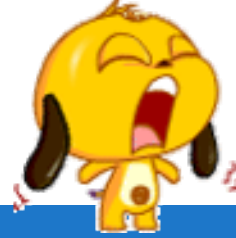




中山大學
SUN YAT-SEN UNIVERSITY

Hello, everybody



Phylogeographical studies of *Angiostrongylus cantonensis* and its host *Achatina fulica*

Based on mt ND2 and Cytb genes.

Yang Tingbao, He Zhangping, Lun Zhaorong, Wu Zhongdao
School of Life Sciences, Sun Yat-sen University



Introduction

Phylogeography

Phylogeography: Study of the relationship between genealogy and (Avice, et al 1987), to identify the intraspecific evolutionary processes based on the present phylogeographical patterns.

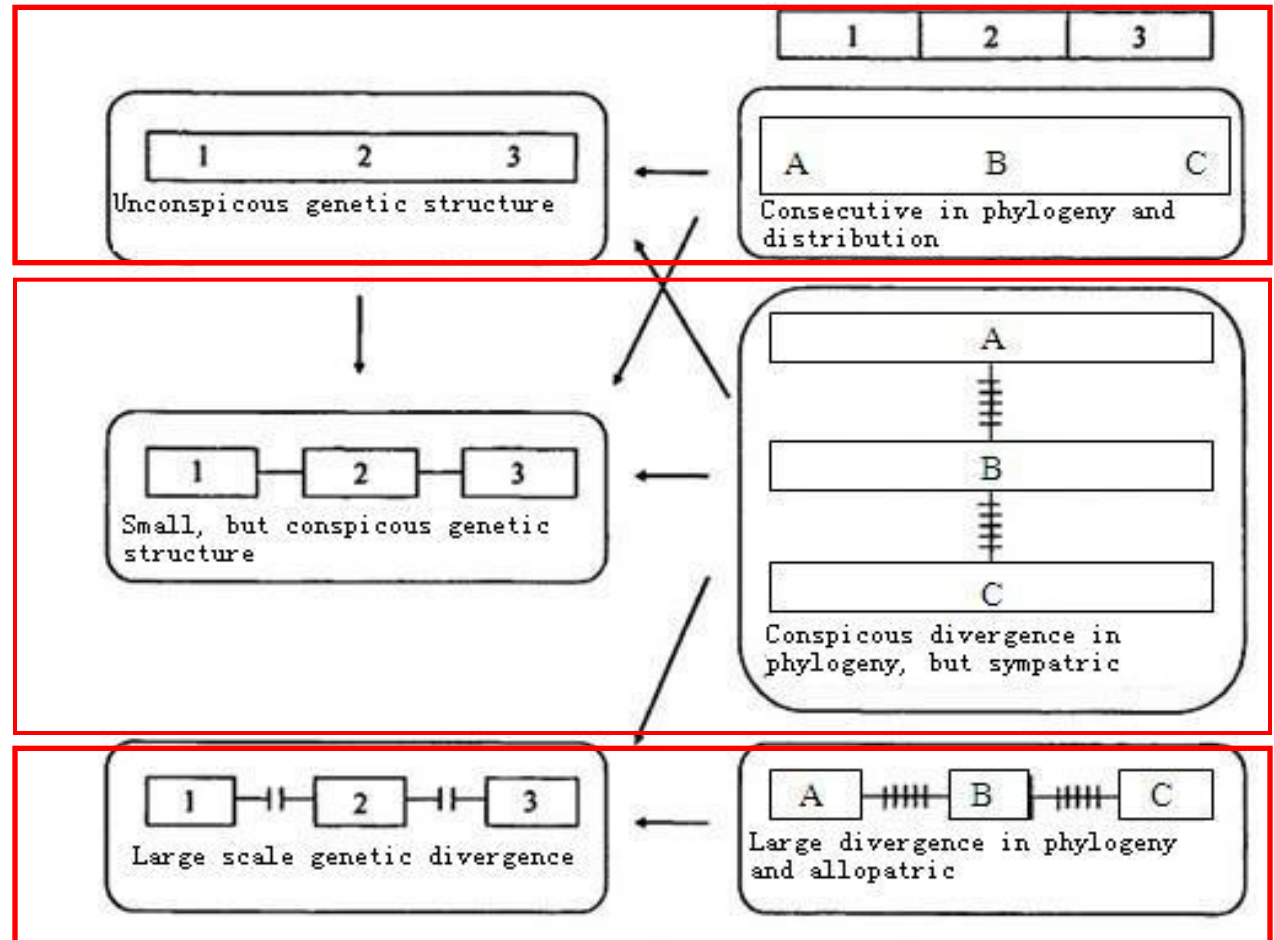
Molecular phylogeography: Based on different molecular markers, such as RAPD, RFLP, AFLP, SSR, ISSR, and mtDNA sequences such as CO1 and ND2 of mtDNA。

General objective: From intraspecific micro-evolution to interspecific macroevolution (Avice, 2000)

General Phylogeographic Models

Terrestrial environment:

Existing some physical barriers, organisms can be transported by human activities, related unstable environmental effects especially human intervene.



Phylogenetic studies on *A. cantonensis*

A. cantonensis is endemic in Asia, the Pacific Islands, the Caribbean Islands, USA, and Africa. However, endemic regions are now thought to be more widely distributed (Foronda et al., 2010);

The phylogeny inferred from cytochrome c oxidase subunit I (coxI) revealed three distinct lineages among *A. cantonensis* collected from Thailand, Hawaii, and Mainland China (Eamsobhana et al., 2010);

The spatial distribution of *A. cantonensis* was closely associated with the spread of the intermediate hosts, *Achatina fulica* in the Pacific Islands [Alicata, 1966], Mainland China [Lv et al 2009] and Brazil;

The partial coxI sequences were determined for 83 worms from 18 different geographical localities from Japan, Mainland China, Taiwan, and Thailand (Tokiwa et al, 2012).

Phylogenetic studies on *A. cantonensis*

These phylogenetical studies emphasized the phylogenetic relationship although they used materials from different locations, but samples does not represent a population (Table below from Tokiwa et al, 2012), while phylogeographic study were carried out at the population level.

Angiostrongylus cantonensis samples (n = 83) used in the present study.

Locality	No. of individuals	Worm stage	Origins
Chiba, Chiba , Japan	15	Adult	10 <i>Rattus norvegicus</i>
Kawasaki, Kanagawa , Japan	3	L3	1 <i>Limax marginatus</i>
Yokohama, Kanagawa , Japan	4	Adult	1 <i>Rattus norvegicus</i>
Minato-ku, Tokyo , Japan	2	Adult	1 <i>R. norvegicus</i>
Hahajima Island , the Ogasawara Island, Japan	8	L3	8 <i>Achatina fulica</i>
Chichijima Island , the Ogasawara Island, Japan	2	Adult, L1	2 <i>R. rattus</i>
Chichijima Island , the Ogasawara Island, Japan	6	Adult, L1, L3	Laboratory maintained
Sendai, Miyagi , Japan	4	Adult	2 <i>R. norvegicus</i>
Kanazawa, Ishikawa , Japan	4	Adult	1 <i>R. rattus</i>
Nagoya, Aichi , Japan	6	Adult	2 <i>R. norvegicus</i>
Amamioshima Island , the Nansei Islands, Japan	4	L1, Adult	2 <i>R. rattus</i>
Naha, Okinawa Island , the Nansei Islands, Japan	10	Adult	6 <i>R. norvegicus</i>
Nanjo, Okinawa Island , the Nansei Islands, Japan	1	L3	1 <i>Achatina fulica</i>
Taichung , Taiwan	4	Adult	3 <i>R. norvegicus</i>
Shenzhen , Guangdong, China	3	Adult	3 <i>R. norvegicus</i>
Wenzhou , Zhejiang, China	2	Adult	Laboratory maintained
Lianjiang , Fujian, China	3	Adult	Laboratory maintained
Bangkok , Thailand	2	L1	Laboratory maintained
	83		

Introduction

Objectives of the present study

- (i) To evaluate the evolving rates or resolution of Mito-genes ND2 and cytb for both the nematode and host, *Achatina fulica*, at intraspecific level;
- (ii) To assess the genetic homogeneity of this nematode and the host snail in South China area;
- (iii) Further, to elucidate population genetic structure of this nematode and the host snail in south China, effect of vicariance, and possible population develop route by natural spread or human related transportation.

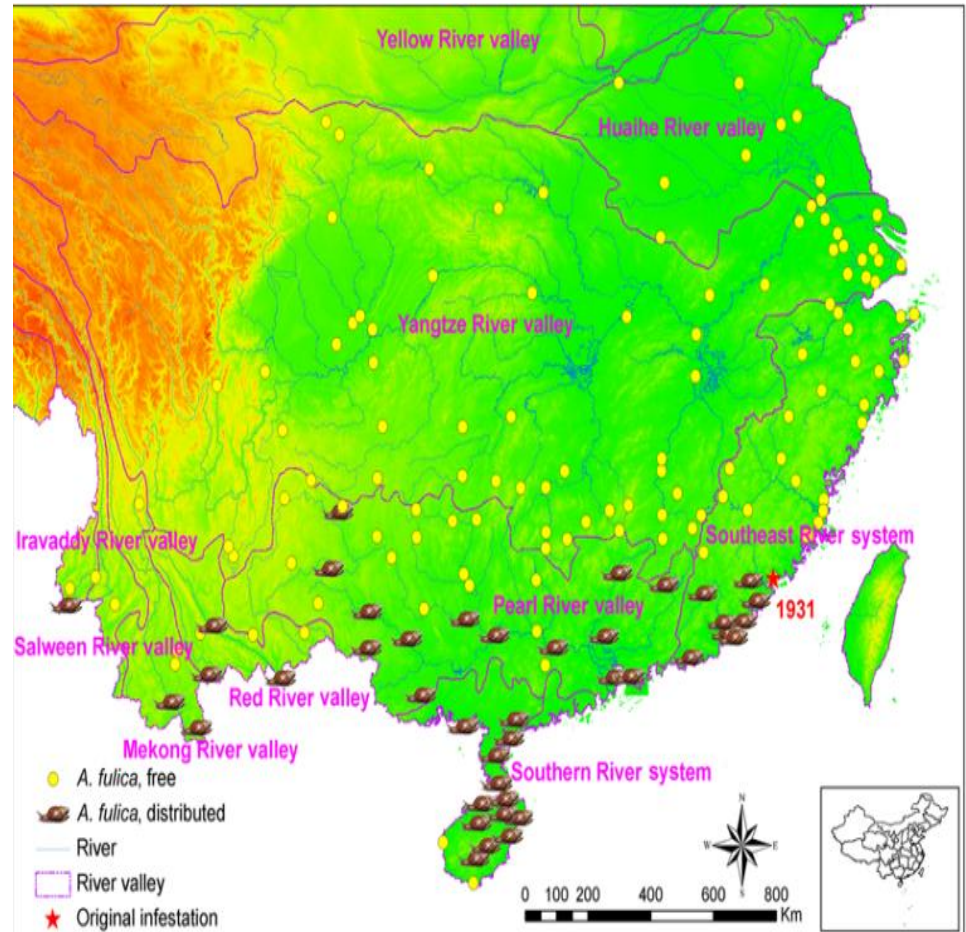
The host of the parasite



The host we sampled is giant african snail, *Achatina fulica*

Reported distribution of *Achatina fulica* in China

► At present, *A. fulica* is known to occur in the province 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 (Ref. Lv et al 2009).



Parasites sampling

Angiostrongylus cantonensis collection

Hosts: *Achatina fulica*

Time: Jun to Nov, 2013

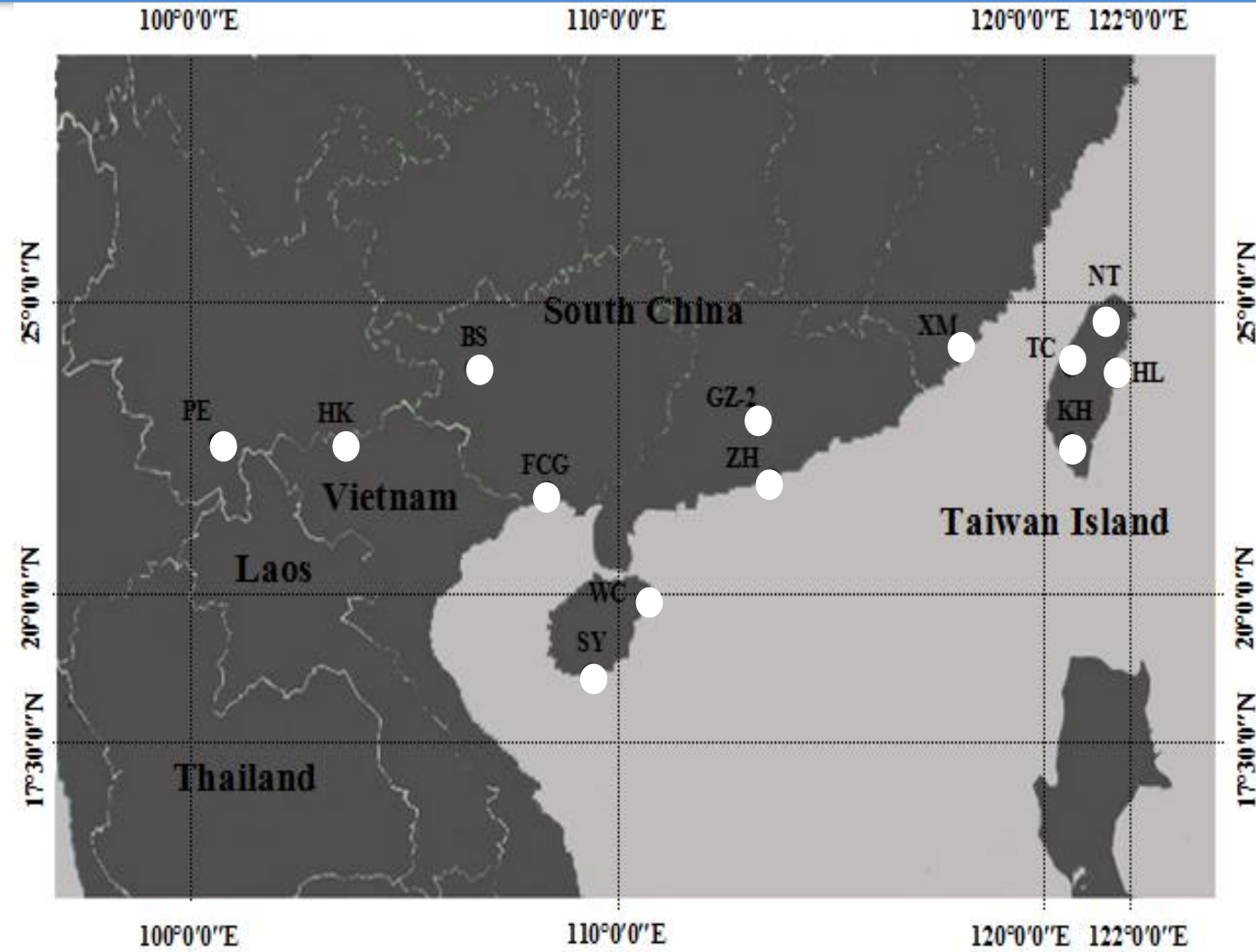
More than 20 samples of parasites were individually collected from different snail individuals at each sites except at HD where the prevalence is extremely low.



A. cantonensis (3rd larvae) in the course of ecdysis



Methods— Locations of sampling



GZ1 (Other areas in Guangzhou, Guangdong), **GZ2** (SYSU-south, Guangzhou, Guangdong), **ZH** (Zhuhai, Guangdong), **XM** (Xiamen, Fujian), **FCG** (Fangchenggang, Guangxi), **HK** (Hekou, Yunnan), **WC** (Wenchang, Hainan), **SY** (Sanya, Hainan), **NT**(New Taipei), **TC**(Taizhong), **HL**(Hualian), **KH** (Gao'iong).

Methods— Sample collection and treatment

Parasites were collected by routing methods

Parasite were Individually stored

DNA Extraction

Amplification of objective sequences

Purification, Recycling and sequencing of DNA

Sequence analyses

Primers of amplification of two genes

Primers used for PCR were designed according to the complete mitochondrial genomes of *A. cantonensis* (Genbank accession number NC013065.1) and *A. fulica* (Genbank accession number NC_024601.1)

Taxa	gene	Primer name	Sequence (5'-3')
<i>Angiostrongylus cantonensis</i>	ND2	Ac-N2F	GAGGG AGTGT TAGGT GATGG TTATG
		Ac-N2R	CTCCT GGGCA AAGTA ACCAC TAAAG
	Cytb	Ac-CBF	GATTA CGGTT CAGAA AGGTG
		Ac-CBR	GGCAA ATACA CCCCA AACTT
<i>Achatina fulica</i>	ND2	Af-N2F	ACGCA ATAAT AGTAG TTCTT
		AfN2R	AACCC ACATC ATACA CCA
	Cytb	AfCBF	TTACC CTTCT GGTTT CGG
		AfCBR	ACTGC CCTCA AAGTC TCA

Softwares used for the present studies

- 1 MEGA 4.0 → Sequence alignment Phylogeny
Sequence variation
- 2 Dnasp 5.0 → Diversity of haplotype and nucleotide
- 3 Modeltest 3.7 → Nucleotide substitution model
- 4 Network 4.5 → Network
- 5 Arlequin 3.5 → Genetic differentiation F_{ST} AMOVA
Exact test

Results

Genetic diversity of *Achintina fulica* based on Cytb and ND2 gene sequences

Cytb

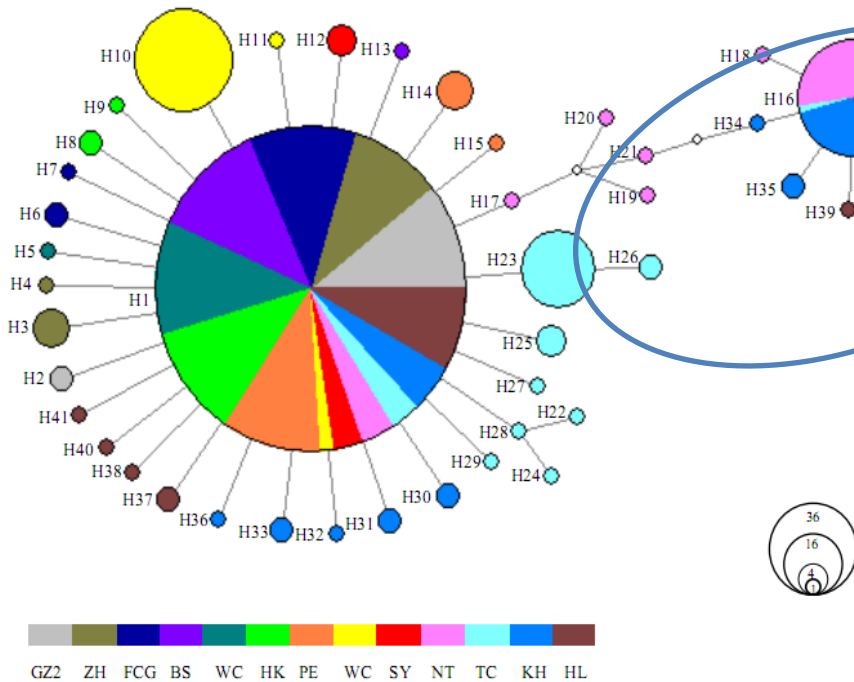
Site	Abbr.	No.sa mples (N)	No.hapl otypes (H)	Haplotype diversity ($h \pm SD$)
Guangzhou 2	GZ-2	38	2	0.1024 ± 0.0649
Zhuhai	ZH	38	3	0.2802 ± 0.0868
Xiamen	XM	39	2	0.0513 ± 0.0481
Fangchenggang	FCG	40	3	0.1449 ± 0.0737
Baise	BS	40	2	0.0500 ± 0.0469
Hekou	HK	40	3	0.1449 ± 0.0737
Puer	PE	40	3	0.2679 ± 0.0843
Wenchang	WC	40	3	0.2679 ± 0.0843
Sanya	SY	13	2	0.3846 ± 0.1321
New Taipei	NT	40	7	0.5910 ± 0.0631
Taizhong	TC	40	10	0.7179 ± 0.0594
Kaohong	KH	40	9	0.7410 ± 0.0489
Hualian	HL	40	7	0.5218 ± 0.0855
Total		488	41	

ND2

No.haplotypes (H)	Haplotype diversity($h \pm$ SD)
5	0.2475 ± 0.0914
2	0.0526 ± 0.0492
4	0.3225 ± 0.0927
1	0.0000 ± 0.0000
4	0.1910 ± 0.0820
9	0.8449 ± 0.0247
3	0.5013 ± 0.0761
4	0.2756 ± 0.0897
3	
8	0.5064 ± 0.0942
6	
4	0.5564 ± 0.0388
8	0.4346 ± 0.0958
45	

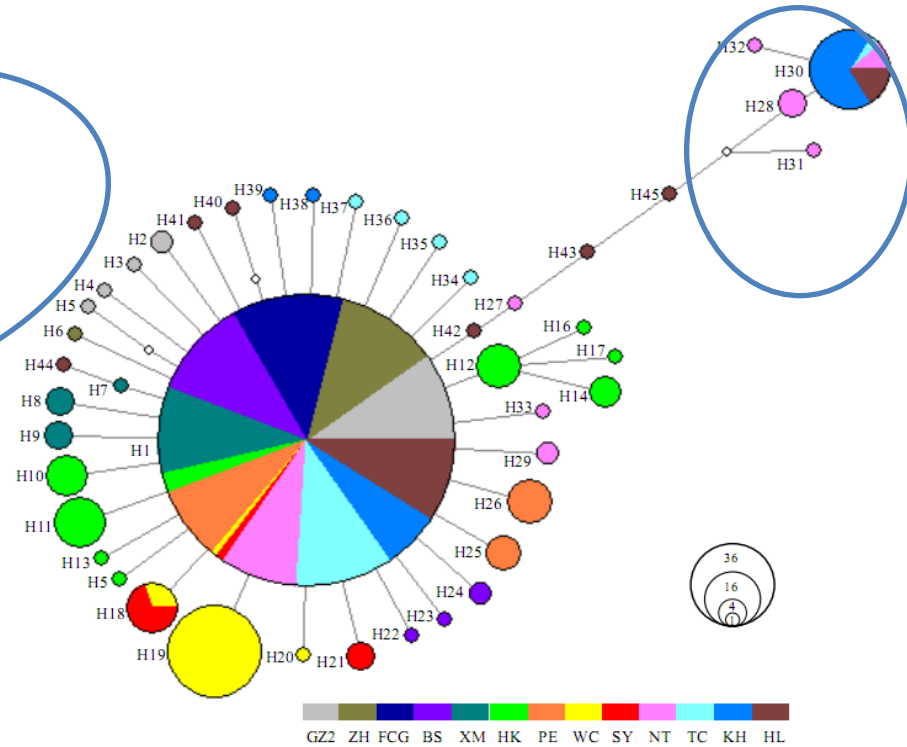
Results

Phylogenetic analyses of haplotypes based on sequences of cytb and ND2



Based on Cytb sequences

Two diverged branches



Based on ND2 sequences

Star shaped structure implies the existence of population expansion

Results

Genetic structure of *Achintina fulica* based on Cytb and ND2 gene sequences

Table 3 Significance of Exact test between different populations base on cytb and ND2

	GZ-2	ZH	XM	FCG	BS	HK	PE	WC	SY	NT	TC	KH	HL
GZ-2		*	*	*	—	*	*	*	*	*	*	*	*
ZH			*	*	*	*	*	*	*	*	*	*	*
XM				*	*	*	*	*	*	*	*	*	*
FCG					—	*	*	*	*	*	*	*	*
BS						*	*	*	*	*	*	*	*
HK							*	*	*	*	*	*	*
PE								*	*	*	*	*	*
WC									*	*	*	*	*
SY										*	*	*	*
NT											*	*	*
TC												*	*
KH													*
HL													

Results

Genetic structure of *Achintina fulica* based on Cytb and ND2 gene sequences

Table 4 Statistics of analysis molecular variance (AMOVA) between different populations base on cytb (and ND2)

Source of variation	Percentage of variation	<i>P</i> value
Among populations	40.60 (27.71)	0.00000 (0.00000)
Within populations	59.40 (72.29)	0.00000 (0.00000)
Total		

Certain percentages of variation are from among populations

Results

Genetic structure of *Achintina fulica* based on Cytb and ND2 gene sequences

Table 5 Tajima's D and F_u 's F_s tests of variation of population of *Achintina fulica* based on sequences of *Cytb* and *ND2*

	Tajima's D		F_u 's F_s	
	D	P	F_s	P
Total	-1.96911 (- 2.00727)	0.00100 (0.00110)	-27.21716 (- 26.51680)	0.00000 (0.00000)

The population was deduced to have experienced historical demographical expansion.

Results

Genetic Diversity of *Angiostrongylus cantonensis*

Table 6 Genetic diversity of *Angiostrongylus cantonensis* based on Cytb and ND2 gene sequences

Cytb

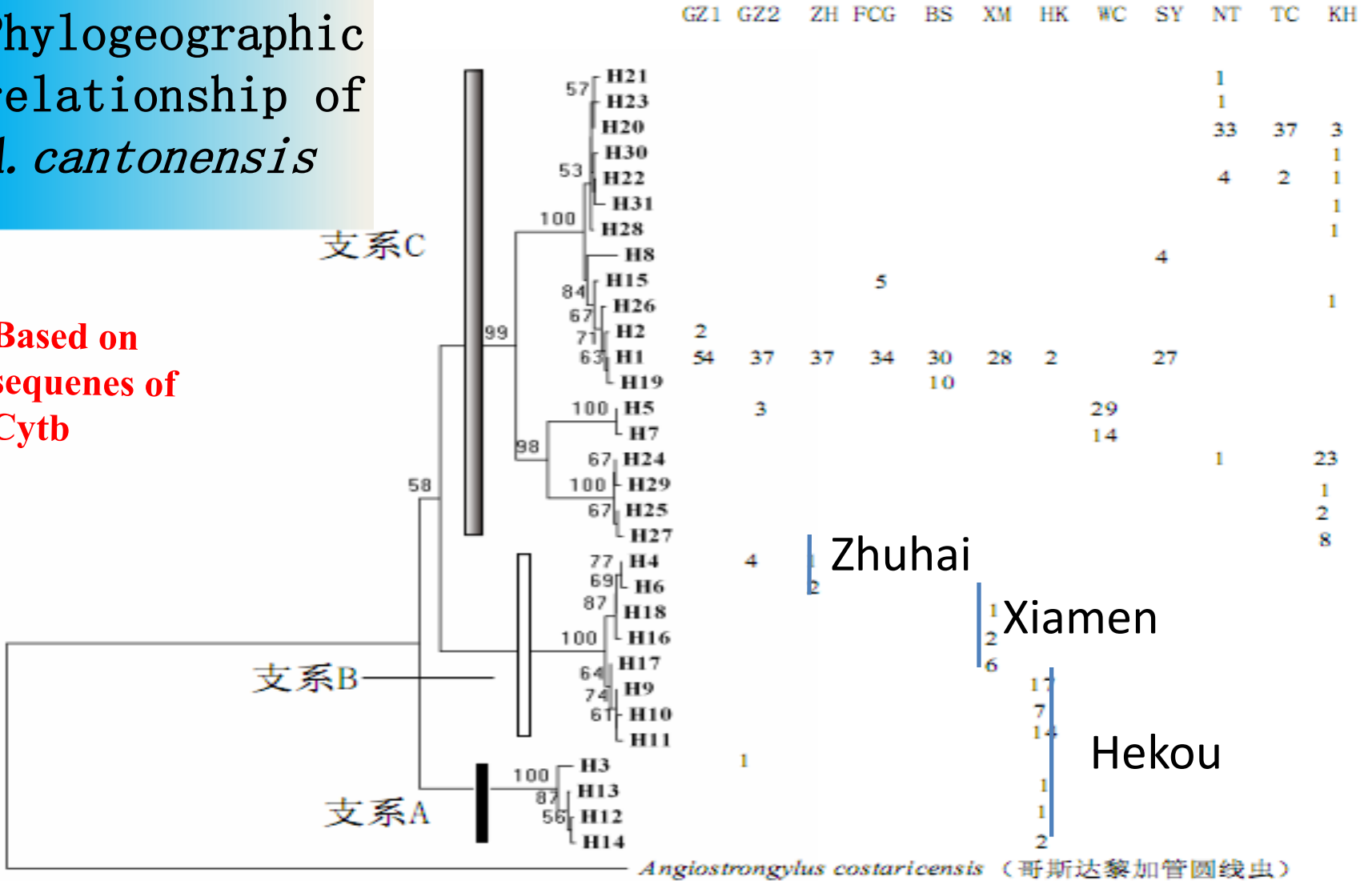
Site	Abbr	No. samples (N)	No. haplotypes (H)	Haplotype diversity ($h \pm$ SD)
Guangzhou 1	GZ-1	56	2	0.0701 \pm 0.0461
Guangzhou 2	GZ-2	45	4	0.3182 \pm 0.0858
Zhuhai	ZH	40	3	0.1449 \pm 0.0737
Xiamen	XM	37	4	0.4084 \pm 0.0906
Fangchenggang	FCG	39	2	0.2294 \pm 0.0800
Baise	BS	40	2	0.3846 \pm 0.0698
Wenchang	WC	43	2	0.4496 \pm 0.0520
Sanya	SY	31	2	0.2323 \pm 0.0899
New Taipei	NT	40	5	0.3154 \pm 0.0914
Taichong	TC	39	2	0.0999 \pm 0.0635
Kaohong	KH	42	10	0.6690 \pm 0.0709
Hekou	HK	44	7	0.7357 \pm 0.0395
Total		496	31	

ND2

No. samples (N)	No. haplotypes (H)	Haplotype diversity ($h \pm$ SD)
56	1	0.0000 \pm 0.0000
45	6	0.3919 \pm 0.0901
40	3	0.1885 \pm 0.0797
40	3	0.4449 \pm 0.0672
40	2	0.0974 \pm 0.0621
40	2	0.0974 \pm 0.0621
43	1	0.0000 \pm 0.0000
31	2	0.2323 \pm 0.0899
40	4	0.4064 \pm 0.0817
39	2	0.0513 \pm 0.0481
40	6	0.3974 \pm 0.0958
44	6	0.6543 \pm 0.0520
498	28	

Phylogeographic relationship of *A. cantonensis*

Based on sequences of Cytb



Angiostrongylus costaricensis (哥斯达黎加管圆线虫)

0.02

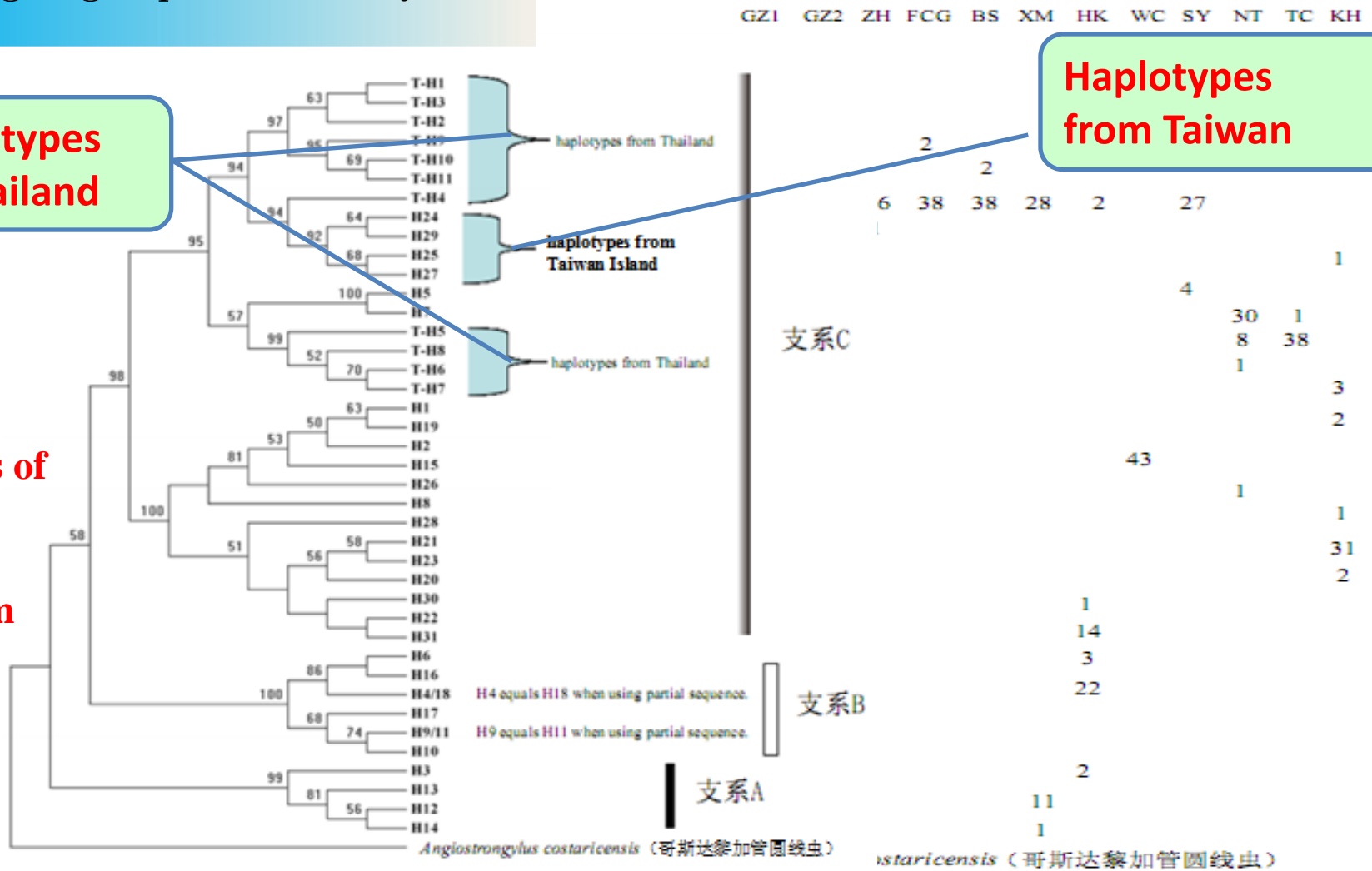
Results

Phylogeographic analyses

11 haplotypes from Thailand

Haplotypes from Taiwan

Based on partial sequences of cytb including some from thailand



Angiostrongylus costaricensis (哥斯达黎加管圆线虫) *costaricensis* (哥斯达黎加管圆线虫)

0.05



Results—Genetic structure of *A. cantonensis*

Table 7 Significance of Exact test between different populations base on cyt b and ND2

	GZ-1	GZ-2	ZH	XM	FCG	BS	HK	WC	SY	NT	TC	KH
GZ-1		*	*	*	*	*	*	*	*	*	*	*
GZ-2	0.10571* (0.07275*)		*	*	*	*	*	*	*	*	*	*
ZH	0.06746 (0.06956*)	-0.00192 (-0.00559)		*	*	*	*	*	*	*	*	*
XM	0.26725* (0.32487*)	0.03378 (0.13506*)			*	*	*	*	*	*	*	*
FCG	0.11314* (0.03884)	0.07693* (0.05566*)				*	*	*	*	*	*	*
BS	0.22876* (0.00000)	0.08917* (0.05568*)					*	*	*	*	*	*
HK	0.87284* (0.94869*)	0.69908* (0.82410*)						*	*	*	*	*
WC	0.99418* (1.00000*)	0.75181* (0.81368*)							*	*	*	*
SY	0.14059* (0.15114*)	0.06237* (0.02952)								*	*	*
NT	0.87166* (0.89756*)	0.31517* (0.46004*)									*	*
TC	0.98668* (0.99740*)	0.33798* (0.53175*)										*
KH	0.84372* (0.89636*)	0.58248* (0.73747*)	0.69297* (0.78096*)	0.55466* (0.64750*)	0.80932* (0.87642*)	0.81520* (0.87759*)	0.78505* (0.89663*)	0.78961* (0.87164*)	0.77115* (0.84686*)	0.75337* (0.82610*)	0.78771* (0.86143*)	

Haplotypes from Taiwan



Results—Genetic differentiation of *A. cantonensis*

Table 8 Statistics of analysis molecular variance (AMOVA) between different populations base on cytb (and ND2)

Source of variation	Percentage of variation	<i>P</i> value
Between HK population and all other groups as a whole	67.59 (65.60)	0.08016 (0.08472)
Among populations	19.93 (24.28)	0.00000 (0.00000)
Within populations	12.48(10.12)	0.00000 (0.00000)
Total		

More than 2/3 of variation from btween HK population and other population as a whole

Variation is over 80% among different populations



Results of Neutrality test

Table 9 Tajima's *D* and Fu's *F_s* tests of variation of population of *A. cantonensis* based on sequences of *Cytb* and *ND2*

	Tajima's <i>D</i>		Fu's <i>F_s</i>	
	<i>D</i>	<i>P</i>	<i>F_s</i>	<i>P</i>
HKW	-0.47781(-0.12052)	0.37650(0.53960)	20.30381(19.05730)	0.97080(0.97170)
HK	-0.78714 (-1.93366)	0.23030 (0.00870)	19.65693 (6.17550)	0.99990 (0.96890)
Total	0.51692 (0.70941)	0.77640 (0.82270)	29.37733 (24.00816)	0.98050 (0.97750)

Tests for both Hekou population and that outside Hekou revealed insignificant Tajima's *D* and *F_s* values, which implies that *A. cantonensis* did experience demographical expansion.

1. Genetic diversity and historical demography of *Achatina fulica*

- ***A. fulica* population in Taiwan generally have higher genetic diversity and were detected to have experience historical population expansion.**

Re: In general, this snail was considered introduced from Taiwan via Xiamen.

- ***A. fulica* population in Hekou area, Yunnan Province with high genetic diversity**

Re: The snail population in Hekou area might be artificially introduced or migrated naturally from Southeast countries, such as Vietnam.

2. Genetic structure of *Achatina fulica*

- *A. fulica* population in Taiwan is comprised by two slightly differentiated groups, with genetic differentiation, was considered as nonrandom mating population.

Re: The snail might be introduced from different sources, one of which was lately introduced to mainland China.

Both high mountain and water separation might have complicated impact to the genetic separation and differentiation.

A. fulica populations in south part of mainland China were also found to be with genetic differentiation and are not random mating population due to the isolation of water and high mountains.

3. The origin of *A. cantonensis* in southern mainland China.

- Hekou, Yunan Province might be the origin or at least one of the origins of *A. cantonensis* in China.

Re: *A. cantonensis* population in Hekou area have obvious higher genetic diversity than other regional populations.

It was reflected on the phylogeographic relationship (based on *cytb* gene sequence) because some of haplotypes collected from Hekou formed a clade near the bottom although some of its members are also appears in other clades.

Drozdz et al (1975) suggested that south east asian countries are the origin of *A. cantonensis* while Hekou area borders on Vietnam.

Tokiwa et al (2012) proposed that *A. cantonensis* was multiple origin based on sample from different areas including some parts in China.

I. For *Achatina fulica*

- With high genetic diversity, nonrandom mating population, but without significant structures,.
- Population experienced demographical expansion.

2. For *Angiostrongylus cantonensis*

- Hekou area in Yunnan Province is with higher genetic diversity than other areas, nonrandom mating population, but without significant structures.
- Population did not experience demographical expansion.

3. Role of *Achatina fulica* in the spread of *Angiostrongylus cantonensis* is still unclear, needs sample from more locations.



thanks

謝謝

for your attention