namely, introduction, establishment and spread (Jeschke and Strayer, 2005). For the establishment and spread phases, a growing population is required (Arim et al., 2006). Many environmental factors such as temperature can influence the growth of a population (Crooks and Soule, 2001). Suboptimal temperature may result in a lag in the population growth, and hence interrupt further spread. Rising temperatures may change the vulnerable area to a spread region.

Annual variation of the mean January temperature accounts for the current distribution of *P. canaliculata* in P.R. China. January temperatures are highly variable from one year to another as is evident from the large area between the isotherms of the average temperature and plus/minus one SD. In the 'establishment' region, the isotherms showed dramatic shifts between subsequent years in the 1990s. The unstable temperature probably results in a high interannual variation in *P. canaliculata* population sizes, and hence does not provide an environment for stable growth, which is a prerequisite for expansion. In contrast, temperatures in the 'spread' region are more stable and suitable for *P. canaliculata* as suggested by an isotherm of 5.43 °C minus one SD which is close to the 5.43 °C isotherm. The climate data based on SRES A2 suggest a significant increase in winter temperatures by the 2030s. Thus, the 'establishment' areas will become part of the 'spread' region.

We also analysed the variation in the dormant period of *P. canaliculata*, which is relevant to the transmission of *A. cantonensis*. The results show that there is considerable variation between the line of 13.85 and that of 13.85 minus one SD, especially in the central part. This vulnerable area probably becomes *A. cantonensis*-endemic if the dormant period falls below 173.2 days along with rising temperatures. Our findings for the 2020s and 2030s underscore this issue.

### 8.6. Conclusions and research needs

In this study the current distribution of *P. canaliculata* and *A. cantonensis* were fitted using a model built around key biological determinants and environmental temperature. The model was then used to assess a potential impact of climate change on the transmission dynamics of *A. cantonensis*. The most important finding of our investigation is that, in face of climate change, the distribution of an invasive freshwater snail species will change, and hence exacerbate the endemicity of *A. cantonensis*. Our study also suggests that geographical variation analysis is a useful tool to identify areas that are most vulnerable to the spread of intermediate hosts, and might also be applicable for vectors and parasites.

The present study calls for rigorous surveillance and identified two major research needs. With regard to surveillance, a precise knowledge of the actual distributions of the snail and the parasite are needed for further model refinement. Indeed, our findings show that the predicted range of *P. canaliculata* matched well with the known distribution, but that of the parasite requires further validation in the field. Although Yunnan province was included in the national sampling survey in 2006 and 2007, no *A. cantonensis* were discovered there (Lv et al., 2009b). However, a previous field survey demonstrated an endemic focus in a setting where Yunnan province borders Vietnam (Li et al., 1989). Hubei province was also reported endemic for *A. cantonensis* in the early 1990s (Zhou et al., 1993), but there were no further reports thereafter. Interestingly, southeastern Hubei is a region of predicted variable *A. cantonensis* endemicity according to our study. If endemicity of the parasite is confirmed in this area, it is conceivable that the parasite may also be present in the Sichuan basin, where *P. canaliculata* has been well established.

With regard to research needs, it is necessary to confirm the potential transmission pattern of *A. cantonensis* along with the spread of *P. canaliculata* in face of climate change. In case the *A. cantonensis*-*P. canaliculata* pattern holds, the worm burden of wild rats must be determined in areas where *A. fulica* is absent and terrestrial slugs are scarce. The northern part of Fujian province and southern parts of Zhejiang, Jiangxi and Hunan provinces possibly are suitable for this validation (Lv et al., 2009b).

A second research need is to develop social-ecological models to predict the impact of climate change on infectious diseases. Our biological model emphasised rising temperatures as the main effect of climate change. However, climate change goes far beyond rising temperature. The spread of vectors and intermediate hosts, and hence the transmission of a parasitic disease, is a complex process and other factors (e.g. biodiversity, water velocity and land use patterns, as well as human behaviour and socio-cultural factors) may play equally important roles in the transmission dynamics of vector-borne diseases. There is a need to identify key factors deriving from ecological niche modeling and ecosystem health approaches to address large scale effects.

In conclusion, we have shown that the interplay of an invasive species in face of climate change may result in a significant expansion of the current endemic area of an emerging parasitic disease in P.R. China. Given the public health importance of angiostrongyliasis, awareness of this disease risk and related research and surveillance must be further strengthened, and interventions implemented to halt the spread and establishment of *P. canaliculata*.

### 8.7. Acknowledgement

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# 9. Discussion and conclusions

## 9.1. Discussion

This PhD thesis in epidemiology was performed in the frame of joint programme pertaining to angiostrongyliasis research between the National Institute of Parasitic Diseases (NIPD), Chinese Center for Disease Control and Prevention in P.R. China and the Swiss Tropical and Public Health Institute (Swiss TPH). The nationwide sampling survey on *Angiostrongylus cantonensis* was carried out in 2006 and 2007 shortly after the largest outbreak of angiostrongyliasis that occurred thus far in P.R. China, involving 160 cases (see Chapter 4). During the nationwide survey, a novel detection technique (Liu et al., 2007) and the stage-specific morphology of *A. cantonensis* larvae within intermediate host (Lv et al., 2009b) were employed., These techniques greatly facilitated a smooth conduct of the survey. Findings obtained from this national survey deepened our understanding of the epidemiology of *A. cantonensis* and the techniques utilized in this survey were readily available for the subsequent emergencies, e.g. angiostrongyliasis outbreaks in Dali (2008). Additional outbreak investigations provided an opportunity to understand the entire process of outbreaks, including diagnosis and treatment (see Chapter 3). In turn, this insight accelerated the revision of diagnostic criteria put forwad by Ministry of Health (MOH) in 2006 and also facilitated the establishment of hospital-based surveillance system in eight endemic cities.

Although *A. cantonensis* has been described in P.R. China in the 1930s, little was known regarding its geographical distribution and other related issue in the presumptive “home range” until the turn of the new millennium (Prociv et al., 2000). Of course, some Chinese literature regarding *A. cantonensis* was inaccessible for the international community several decades back. Our results pertaining to the distribution of *A. cantonensis* and the key intermediate hosts have now filled some of these gaps. Additionally, we also used some worms to study the intraspecific differentiation of *A. cantonensis* in its presumptive “home range” (see Chapter 6), which now provides a basis for the studies regarding the true origin and global spread patterns. As the first step of the study of intraspecific differentiation, we determined the complete mitochondrial (mt) genomes of two rodent intra-arterial *Angiostrongylus species* (*A. cantonensis* and *A.*

*costaricensis*) which can cause human ill-health (see Chapter 5).

*Pomacea* spp. became the major source of infection in the mainland of P.R. China, and hence drove the emergence of angiostrongyliasis (Lv et al., 2008). However, the real number of species and the potential spread pattern in P.R. China still remained to be fully elucidated. Our genetic population study of *Pomacea* spp. shed new light on these issues (see Chapter 7). Although the current molecular evidence could not conclusively clarify the role of *Pomacea* spp. in the present distribution pattern of *A. cantonensis* in the mainland of P.R. China, its potential impact in the face of climate change is considerable (see Chapter 8).

Several other papers and reviews from our group are not included as separate chapters in this thesis (see CV and publication list towards the end of this document). However, the remainder of this discussion draws on these complementary papers and reviews (i) to update the epidemiology of eosinophilic meningitis, (ii) to reveal the current distribution and potential dynamics of *A. cantonensis* in the “home range”, (iii) to highlight the potential impact of biological invasion on local infectious disease, and (iv) to establish a biology-driven model of *A. cantonensis* in face of climate change.

### *9.1.1. Epidemiology of eosinophilic meningitis with particular consideration to outbreaks*

There are several distinctive characteristics of angiostrongyliasis in P.R. China. First, eosinophilic meningitis due to *A. cantonensis* is emerging, characterized by outbreaks. To date, at least 385 cases have been reported in the mainland of P.R. China. Most of the cases (88%) were involved in nine outbreaks (Table 9.1). Two additional outbreaks of angiostrongyliasis occurred in 2007 and 2008 (Deng et al., 2007; Lv et al., 2009a) and these raised considerable awareness in public health, particularily in view of the large 2006 outbreak in Beijing in 2006. Interestingly, the lasest outbreaks occurred in the same ethnic group. Although there is currently no evidence indicating that certain ethnic groups are more susceptible to *A. cantonensis*, the culture-related habits probably play an important roles in these outbreaks. Deeply rooted habit of food consumption therefore is a formidable challenge to angiostrongyliasis control. Similar observations have been made situation occurs in other countries or territories in Southeast Asia. Furthermore, the habit also resulted in outbreaks among Asian people beyond their countries (Kliks et al., 1982; Tsai et al., 2001; Lin et al., 2010).

Second, outbreaks frequently occurred among urban residents. Most of the previously reported outbreaks occurred when snails were collected and prepared for consumption by individuals, especially in rural areas. However, several outbreaks in P.R. China, such as the one that occurred in Wenzhou in 1997 (Zheng et al., 2001), the large 2006 Beijing outbreak (He et al., 2007) and the most recent one in Dali in 2008 (Lv et al., 2009a), could be traced back to restaurants. This shows that the increasing diversification of the diet and convenient long-distance transportation of foodstuffs now also puts urban consumers at risk of angiostrongyliasis. In such cases, more individuals are involved and the epidemiological picture is more complex.

Third, the major source of infection is through consumption of two invasive snail species, i.e. *Pomacea* spp. and *A. fulica*. Indeed, more than three-quarter of cases are directly related to consumption of these snails and all the nine outbreaks in P.R. China were owned to these two snail species, most importantly *Pomacea* spp. The African land snail, *A. fulica*, appeared in P.R. China as early as 1931. However, cases of angiostrongyliasis were not reported until 1984. The first case, a child in Xuwen probably acquired an infection due to inappropriate hygiene behaviour (e.g. crushing *A. fulica* and feeding the poultry with snail soft body). While *A. fulica* can be occasionally observed on markets today (see Chapter 4), the number of cases linked to the consumption of this snail species are far lower than those owning to *Pomacea* spp. The introduction of apple snails into P.R. China not only changed the epidemiology of angiostrongyliasis but also switched the manifestation spectrum, which brought about the fourth characteristic.

Fourth, moderate infection and mild or transient symptoms are more frequent. There is a striking contrast in incubation period between cases due to terrestrial snails or slugs and those owning to freshwater snails (Lv et al., 2010). The difference in infection intensity of mollusk species is probably the key driver of this observation. Normally, the natural infection intensity in terrestrial mollusk, including *A. fulica*, is higher than that in freshwater snails, e.g. *Pomacea* spp. (Lv et al., 2008). Indeed, severe or fatal cases are significantly associated with the consumption of terrestrial snails or slugs (Kliks et al., 1982; Hwang and Chen, 1986; Li et al., 2006; Liu et al., 2006). In these cases, the discovery rate of parasite from cerebrospinal fluid is higher (Yii et al., 1975). In contrast, cases due to *Pomacea* spp. usually manifest moderately, which is notable in the most recent outbreaks (Table 9.1). For example, in an outbreak occurring in Guangning in 2007, 14 individuals had consumed raw *P. canaliculata*, but only six of them reported relevant symptoms and only two sought medical care in a hospital (Deng et al., 2007).

Mild infection and low discovery rate of parasites are diagnostic challenge. Outbreak identification differs from individual diagnosis because additional information regarding exposure history and shared clinical manifestations can shed additional light for more accurate diagnosis (Odermatt et al., 2010). However, prompt and accurate diagnosis of index patients is a challenge in angiostrongyliasis outbreaks. Parasitological diagnosis is generally rare, especially in outbreaks (Table 9.1), although the patient's position before lumbar puncture and the technique employed in spinal tap may affect the parasite recovery rate (Tsai et al., 2001; Yii, 1976). Immunological assays are important alternatives, but their sensitivity and specificity need to be further improved. We proposed a set of stratified diagnostic criteria (see Chapter 3). Three stratifications, i.e. (i) suspected (ii) clinically diagnosed and (iii) parasitologically confirmed, are considered according to the strength of the available evidence. These criteria can direct clinicians towards a diagnosis, but improved diagnostic techniques are urgently needed.

### *9.1.2.* Angiostrongylus cantonensis *in its presumptive home range and global implications*

We performed a nationwide sampling survey on *A. cantonensis* and their hosts. The findings reveal the current distribution of the rat lungworm in P.R. China; seven provinces were identified as endemic areas of *A. cantonensis*. Although five of them were previously reported, our results provide new insights. For example, the two newly-identified endemic provinces are Jiangxi and Hunan closed to Guangdong and Fujian provinces, where the epidemiology of human angiostrongyliasis is well known. Our findings indicate the risk of angiostrongyliasis among the local people. We also note one exception, i.e., Yunnan province. We failed to discover any larvae or adult worm from host animals in this province (see Chapter 4). However, *A. cantonensis* was reported in one county (Hekou) on the boundary between P.R. China (Li et al., 1989) and Vietnam and another (Ruili) on the boundary between P.R. China and Myanmar (Huang, 2000). A considerable prevalence of

**Table 9.1** Angiostrongyliasis outbreaks in the mainland of P.R. China

| Year | Location | No. cases | Population | Source of infection | Parasitological diagnosis (%) | Outcome (fatality rate%) | References |
|---|---|---|---|---|---|---|---|
| 1997 | Wenzhou | 65 | Local residents | *Pomacea canaliculata* | 0 (0) | No death (0) | (Zheng et al., 2001) |
| 2002 | Changle | 8 | Local residents, eight children | *Pomacea canaliculata* | 1 (12.5) | No death (0) | (Lin et al., 2003) |
| 2002 | Fuzhou | 9 | Staff from the same institute | *Pomacea canaliculata* | 0 (0) | No death (0) | (Wu and Yan, 2004) |
| 2002 | Fuzhou | 13 | Staff from the same institute | *Achatina fulica* | 0 (0) | No death (0) | (Yang et al., 2004) |
| 2004 | Kunming | 25 | Local residents | *Pomacea canaliculata* | 0 (0) | No death (0) | (Han et al., 2005) |
| 2005 | Kunming | 9 | Local residents | *Pomacea canaliculata* | 0 (0) | No death (0) | (Wei et al., 2005) |
| 2006 | Beijing | 160 | Local residents | *Pomacea canaliculata* | 0 (0) | No death (0) | (He et al., 2007) |
| 2007 | Guangning | 6 | Bai migrant workers | *Pomacea canaliculata* | 0 (0) | No death (0) | (Deng et al., 2007) |
| 2008 | Dali | 41 | Local residents | *Pomacea canaliculata* | 0 (0) | No death (0) | (Chapter 3) |

* An outbreak is defined as more than five patients with the same exposure.

*A. cantonensis* among mollusks was observed; 37.2% and 23.1% in *A. fulica* and *Vaginulus* spp., respectively (Li et al., 1989). Furthermore, the research also declared another rat lungworm species, *A. malaysiensis*. Subsequent investigations in our surveillance frame revealed very low prevalence in snails collected from both field and markets; only 0.6% and 0.3% in two field sites (Wang et al., 2010) and 1.44% in markets (Chen et al., 2011), which were much lower than the average prevalence in the country (see Chapter 4). Although the data were only derived from the examination of *Pomacea* spp., the prevalence in terrestrial mollusks and rats are expected lower than those in other settings. The inconsistency indicated a potential change in the endemicity of *A. cantonensis*.

The two invasive snail species, *A. fulica* and *Pomacea* spp., are the leading intermediate hosts of *A. cantonensis* in P.R. China, although the roles of other mollusk species must not be neglected. The striking compatibility of *A. fulica* and *A. cantonensis* was discovered in the 1960s through extensive field work. The role of *A. fulica* in the transmission of angiostrongyliasis and a series number of case reports and outbreaks on Pacific Islands was clarified in the 1960s and 1970s (Alicata and Jindrak, 1970). In the mainland of P.R. China the real number of cases linked to this snail species was not clear, since little attention was paid on angiostrongyliasis during the period of civil war and World War II and subsequent social and economic development. The misdiagnosis and omission due to rare medicine resource were expected. Fortunately, the consumption of these snails is less common today. Furthermore, an albinistic variant normally cultured in breeding yard is the substitution of wild snails. In this case the contact between rats and snails is less likely and hence the food safety is guaranteed. However, a particular concern arises when African land snail meets the Asian rat lungworm on the Africa continent. Although the rat lungworm and angiostrongyliasis were reported as early as the 1960s on the islands (Madagascar, Mauritius and Sarawak) of Southeast Africa (Alicata, 1965) and the 1970s in West Africa (Kliks and Palumbo, 1992) and Egypt (Yousif and Ibrahim, 1978), the recent findings in South Africa (Archer et al., 2011) and the Canary Islands (Foronda et al., 2010) indicated the wide establishment in this region. *A. fulica* was once widely used both fresh and preserved as a source of dietary protein in West Africa (Kliks and Palumbo, 1992); probably this habit exits today particularly in rural areas. Therefore, more attention should be paid on the the risk of angiostrongyliasis.

The potential importance of freshwater snails as intermediate host of *A. cantonensis* has been proposed in the early 1960s (Richards, 1963). Soon the natural infection in *Cipangopaludina chinensis* was found in Taiwan and implicated in a human case (Chang et al., 1968). Therefore, the native snail species *C. chinensis* was considered as an important source of infection in Taiwan. However, the essential role of aquatic snail species was realized in Thailand since 1965 when several patients with eosinophilic meningitis complained of the consumption of big freshwater snails (*Pila* spp.) (Punyagupta et al., 1968). Subsequent studies highlighted the importance of this snail species in public health (Punyagupta et al., 1970, 1990). *Pila* native to Southeast Asia is the second largest genus in the known genera of Ampullariidae (apple snail) (Cowie et al., 2006). Although the species from this genus was previously reported in the south part of P.R. China, we failed to discover this snail species during the national sampling survey, which indicated that these snails do not play a dominant role.

Interestingly, another apple snail species (*Pomacea* spp.) indigenous to the South Americas showed the potential in transmission of angiostrongyliasis in P.R. China soon after it had been introduced. Conclusive evidence dates back to 1985 when a small outbreak involving four children occurred in Taiwan (Hwang and Chen, 1986). Subsquently, a following systematic investigation regarding natural infection of this snail species was carried out in Japan (Nishimura et al., 1986). The results showed the heterogeneous distribution of infected snails. Indeed, the prevalence in some place could reach up to 12.5%, and the highest infection intensity was 1827 larvae per snail. The experiment also showed the remarkable susceptibility of *P. canaliculata*. The importance of *Pomacea* spp. in the transmission of angiostrongyliasis was highlighted by a series of outbreaks both in Taiwan and the mainland of P.R. China ( Tsai et al., 2001; Lv et al., 2008; Lin et al., 2010). Of special concern is that the snail species probably play the same role in the native range, the South Americas. Recently, the outbreaks in Ecuador (Pincay et al., 2009) and sporadic cases in Brazil (Caldeira et al., 2007; Lima et al., 2009; Thiengo et al., 2010) highlighted the risk of eosinophilic meningitis due to *A. cantonensis*. The native snail *Pomacea lineata* as well as the invasive snail *A. fulica* have been found naturally infected in this region (Caldeira et al., 2007; Maldonado et al., 2010; Thiengo et al., 2010). Fortunately, freshwater snails are less frequently consumed in the South Americas than in Southeast Asia (Thiengo et al., 2010). Nevertheless, the particular attention should be paid to the conjecture, especially for those migrants from Southeast Asian countries.

Three closely related species of rodent intra-arterial nematodes, i.e., *A. cantonensis*, *A. mackerrasae* and *A. malaysiensis*, were distinguished. The coevolution between *Angiostrongylus* and the rat host (mainly *Rattus*) was considered as the major explanation for the divergence of these three species (Prociv et al., 2000). The similarity among the species obviously exceeds the difference, but only *A. cantonensis* was confirmed in both human eosinophilic meningitis and animal ill-health. There are many factors (eating habits, sanitation, etc.) involved in the occurrence of angiostrongyliasis. Pathogenicity should be considered as one important explanation. Even in the endemic areas, e.g., P.R. China, angiostrongyliasis does not equally occur in different geographical regions. For instance, human cases were not reported until recently on the Hainan Island (Chen et al., 2010), although the immunological arrays performed in the min-1980s indicated insidious infections (He et al., 1985). Furthermore, the consumption of *Pomacea* spp. and *A. fulica* is not uncommon on this island still today. Indeed, in our study pertaining to intraspecific differentiation, the major isolate from Hainan Island is genetically distinctive. However, additional research is warrated to clarify the potential difference in pathogenicity of different isolates.

The distribution of seven clades identified in this study showed no significant isolation by distance. However, isolation probably was obscured by the wide distribution of the two clades Mainland and Tiane. In contrast, the other clades including Hainan, Sanya, Nanao, Zixing as well as Thailand, showed the geographical clustering. Our study failed to address the issue of how the current distribution of the two clades Mainland and Tiane formed. The migration of rat definitive hosts and probably the current transportation of some snail species may play key roles, but the appropriate evaluation should be done according to both the geophylogeny of parasite and those of hosts. Our findings in intraspecific differentiation provided molecular evidence for an Asian origin of *A. cantonensis*, since the current geographic distribution pattern of this rat lungworm species refutes the expected homogeneous spread driven by biological invasion of *A. fulica*. There are two major hypotheses for the spread of *A. cantonensis* from Southeast Asia to the Pacific Islands and then Caribbean region (Figure 1.2). However, these two hypotheses have not yet been confirmed. Moreover, the new ranges (e.g., the South Americas and

South Africa) as well as the opposite dispersal from Southeast Asia to South Asia and Southeast African Islands need further interpretation. Our study can serve as a starting point to further studies pertaining to the global dispersal of *A. cantonenis*.

### *9.1.3. Invasive species and emerging infectious diseases*

Biological invasion never stop its process and is being accelerated following human footprints. Invasive species pervade the invaded ecosystem and try to reshuffle the established patterns either in a radical or an imperceptible manner. One hundred species are listed as top invasive species in the world according to either their serious impact on biological diversity or their illustration of important issues surrounding biological invasion (Lowe et al., 2000). They include microorganism (mainly pathogens), plant, invertebrate, and vertebrate. *P. canaliculata*, *A. fulica*, and *R. rattus* are in the list. Notably, only one species from each genus was included in this list of the top 100 invasive species (Lowe et al., 2000), which does not mean that *P. insularum* and *R. norvegicus* are not the global invasive species. From a regional point of view, the number and extent of invasive species may be different. For example, there are 283 invasive species in P.R. China (Xu et al., 2004). Consistent with the global list, *A. fulica*, *P. canaliculata* and *R. norvegicus* are included, although the latter is conventionally considered originating from the north part of P.R. China (Gibbs et al., 2004). In this case, the endoparasite *A. cantonensis* therefore becomes the invasive species along with the hosts. Indeed, many countries outside Southeast Asia reported this parasite, particularly in within those invasive hosts (Figure 1.2).

Loss of native parasite was considered as one of factors resulting in success of biological invasion (Kim et al., 2002; Clay, 2003; Torchin et al., 2003). Unfortunately, the two invasive snail species, *A. fulica* and *Pomacea* spp., meet the new parasite (*A. cantonensis*) in the invaded range, and moreover *R. norvegicus* presumptively native to Asia fails to get rid of the parasite. There is no evidence that the native parasite increases the mortality of invasive snails and hence mitigates their invasion, although striking infection intensities have been observed frequently (Wallace and Rosen, 1969; Lv et al., 2006a). This observation partly explains that invasive species can become established and therefore key intermediate hosts of *A. cantonensis* in P.R. China. *R. norvegicus* has been confirmed as the most suitable host by both field investigations and laboratory studies. In contrast to infections in human and other mammals, an infection rarely influence the function of central nervous system and respiratory system of *R. norvegicus* (Mackerras and Sandars, 1954; Kino, 1984), although heavy infections can cause the blockage of the common pulmonary artery and thus death of rats. Furthermore, the duration (average 414.2 days) of larval output of female worms approaches the maximum estimated mean life-span of wild rats (Kino, 1984). Regardless of the probability that *R. norvegicus* and *R. rattus* invade the new range coupling with the lungworm, the extant distribution of these rat species is ready for the global establishment of *A. cantonensis*.

Because of the "domiciliation", the dispersal of *R. norvegicus* and *R. rattus* is related to human activities. Their origin and spread routes are still controversy due to the lack of early detailed records. However, the process of spread is not ceased, which can be illustrated by the current findings of *A. cantonensis* in harbour or port cities (Figure 1.2). In contrast to *Rattus* spp., the invasion process of *A. fulica* and *Pomacea* spp. are precisely recorded. *A. fulica* was introduced to Madagascar and offshore islands and then to South Asia and Southeast Asia (Figure 9.1). The Pacific War accelerated the spread to Pacific Islands and the Americas. Recently, one study pertaining to the population genetics of *A. fulica* confirmed this spread route, while the multiple introduction in the invaded range was indicated (Fontanilla, 2010). For example, the samples from New Caledonia and Barbados share the same haplotype, but are different from the major haplotype in Asia and Pacific islands. However, the automatic mutation during the invasion is not ruled out. The haplotype of New Caledonia and Barbados as well as the unique haplotype identified in the Philippines are different from the major haplotype only in one mutation site, respectively. Furthermore, these two haplotypes were not discovered in the native range. In this case, the secondary introduction in invaded range rather than multiple introductions from the original range is more likely to occur.

In contrast to the obvious genetic bottleneck during biological invasion of *A. fulica*, *Pomacea* spp. showed the considerable genetic variation in the invaded range (see Chapter 7 and Hayes et al., 2008). Additionally, the clades B and F only occurring in P.R. China and D and E simultaneously occurring in several Southeast Asian countries are distinct from other clades and hence the automatic mutation as indicated in *A. fulica* is unlikely to

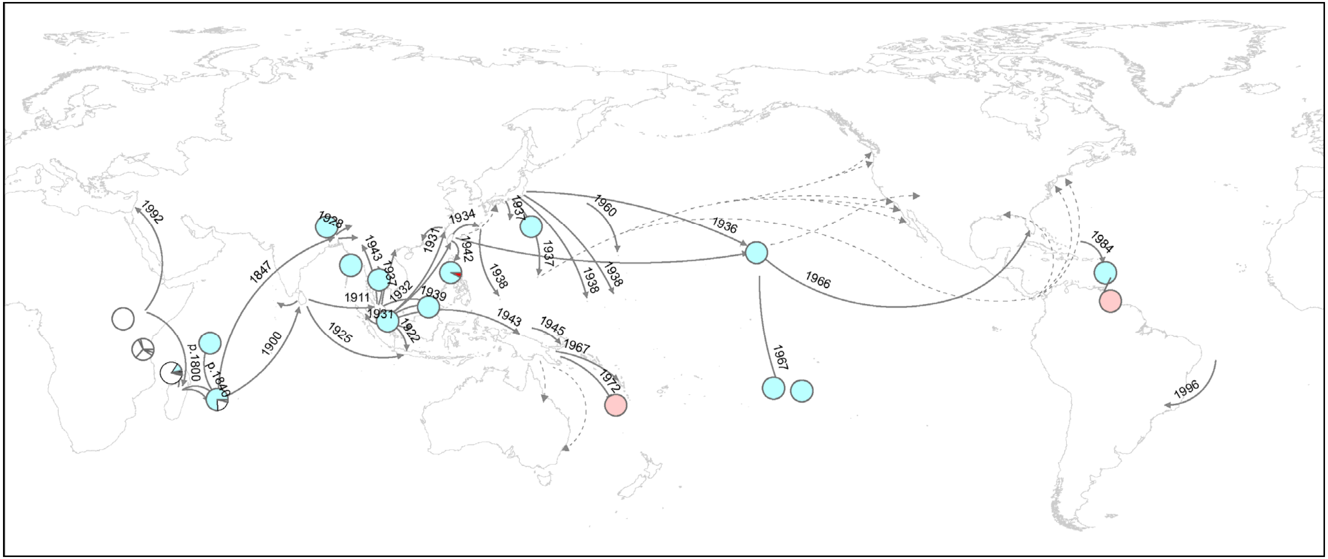

**Figure 9.1** The spread route and population genetic evidence of *Achatina fulica* (after (Fontanilla, 2010; Mead, 1961; Raut and Barker, 2002)). Different color pies denote the haplotypes. The non-invasive haplotypes in Africa are indicated white pies. Dot lines denote the interception.

happen during the invasion. As intentionally introduced food resource in Southeast Asia, multiple introductions from the South Americas probably occurred independently in the 1980s, although the secondary introduction from Taiwan (where apple snail was first introduced) to other areas in Southeast Asia is possible. Subsequently, the secondary introduction occurred within countries (e.g. P.R. China). Therefore, the kind of introduction, i.e. multiple and secondary introduction, accounts for the current distribution pattern of *A. fulica* and *Pomacea* spp.

The global spread of *A. cantonensis* was considered as the consequence of the expansive range of hosts mentioned above (Kliks and Palumbo, 1992). Many countries have reported the presence of this parasite based on morphological characteristics. Recently, molecular techniques have been developed and used in several studies for the purpose of diagnosis (Caldeira et al., 2003; Qvarnstrom et al., 2007; Qvarnstrom et al., 2010; Thiengo et al., 2010), which are expected to increase the accuracy of diagnosis of *A. cantonensis*. However, this method may be incapable to distinguish the isolates. The comparative studies of different isolates of *A. cantonensis* and species based on COI gene and a 66-kDa protein gene were conducted (Eamsobhana et al., 2010a, b). Although only few geographical isolates were included, the findings showed the high similarity between Hawaii and P.R. China, or between Hawaii and Japan. Our results from intraspecific differentiation study (chapter 6), however, indicate a more complex answer to the spread of *A. cantonensis* in the world. Therefore, further studies are needed.

### *9.1.4. Biology-driven model in context of climate change*

Climate change is a statistical change in the distribution of weather (Yang et al., 2010). It may be restricted to a specific region, or may occur across the globe. Climate change can influence parasite-host system in many ways. This effect can be numerical (e.g. increased growth rate and thus population density of parasites and hosts), functional (e.g. range expansion or shrink and potential shift of interaction between parasite and host), or microevolutionary (e.g. change of gene frequency through mutation) (Brooks and Hoberg, 2007). The numerical effect is easy to evaluate under laboratory conditions. For instance, the growth and development rate of *A. cantonensis* larvae in mollusk host is significantly affected by temperature (Ishii, 1984; Lv et al., 2006b). Similarly, temperature can also

**Table 9.2** Biological feature in schistosomiasis japonica and angiostrongyliasis cantonensis transmission dynamics for P.R. China

| Risk factor | *Schistosoma japonicum* transmission model | | *Angiostrongylus cantonensis* transmission model | |
|---|---|---|---|---|
| | Species | Biological features | Species | Biological features |
| Parasite | *Schistosoma japonicum* | Trematode | *Angiostrongylus cantonensis* | Nematode |
| | | High specificity to intermediate host and low specificity to definitive host | | High specificity to definitive host and low specificity to intermediate host |
| | | Asexual multiplication in snail | | Non-multiplication mode |
| | | Cercariae shed from snail | | Larvae harboured within snail |
| | | Infection via skin of definitive host | | Infection via mouth of definitive host |
| | | Adult worms parasitizing intestinal vessels | | Adult worms parasitizing pulmonary arteries |
| Intermediate host | *Oncomelania hupensis* | Pomatiopsidae | *Pomacea canaliculata* | Ampullariidae |
| | | Native freshwater snail species | | Invasive freshwater snail species |
| | | Small size (6.09-9.73 mm in length, 2.57-4.24 mm in width) | | Large size (up to 170 mm in length) |
| | | Tropical and subtropical | | Tropical and subtropical |
| | | Amphibious, mainly on wet soil | | Amphibious, mainly in shallow water |
| Definitive hosts | Human, domestic and wild mammals | Longevity (>2 years) | Rats | Longevity (<2 years) |
| | | Longevity somewhat shortened by infection | | Longevity significantly shortened by infection |

impact on the growth rate of mollusk intermediate hosts, including *P. canaliculata* and *A. fulica* (Zhou et al., 1998, 2003). In practice, the direct effect due to climate change has not yet been observed in field. However, some parasite-host systems, e.g., nematode–gastropod–ungulate systems in Arctic region which are more vulnerable to climate change, confirmed the impact (Kutz et al., 2009a, b).

In this thesis, we assessed the potential functional effect of climate change on *A. cantonensis*-*P. canaliculata*-rat system using both laboratory and climatic data. Although we only took temperature into account in this study, the designation of a biology-driven model was a challenge. In the case of *Schistosoma japonicum*, the extant model does not take into account the aspect of the definitive host (Zhou et al., 2008). Indeed, there are many differences in transmission dynamic of *S. japonicum* and *A. cantonensis* (Table 9.2). For example, the impact of infection on definitive hosts is different. Unlike *S. japonicum*, which employs a range of vertebrates as definitive hosts, *A. cantonensis* normally utilizes *Rattus* as the definitive hosts and occasionally involves other species (Table 9.2). The longevity (<2 years) of rats is much shorter than those of vertebrates in the life cycle of *S. japonicum*. Furthermore, the infection by *A. cantonensis* can significantly reduce the longevity of rats, especially when heavy infection occurs (Kino, 1984). Although the longevity of infected rats is not related to climate change, it should be considered because significantly shortened longevity will reduce the possibility of contact between mollusk and rats. When the average longevity of infected rats is shorter than the dormancy or hibernation period of mollusk, the life cycle of *A. cantonensis* is more likely to interrupt.

Our results show that there will be a considerable expansion of *A. cantonensis* in the context of rising temperature and the main extent will occur in the central part of P.R. China (Figure 9.2). Whereas global warming is a slow tendency, extreme climatic events and dramatic change from one year to another are often perceived. Our climatic data derived from the providing regional climates for impacts studies (PRECIS) model indeed showed heterogeneous change of temperature temporarily and spatially. We identified a vulnerable area of transmission of *A. cantonensis* by using average statistic plus and minus one standard deviation, which was so small that it approached the real change rate of temperature. A monitoring system of parasite or its intermediate hosts can be set in the vulnerable areas.

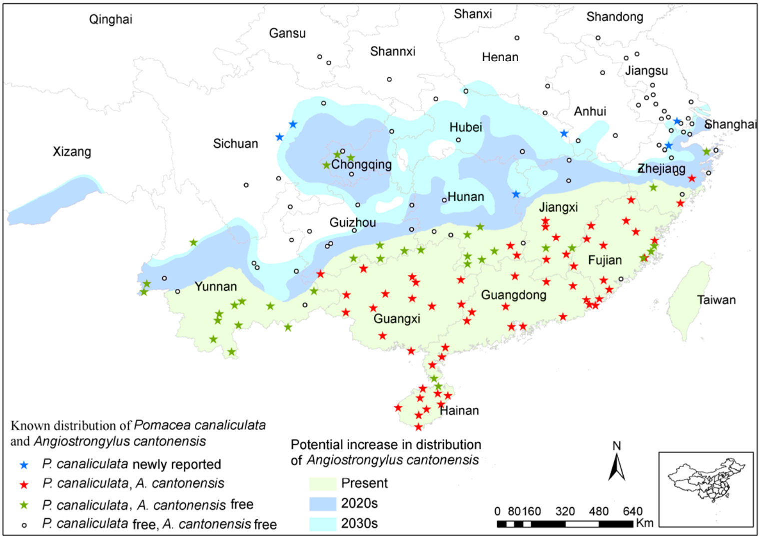

**Figure 9.2** The current distribution of *A. cantonensis* and potential changes in the 2020s and 2030s in P.R. China. This figure integrates a series of maps that have been presented elsewhere (Lv et al., 2011).

## 9.2. Conclusions

*A. cantonensis* was discovered approximately 80 years ago in Guangzhou, P.R. China. However, little was known until a few years ago since frequent outbreaks occurred. This thesis, including a series of field-related surveys and laboratory investigations and the new insights gained at the parasite and human disease level resulted in enhanced knowledge pertaining to *A. cantonensis* and eosinophilic meningitis. Some findings also provide helpful information in control of this disease in other countries. The following five main conclusions were drawn.

1. Key characteristics in the current epidemiology of angiostrongyliaisis in P.R. China include: (i) frequent outbreaks, (ii) urban populations involved, (iii) invasive snails as the major source of infections, and (iv) growing number of mild infections. Investigation of an angiostrongyliasis outbreak in Dali highlights the necessity of categorized diagnosis criteria leading clinicians to find out the most convincible evidences.

2. The nationwide sampling survey of *A. cantonensis* fills a current knowledge gap

pertaining to the distribution in the presumptive home range. The high intraspecific differentiation of *A. cantonensis* supports the Asian origin hypothesis, and provides a platform for further studies regarding the spread in the world. Mitochondrial genetic marker holds promise in developing a clade-specific diagnostic tool. The mutation pattern between clades indicates a high substitution rate between adenine (A) and guanine (G), which potentially play an important role in intraspecific differentiation rather than speciation.

3. The invasive snail species (*Pomacea* spp. and *A. fulica*) identified as the leading intermediate hosts in this survey highlight the impact of biological invasion on distribution and epidemiology of a local parasitic disease, and also raises a particular concern of angiostrongyliasis in their native ranges.

4. There are two *Pomacea* species in the mainland of P.R. China. The current mosaic distribution of haplotypes suggests that multiple and secondary introductions of this snail species occurred. There is no molecular evidence showing that the dispersal of *Pomacea* spp. contributed to the current distribution of *A. cantonensis*. However, the invasive snails will considerably influence the dynamics of *A. cantonensis* in the face of climate change.

5. The complete mitochondrial genome of two rodent intra-arterial nematodes (*A. cantonensis* and *A. costaricensis*) was the smallest characterized thus far in the class of Chromadorea. The mitochondrial-genome-wide analysis deepens the understanding of phylogenetic relationship among nematodes.

## 9.3. Research priorities

Based on the results from this thesis, several research priorities can be recommended.

1. to improve or develop diagnosis criteria ready for the growing number of mild infections;

2. to confirm the origin of clades Sanya, Hainan and Tiane of *A. cantonensis*

3. to look for the origin of clade B of apple snail in the South Americas;

4. to conduct field survey pertaining to *A. cantonensis* infection among apple snails in the South Americas and *A. fulica* in Africa;

5. to develop a sensitive diagnosis of different isolates of *A. cantonensis*.

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# Curriculum vitae

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| 09.2003-06.2006 | M.Sc.<br>Epidemiology and Statistics<br>Chinese Center of Disease Control and Prevention |
| *Thesis title* | The study on compatibility of intermediate host with *Angiostrongylus cantonensis*<br>Supervised by Prof. Xiao-Nong Zhou |
| 09.1998-07.2003 | MD<br>Medicine<br>Zhengzhou University |

## Research experience

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| 01.2011-present | Innovative strategies for sustainable control of Asian schistosomiasis and other helminth zoonoses through socio-ecosystem-based interventions<br>*Supported by Canada International Development and Research Center (Co-PI)* |
| 05.2009-present | The population genetics of *Angiostrongylus cantonensis* and *Pomacea* spp. in P.R. China<br>*Supported by Special Project of National Science and Technology (Key researcher)* |

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| 01.2009-12.2010 | The potential impact of climate change on infectious diseases<br>*Supported by Ministry of Agriculture* |
| 07.2007-06.2009 | The intraspecific differentiation of *Angiostrongylus cantonensis* based on complete mitochondrial genome in P.R. China<br>*Supported by International Society of Infectious Diseases (PI)* |

## Presentations

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| Oct 18-22, 2010 | Ecohealth Emerging Infectious Diseases Workshop<br>Siem Reap, Cambodia<br>Innovative strategies for the sustainable control of Asian schistosomiasis and other helminth zoonoses through socio-ecosystem-based interventions |
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## Publications

**Lv S**, Zhang YI, Steinmann P, Yang GUOJ, Yang KUN, Zhou XN, Utzinger J (2011) The emergence of angiostrongyliasis in the People's Republic of China: the interplay between invasive snails, climate change and transmission dynamics. Freshwater Biol 56: 717-734

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Yang GJ, Sun LP, Hong QB, Yang K, Deng Y, Li SZ, **Lv S**, Zhou XN (2009) Application and perspective of climatic forecasting models related to schistosomiasis transmission. Chin J Schisto Control 21: 432-436 (in Chinese)