Genetic population structure of *Tachypleus tridentatus* in Japan and attempt to comparative phylogenetic study with parasitic planarian species on Asian horseshoe crabs

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and Conservation of Asian Horseshoe Crabs, Hong Kong

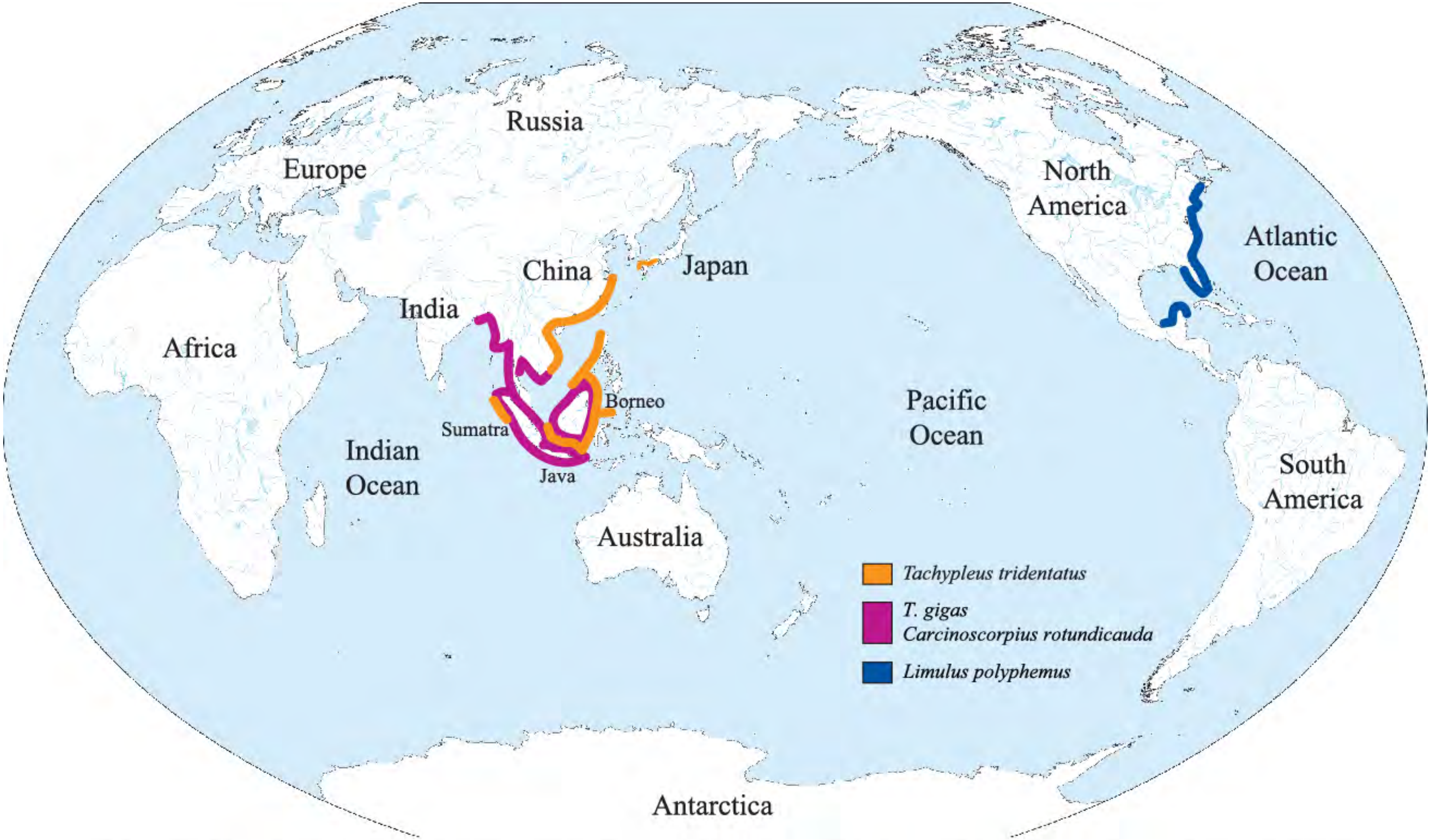
Topics of this presentation

- Mitochondrial (mt) DNA analyses as maternal marker
  (adding new locality: more eastern, Hirao Bay.)
- Microsatellite (MS) DNA analyses with high resolution as biparental marker

- *Sub-topic*: A case of HSC as invader species

- Attempt to…comparative phylogeographic analyses between HSC and their parasitic planarian species.
  ~About outline of this study and sampling strategy~

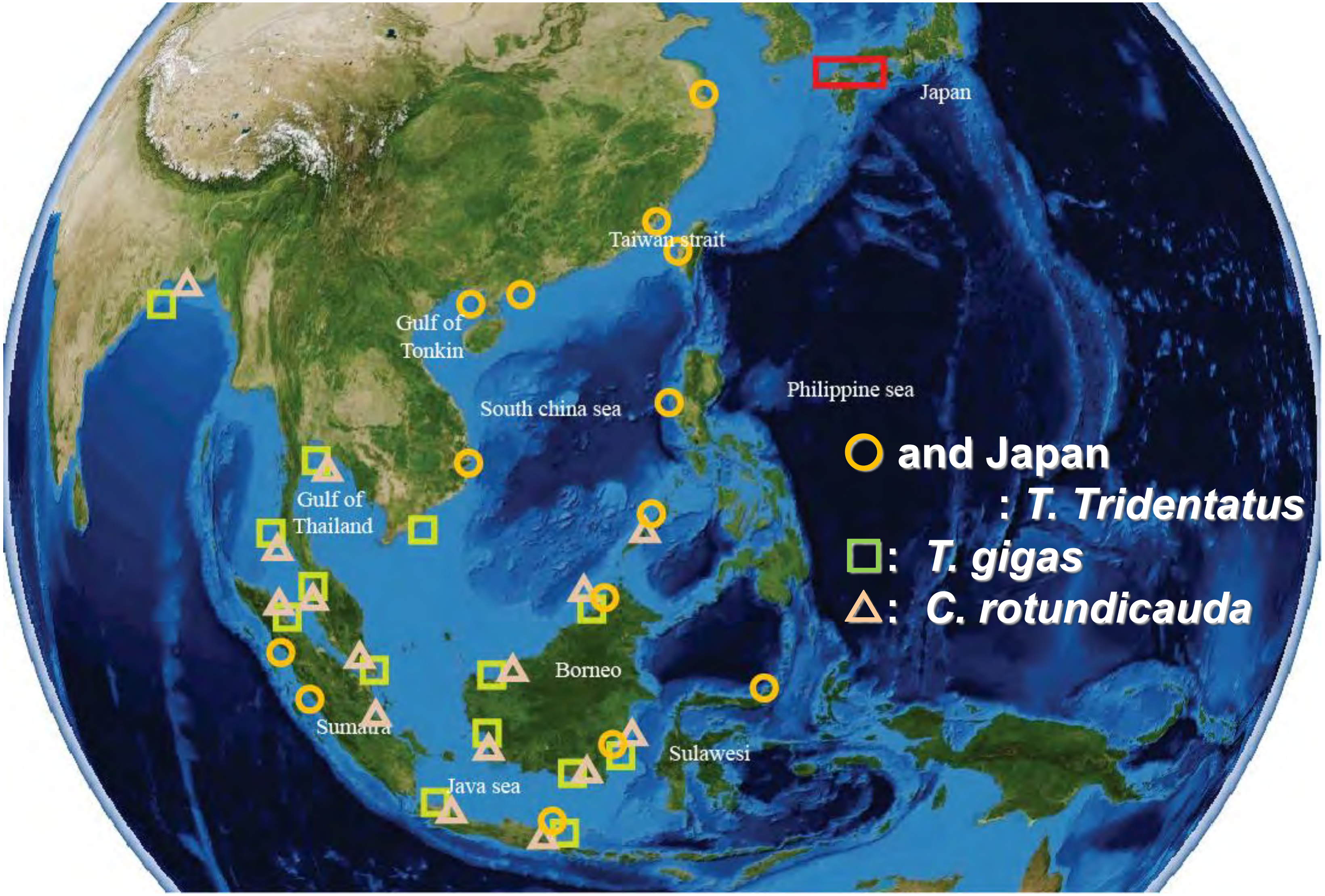
Distributions of HSCs



Distribution of the extant four species of horseshoe crabs

Modified from © *University of Delaware Graduate College of Marine Studies and the Sea Grant College Program and Sekiguchi (1988)*

Distribution of Asian HSCs



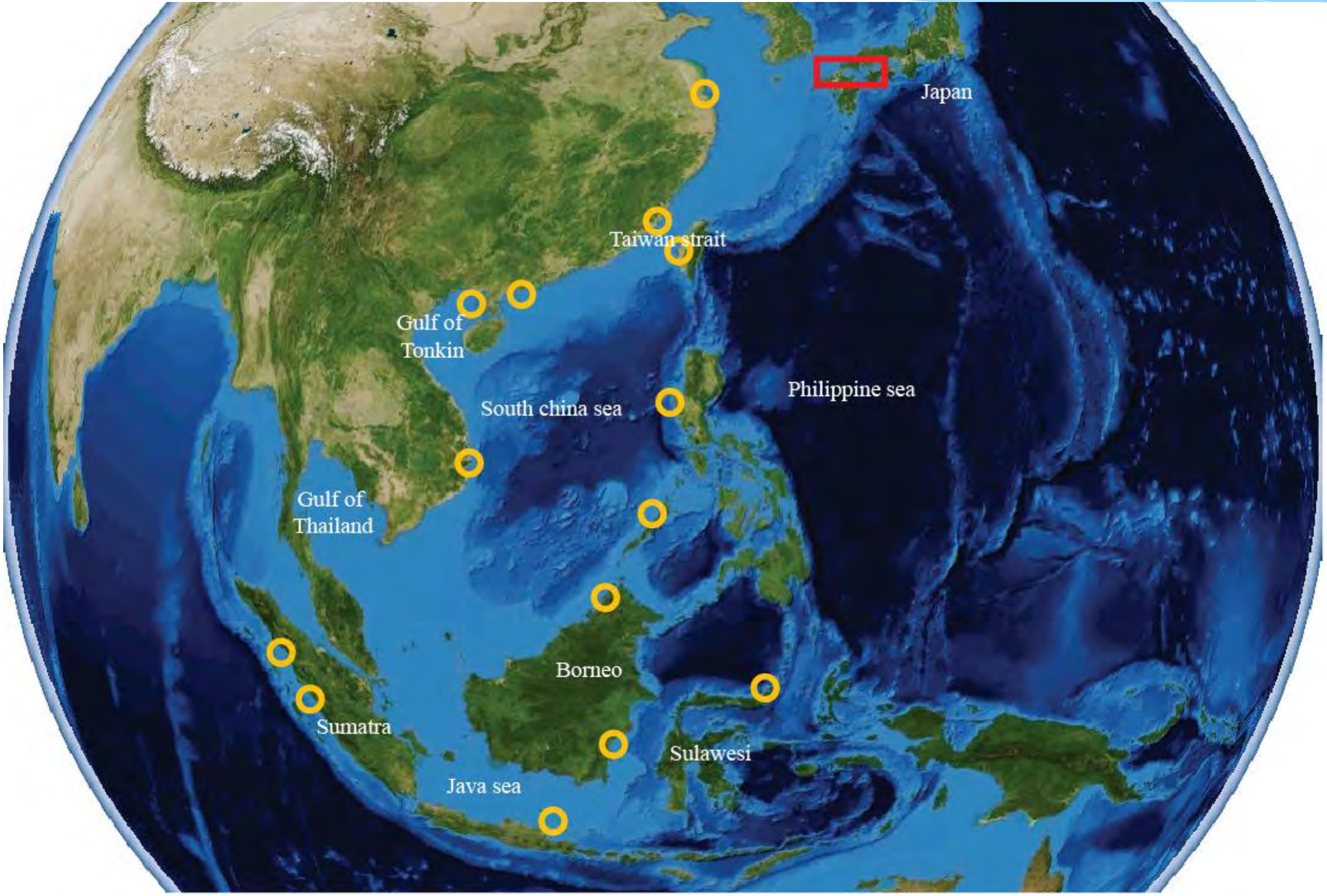
○ and Japan
: *T. Tridentatus*

□: *T. gigas*

△: *C. rotundicauda*

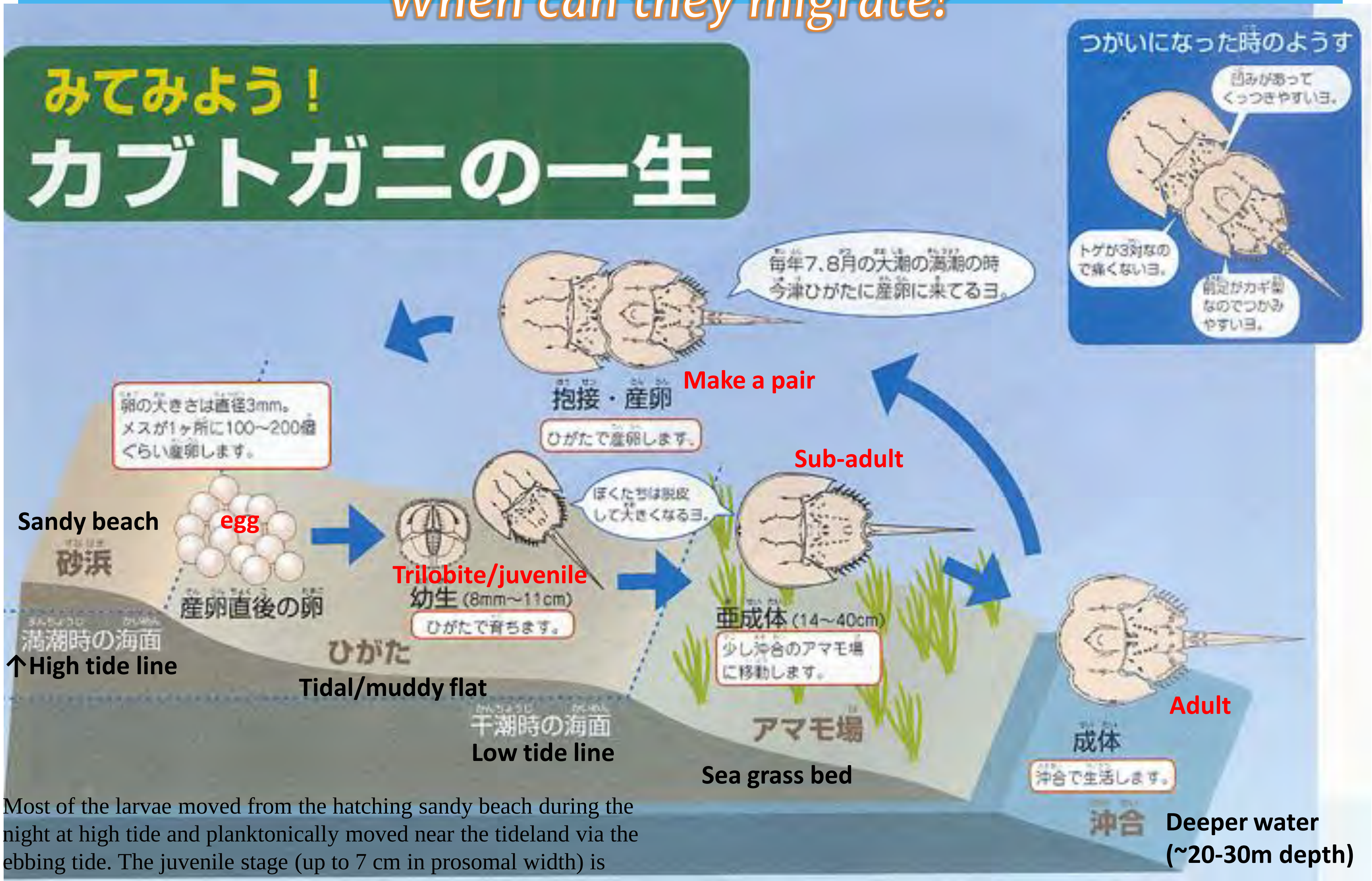
From Sekiguchi 1999 modified

Distribution of *T. tridentatus*



From Sekiguchi 1999 modified

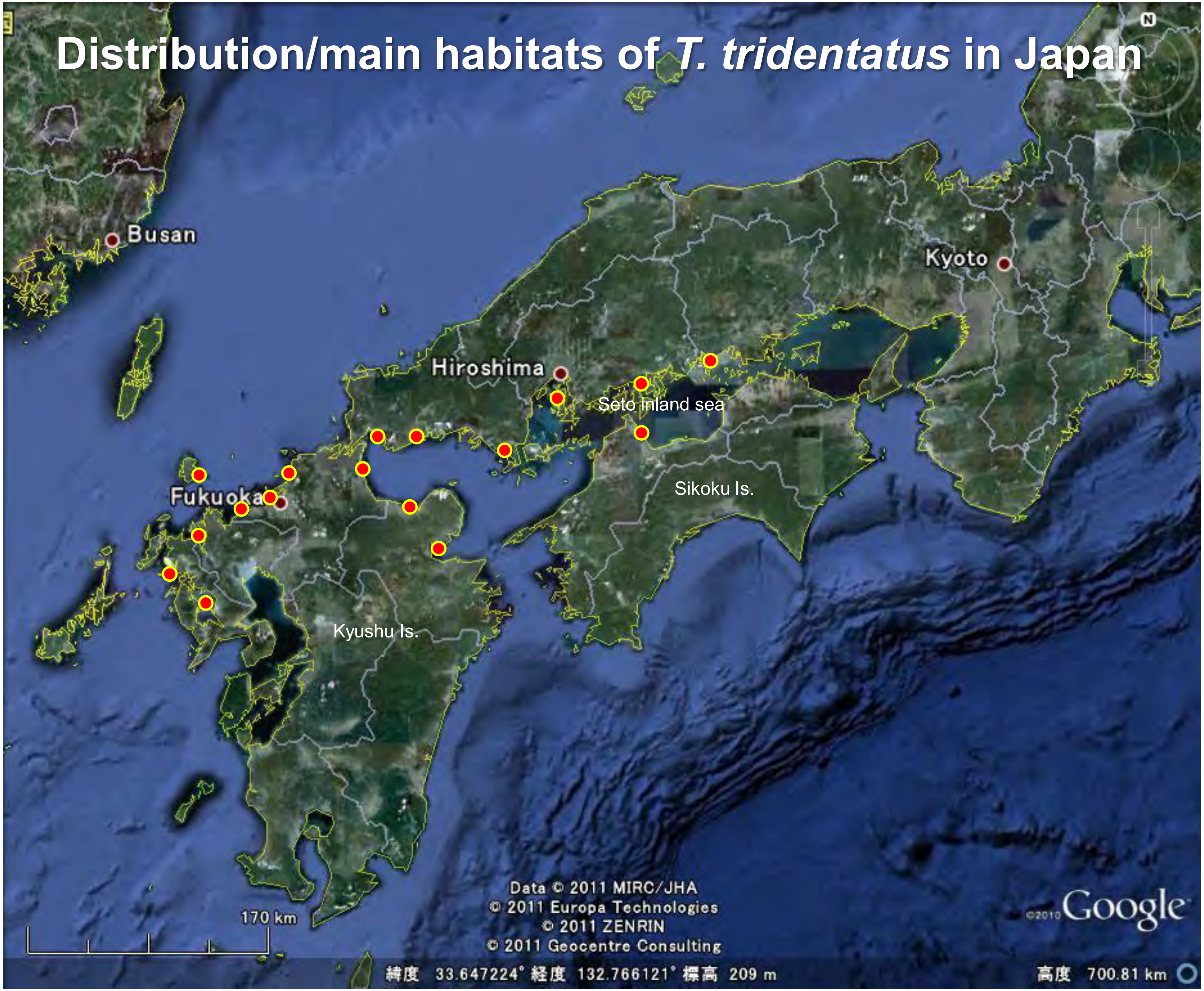
Life cycle of *T. tridentatus*
When can they migrate?



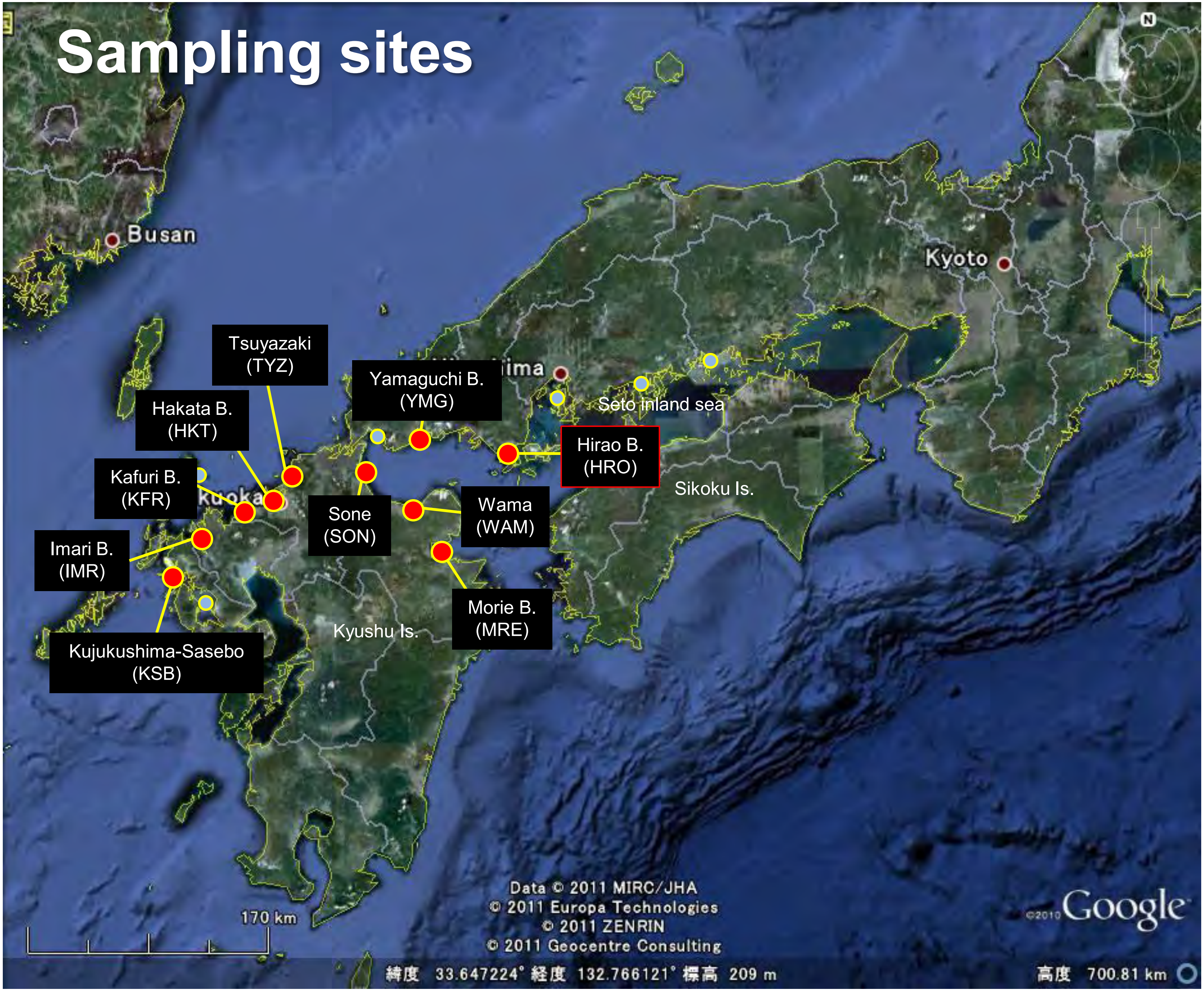
Most of the larvae moved from the hatching sandy beach during the night at high tide and planktonically moved near the tideland via the ebbing tide. The juvenile stage (up to 7 cm in prosomal width) is spent in the tideland near the hatching beach, and locomotive activity is relatively low (50 m/month) (Kawahara 1982).

福岡市環境局･(財)九州環境管理協会「ふくおかのカブトガニ」より

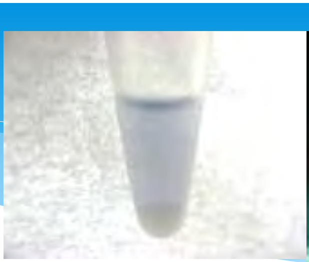
Distribution/main habitats of *T. tridentatus* in Japan



Sampling sites



Materials



Sampling sites / Samples	for mtDNA	for MS
Kujyukushima-Sasebo Bay	99	38
Imari Bay	30	26
Kafuri Bay	51	25
Hakata Bay	71	32
Tsuyazaki tidal flat	19	16
Sone tidal flat	51	25
Wama Bay (Usa)	7	7
Morie Bay (Kitsuki)	29	8
Yamaguchi Bay	22	25
Hirao Bay	37	37
Total	416	239

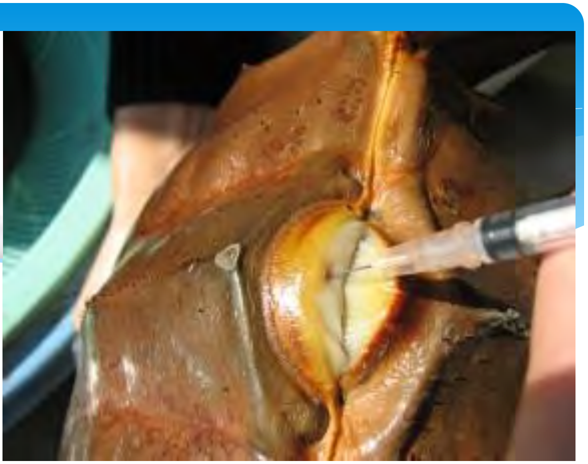


Photo 1 Method of hemolymph sampling. About 0.1 ml of hemolymph was collected from cardiac region by disposable syringe.

Photo 2 Egg samples.
(adjacent hatching)

Methods: region analyzed in this study

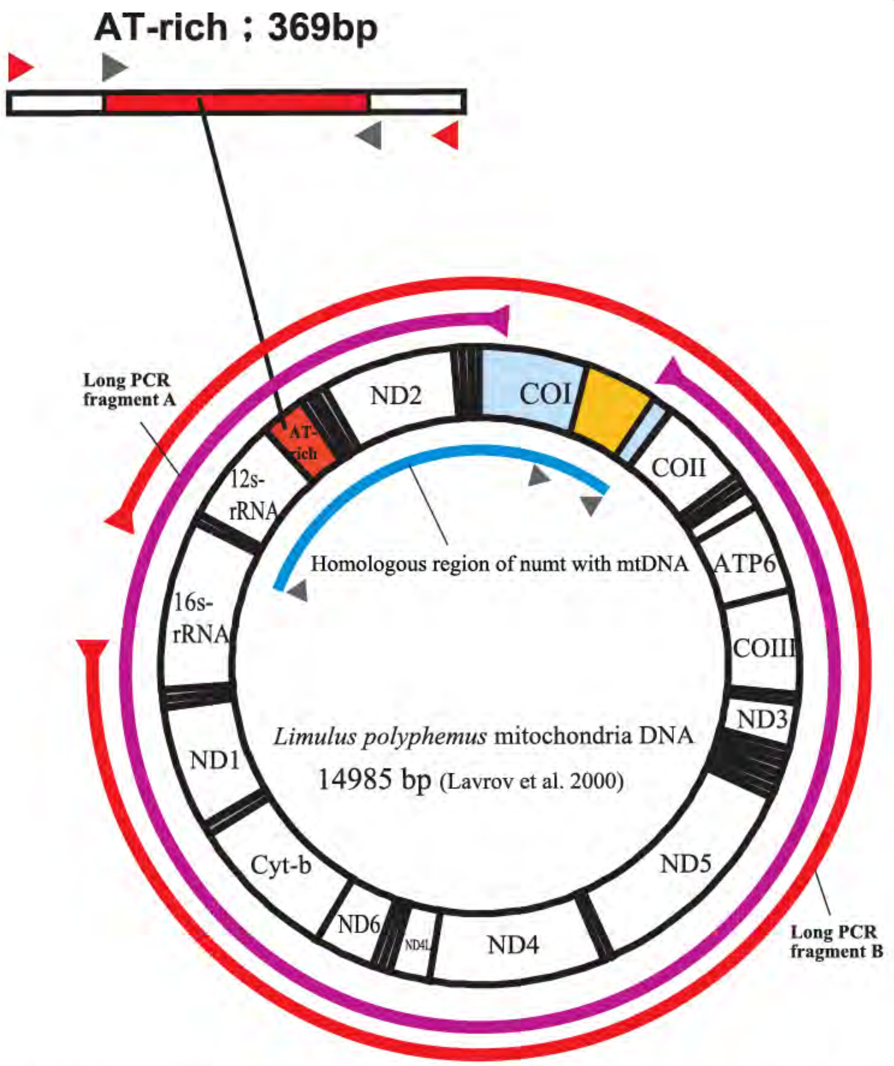
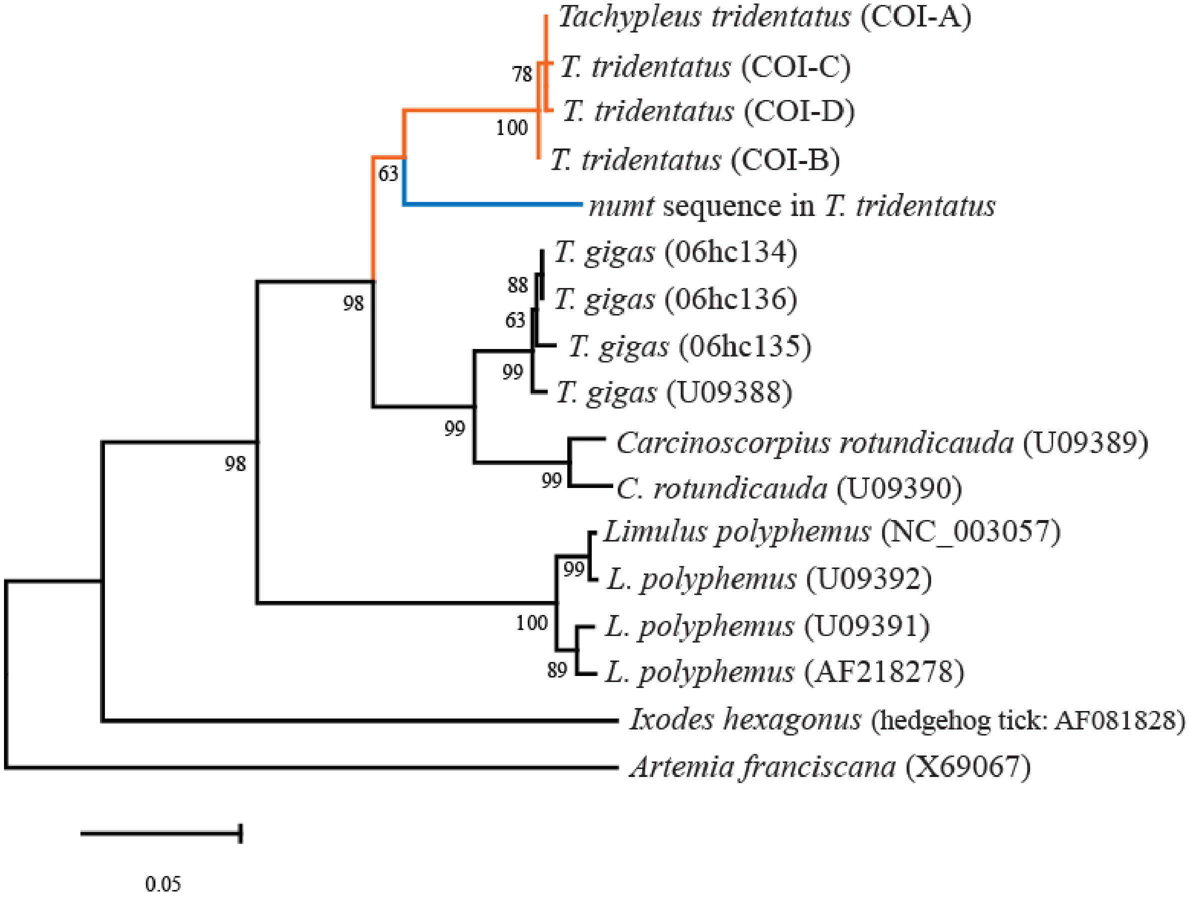


Fig. Analyzed region and primer position in this study.

The mitochondrial AT-rich (369 bp: control region) regions were analyzed. Numt (nuclear integrations of mtDNA) sequence was found in this species, which has high sequence similarity with mtDNA. Therefore, to verify the “true” mtDNA sequence, we conducted the long PCR and amplified the almost complete circular mtDNA. Then specific primers for amplification of the AT-rich region on the mtDNA were designed.

Found "numt" sequence and phylogenetic tree based on COI gene



The neighbor-joining tree for extant four horseshoe crab species and numt in *T. tridentatus* using partial COI sequence (552 bp). The tick and artemia were used as outgroup.

Term: Gene diversity (*h*) and F_{ST}(=R_{ST}/Φ_{ST})

Gene diversity (*h, H*)=

haplotype diversity, heterozygosity (expected):

An individual or population-level parameter for genetic diversity. The proportion of loci expected to be heterozygous in an individual (ranging from 0 to 1.0). In generally, high: over 0.8, middle: 0.8-0.6, low: under 0.6-0.5~.

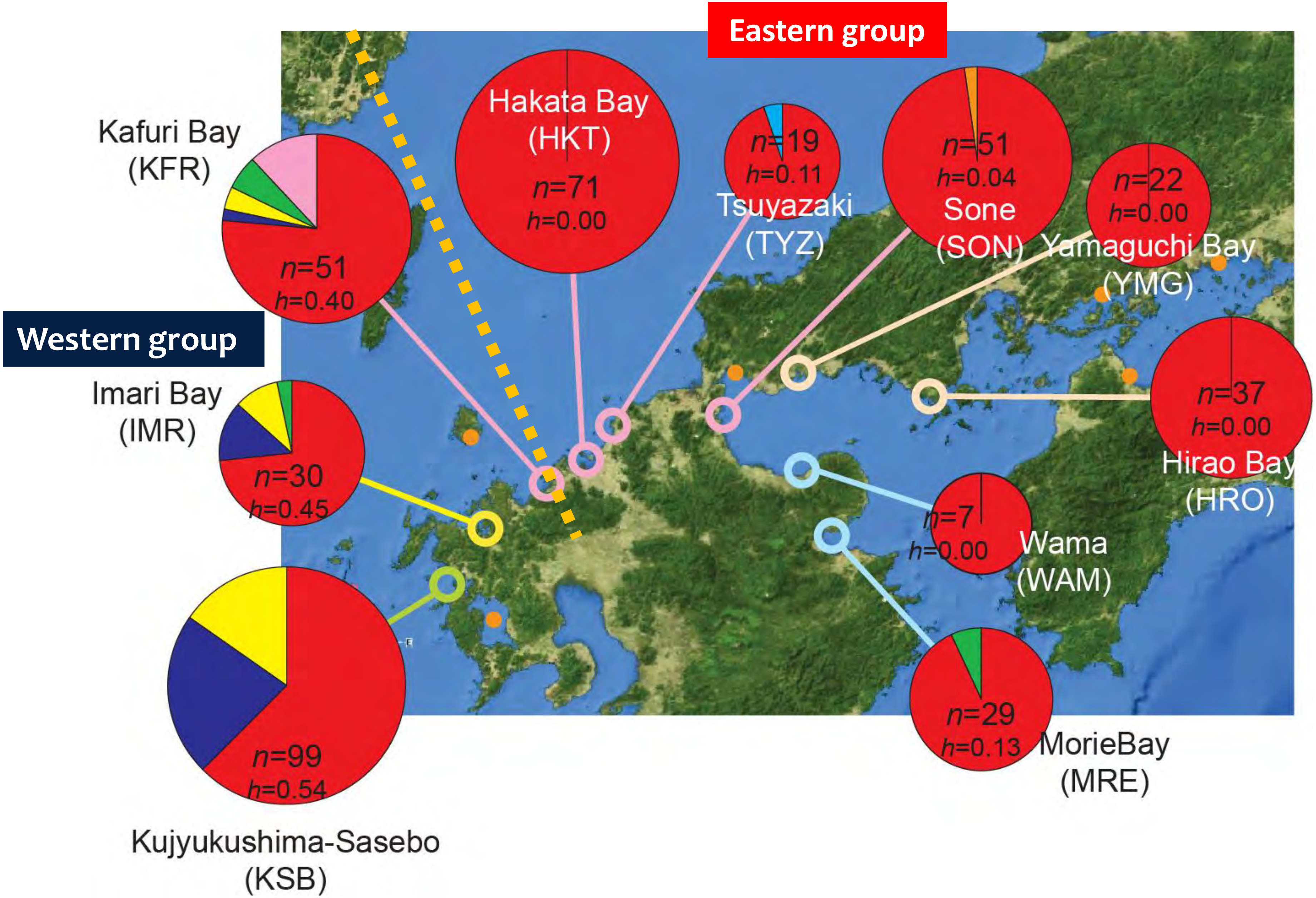
F_{ST} (*F*-statistics, fixation index)

This is the proportion of the total genetic variance contained in a subpopulation (H_S) relative to the total genetic variance (H_T). Values can range from 0 to 1. High F_{ST} (around over 0.15) implies a considerable degree of differentiation among populations.

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

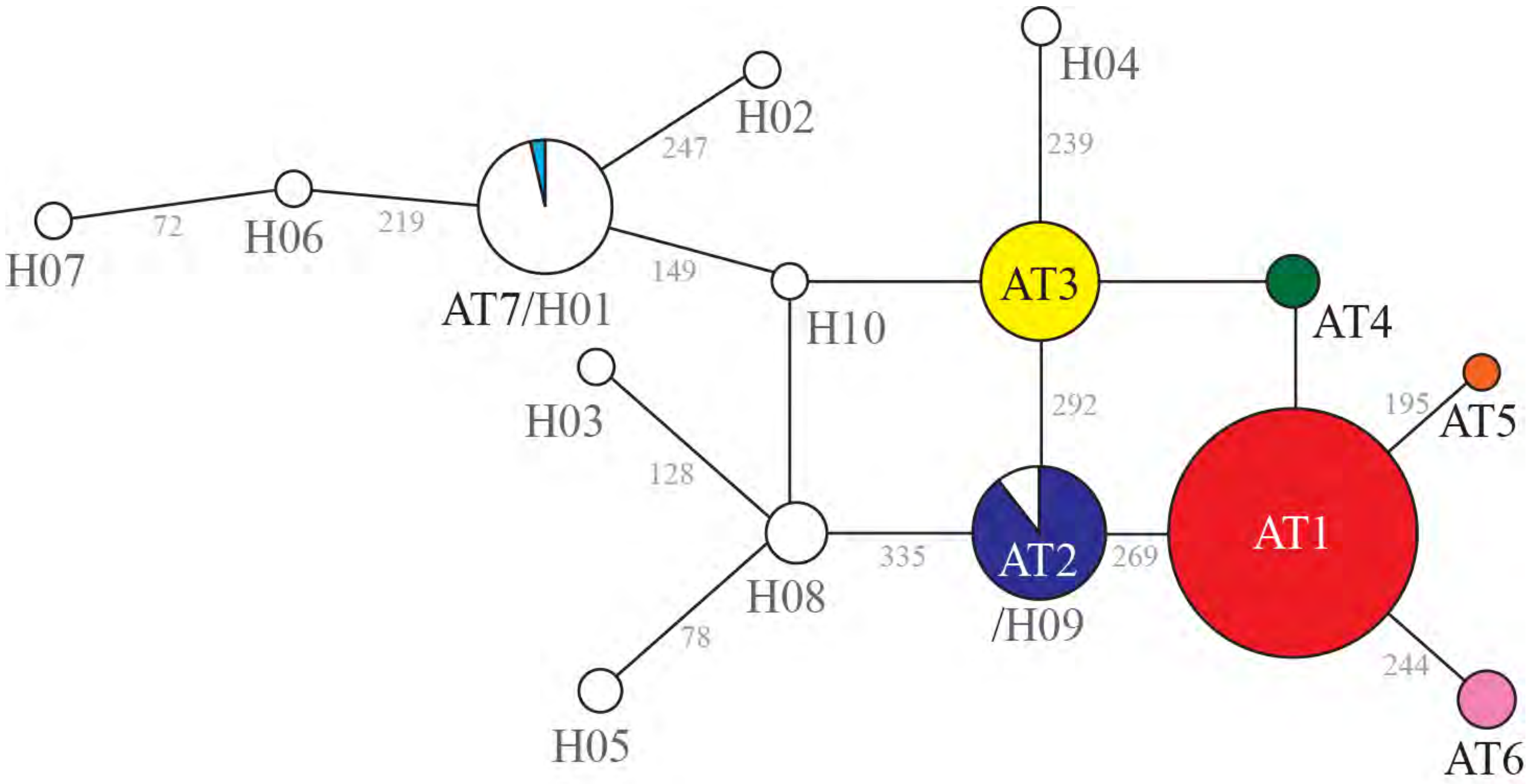
Results

Local populations/haplotypes	AT1	AT2	AT3	AT4	AT5	AT6	AT7	total	*h*	π (%)
Kujukushima-Sasebo Bay	62	22	15					99	0.54 ± 0.04	0.20 ± 0.17
Imari Bay	22	4	3	1				30	0.45 ± 0.10	0.17 ± 0.15
Kafuri Bay	39	1	2	3		6		51	0.40 ± 0.08	0.14 ± 0.13
Hakata Bay	71							71	0.00 ± 0.00	0.00 ± 0.00
Tsuyazaki tidal flat	18						1	19	0.11 ± 0.09	0.11 ± 0.12
Sone tidal flat	50				1			51	0.04 ± 0.04	0.01 ± 0.03
Wama Bay (Usa)	7							7	0.00 ± 0.00	0.00 ± 0.00
Morie Bay (Kitsuki)	27			2				29	0.13 ± 0.08	0.04 ± 0.06
Yamaguchi Bay	22							22	0.00 ± 0.00	0.00 ± 0.00
Hirao Bay	37							37	0.00 ± 0.00	0.00 ± 0.00
Total	355	27	20	6	1	6	1	416		



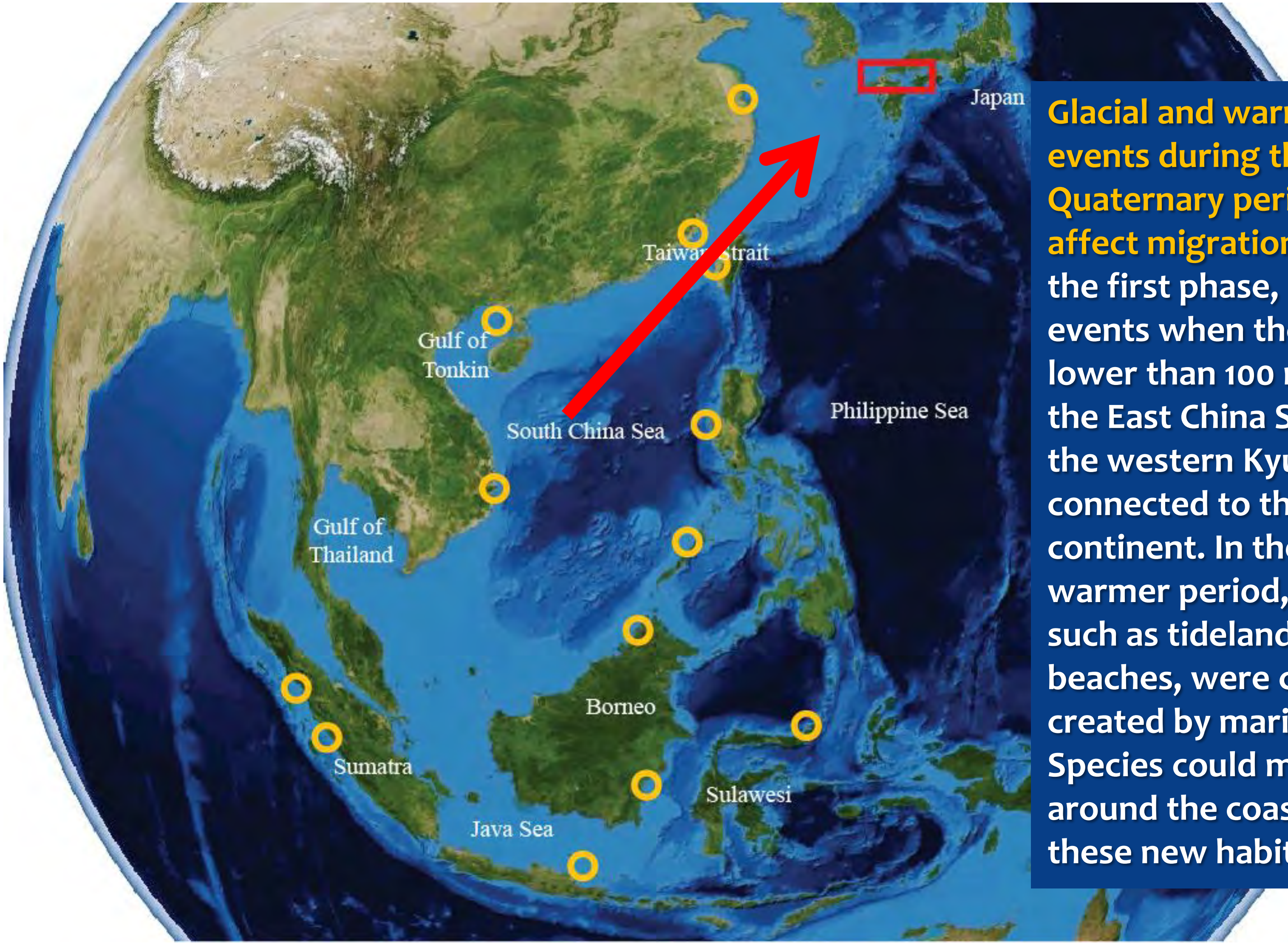
Frequency and distribution of haplotypes for *T. tridentatus* at Japan

h: gene diversity=haplotype diversity, heterozygote

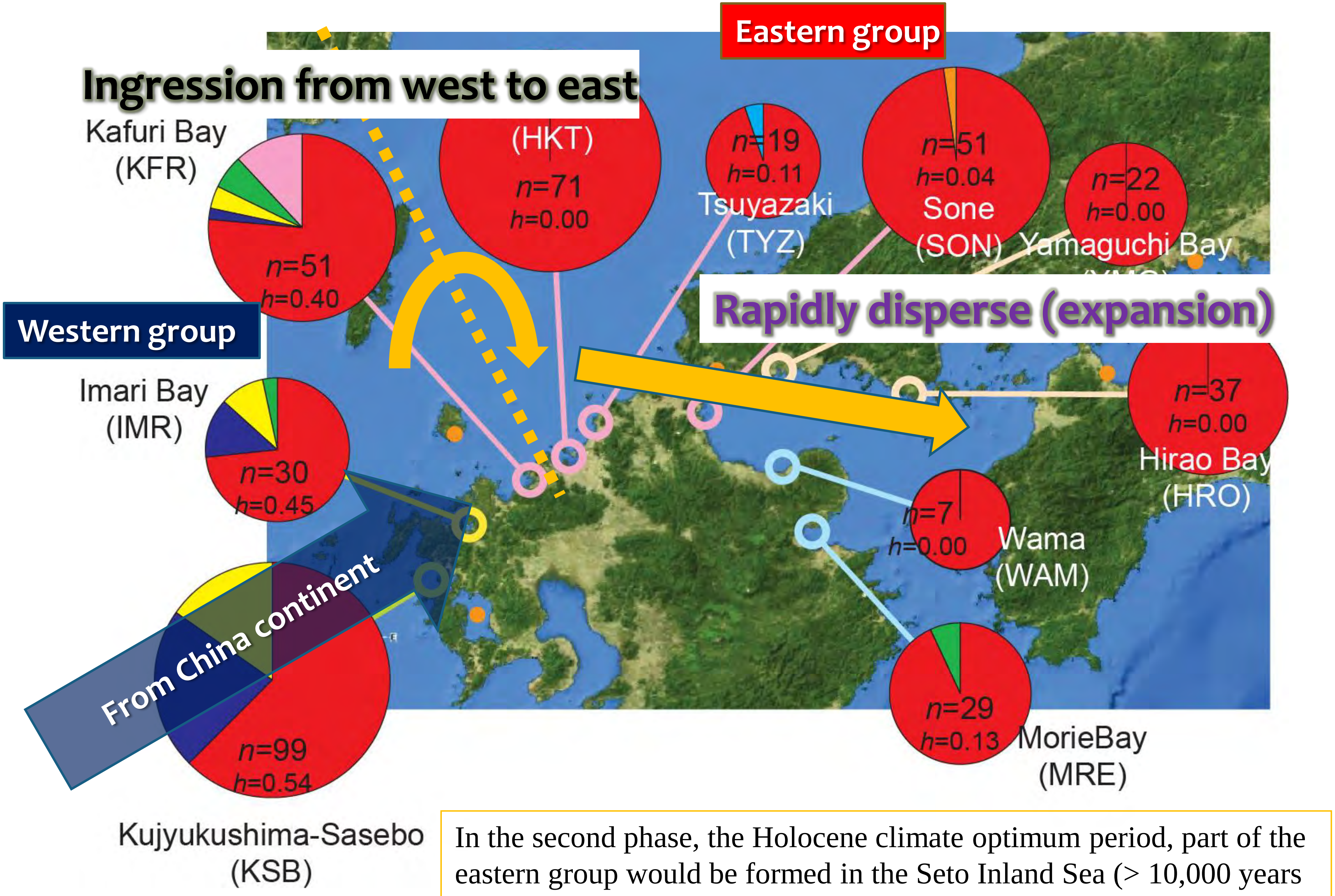


The MJ network for mtDNA AT-rich region haplotypes from Japan (AT1–AT7) and the Taiwan Strait (in white, H01–H10, Yang et al. 2007). The area of the circles is proportional to the frequency of the haplotype. Each line represents a mutational step with the indicated numbers.

When did they come to Japan?



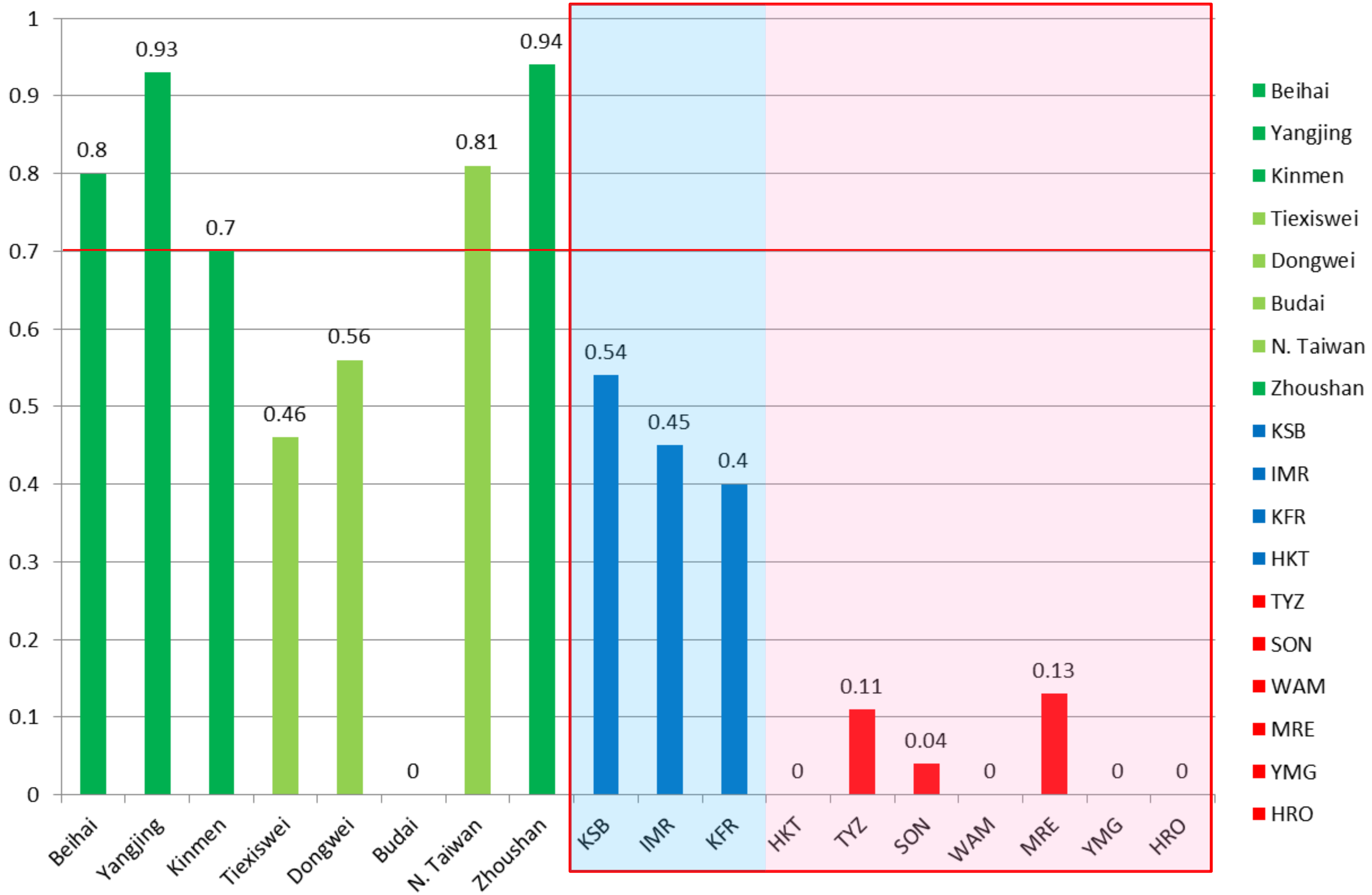
Glacial and warmer interglacial events during the middle to late Quaternary period would strongly affect migration of this species. In the first phase, during the glacial events when the sea level was lower than 100 m (Ohshima 1990), the East China Sea was dry, and the western Kyushu was connected to the southern Asian continent. In the subsequent warmer period, new habitat areas, such as tideland and sandy beaches, were continuously created by marine transgression. Species could migrate into Japan around the coastal line through these new habitats.



In the second phase, the Holocene climate optimum period, part of the eastern group would be formed in the Seto Inland Sea (> 10,000 years ago) , as this marine area emerged during this period.

Scenario estimated of migration and radiation to Japan

Comparison of gene diversities (*h*)



Mainland China and Taiwan
(Yang et al 2009)

Japan

Microsatellite (MS) DNA analyses with high resolution as biparental marker

1. Re-evaluation of genetic structure of Japanese populations by biparental inheritance DNA marker: Western vs. Eastern genetic group.

2. Try to detect population genetic structure in Eastern genetic group, which were almost completely monomorphic on mtDNA diversity, by MS DNA marker with high resolution.

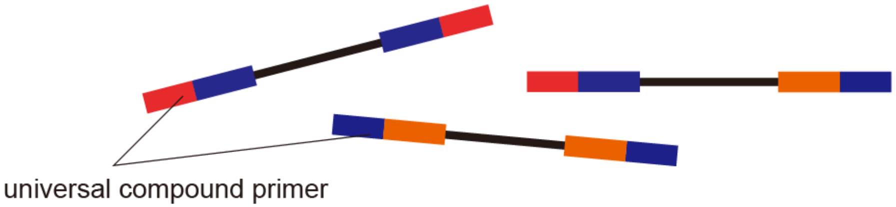
Development of compound microsatellite DNA marker of *T. tridentatus*

Development of new compound microsatellite DNA marker

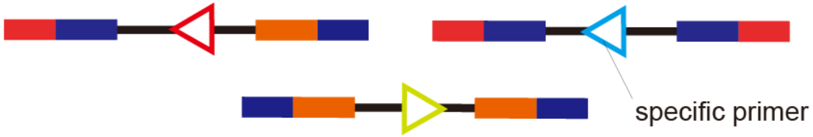
1. Direct PCR using universal compound primer which are designed for border position of repeat motifs (Lian et al 2006).

5′-TCTCTCTCTCACACACACACAC-3′
5′-ACACACACACAGAGAGAGAGAG-3′

2. Amplicons have compound microsatellites for both end. Isolation of each amplicon by TA-cloning and detect their sequence.



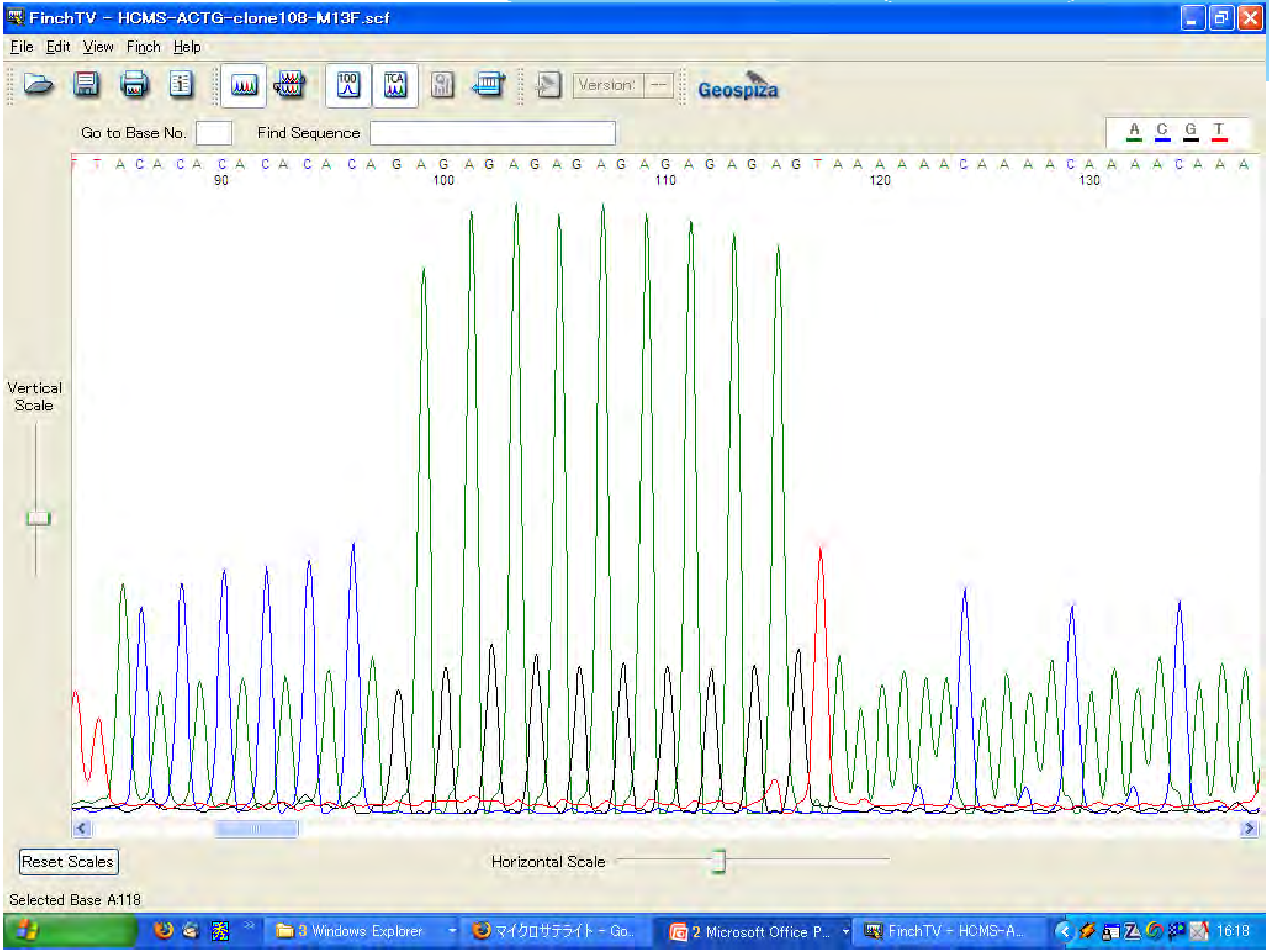
3. Design specific primer on either one side.



◇Success to development new eight compound MS markers.
- No need to construct an DNA libraries.
- All clones had microsatellite repeats.
- Can save a cost, because it is able to use common/share fluorescence dye-labelled compound primers (Lian et al. 2006).
- it has application in multiplex PCR. In this study, two to three loci are amplified at the same time in one tube.

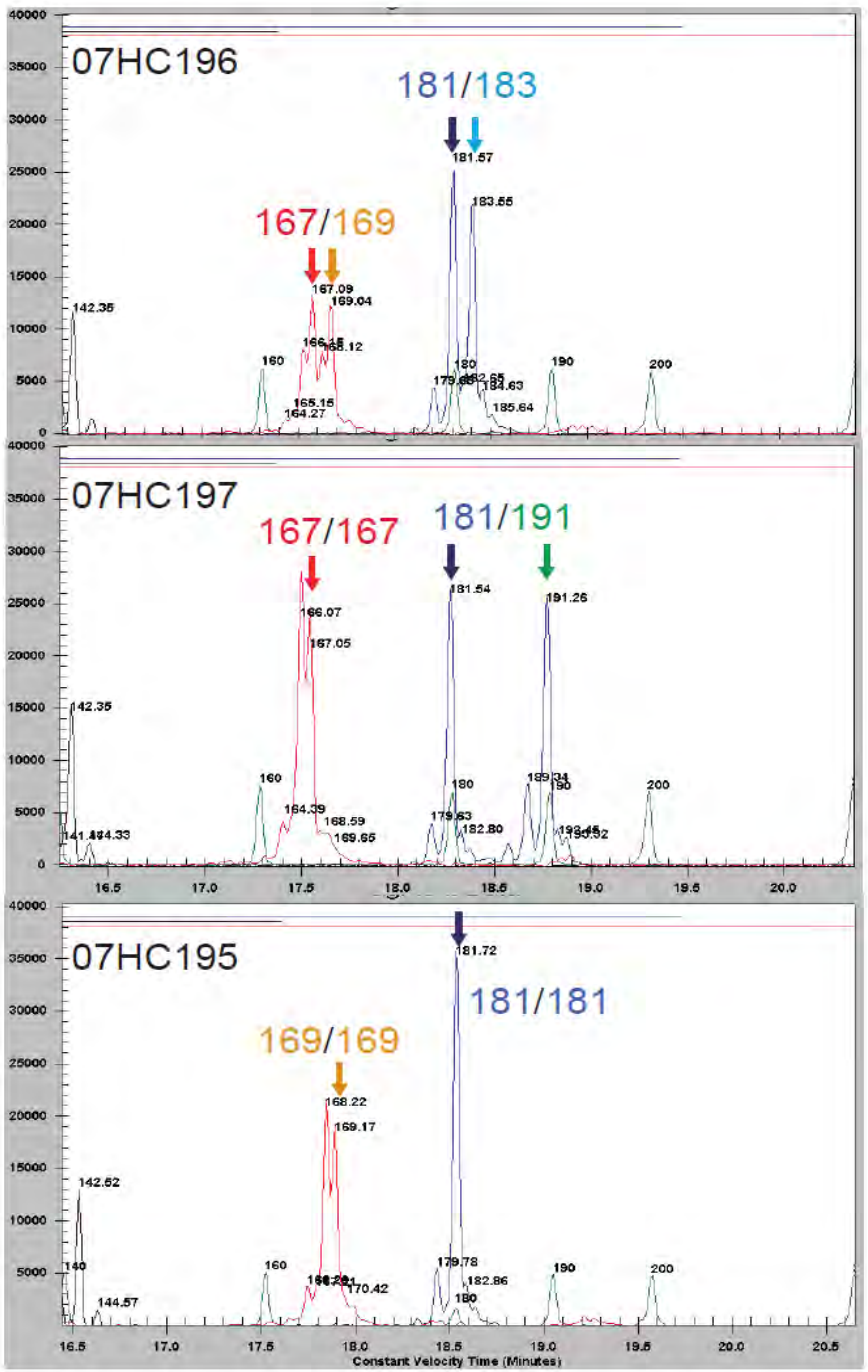
Example of output wave for microsatellite(s)

(STR: short tandem repeat, SSR: simple sequence repeat)



Example of detecting alleles on some microsatellite loci for *T. tridetatus*

These three individuals have different allele patterns: can identify each individuals using by these microsatellite loci.



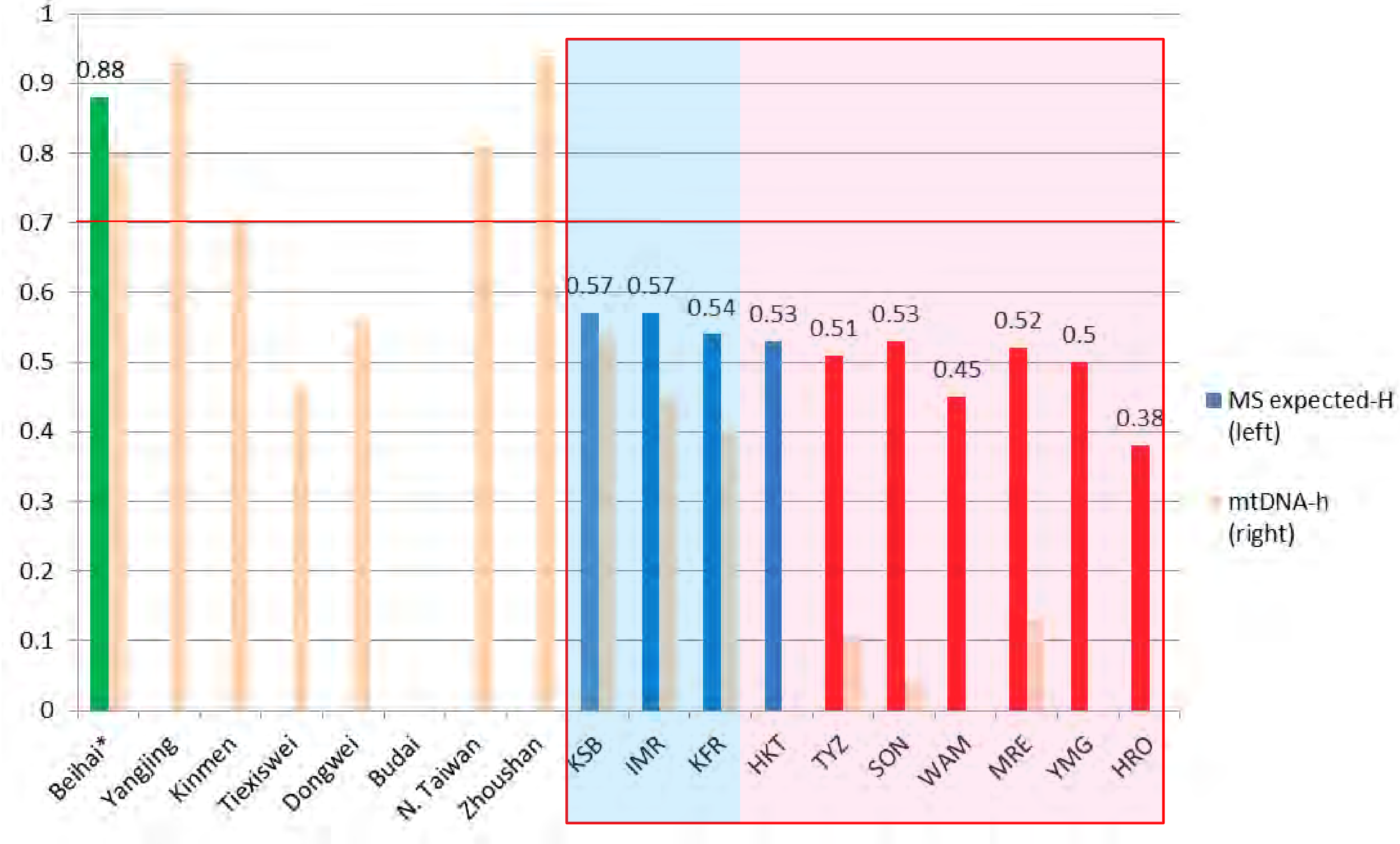
Result of microsatellite analysis

←Western Eastern→

			KSB			IMR			KFR			HKT			TYZ			SON			WAM			MRE			YMG			HRO		
			n=38			*n*=26			*n*=25			*n*=32			*n*=16			*n*=25			*n*=7			*n*=8			*n*=25			*n*=37		
	Na	size range (bp)	*Na*	H_O	H_E	*Na*	H_O	H_E	*Na*	H_O	H_E	*Na*	H_O	H_E	*Na*	H_O	H_E	*Na*	H_O	H_E	*Na*	H_O	H_E	*Na*	H_O	H_E	*Na*	H_O	H_E	*Na*	H_O	H_E
HCMS020	5	88-104	5	0.53	0.63	4	0.46	0.59	3	0.44	0.56	4	0.38	0.57	3	0.38	0.46	3	0.40	0.57	3	0.57	0.58	3	0.50	0.43	3	0.44	0.56	3	0.54	0.52
HCMS065	3	110-116	**KSB (*n*=38)** N_a = 4.0 H_O= 0.58 H_E= 0.57			**IMR (*n*=26)** N_a = 3.3 H_O= 0.50 H_E= 0.57			**KFR (*n*=25)** N_a = 3.1 H_O= 0.53 H_E= 0.54			**HKT (*n*=32)** N_a = 3.0 H_O= 0.52 H_E= 0.53			**TYZ (*n*=16)** N_a = 2.8 H_O= 0.54 H_E= 0.51			**SON (*n*=25)** N_a = 2.8 H_O= 0.45 H_E= 0.53			**WAM (*n*=7)** N_a = 2.5 H_O= 0.41 H_E= 0.45			**MRE (*n*=8)** N_a = 2.6 H_O= 0.45 H_E= 0.52			**YMG (*n*=25)** N_a = 2.8 H_O= 0.49 H_E= 0.50			**HRO (*n*=37)** N_a = 2.5 H_O= 0.42 H_E= 0.38		
HCMS008	2	115-117																														
HCMS069	4	129-145																														
HCMS060	2	132-136																														
HCMS015	3	140-144																														
HCMS068	5	165-177																														
HCMS059	7	171-192	8	0.87	0.82	5	0.54	0.69	5	0.64	0.66	4	0.66	0.67	4	0.69	0.72	4	0.48	0.63	3	0.71	0.62	4	0.50	0.73	4	0.64	0.57	3	0.59	0.64
Mean			4.0	0.58	0.57	3.3	0.50	0.57	3.1	0.53	0.54	3.0	0.52	0.53	2.8	0.54	0.51	2.8	0.45	0.53	2.5	0.41	0.45	2.6	0.45	0.52	2.8	0.49	0.50	2.5	0.42	0.38

Na: number of alleles, *Ho*: observed heterozygosity, *He*: expected heterozygosity

Comparison of gene diversities among pop. from mtDNA and MS analyses.

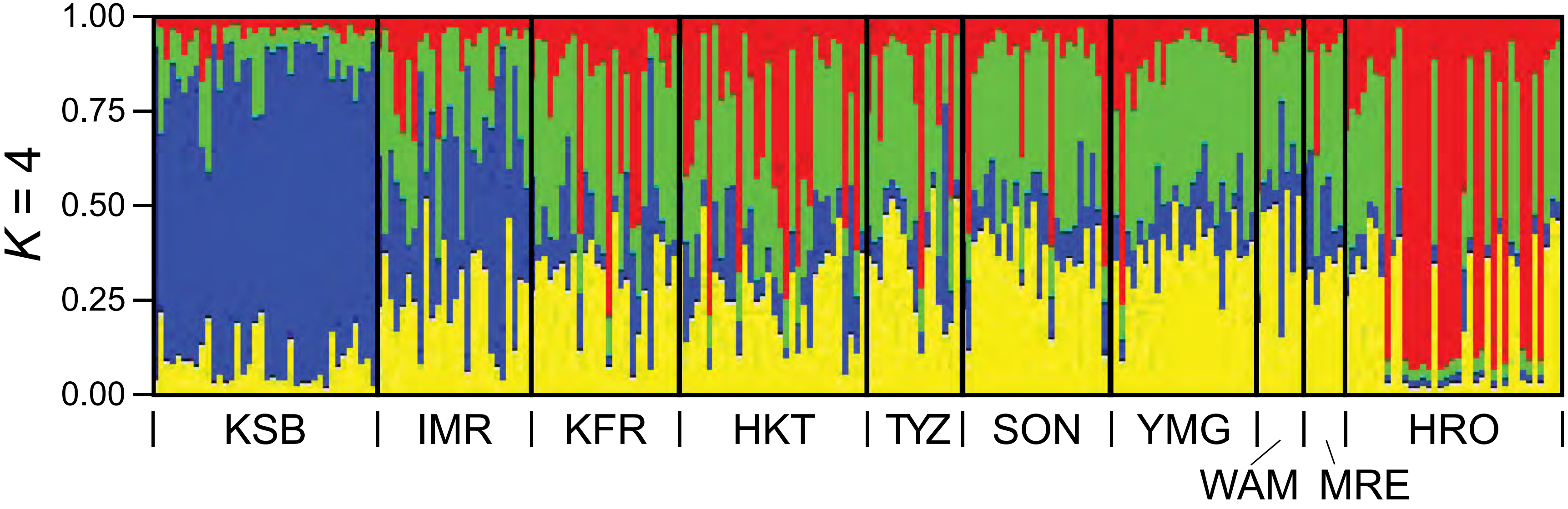


Mainland China and Taiwan
(mtDNA data from Yang et al 2009
MS data from Li et al 2009)

Japan

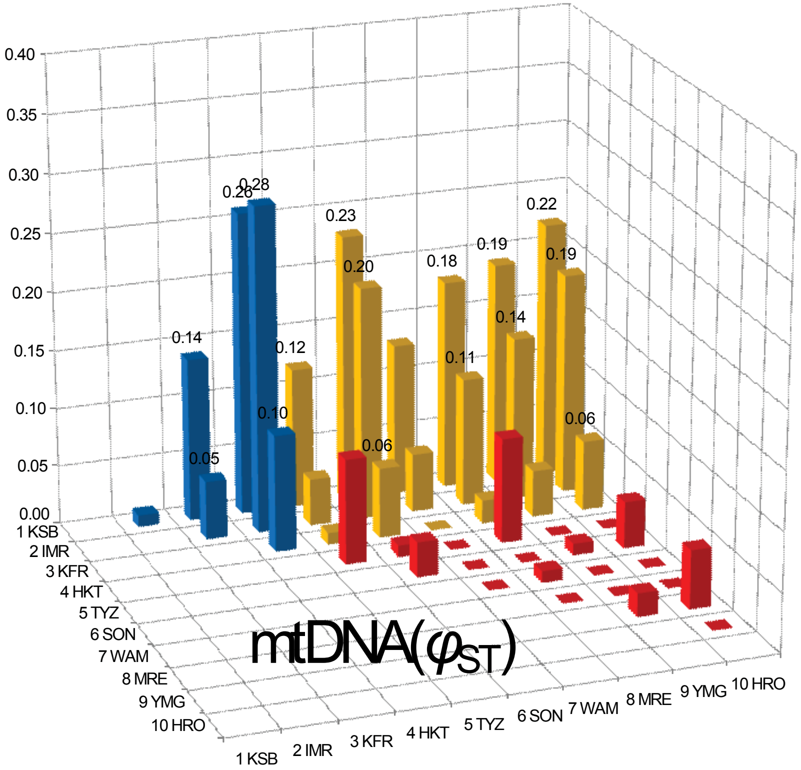
Genetic structure of *T. tridentatus* in Japan by Structure analysis using microsatellite data (Pritchard et al. 2000)

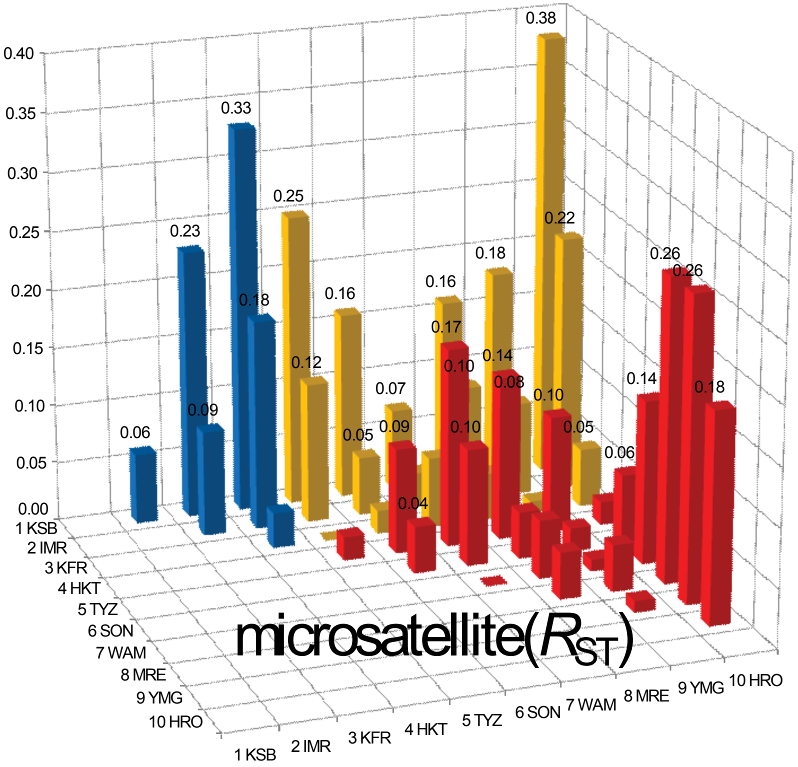
inferring the presence of distinct populations, assigning individuals to populations, studying hybrid zones, identifying migrants and admixed individuals



Estimate population (component) No.= K

Compare of genetic differentiations (*F*-statictics) from different DNA markers

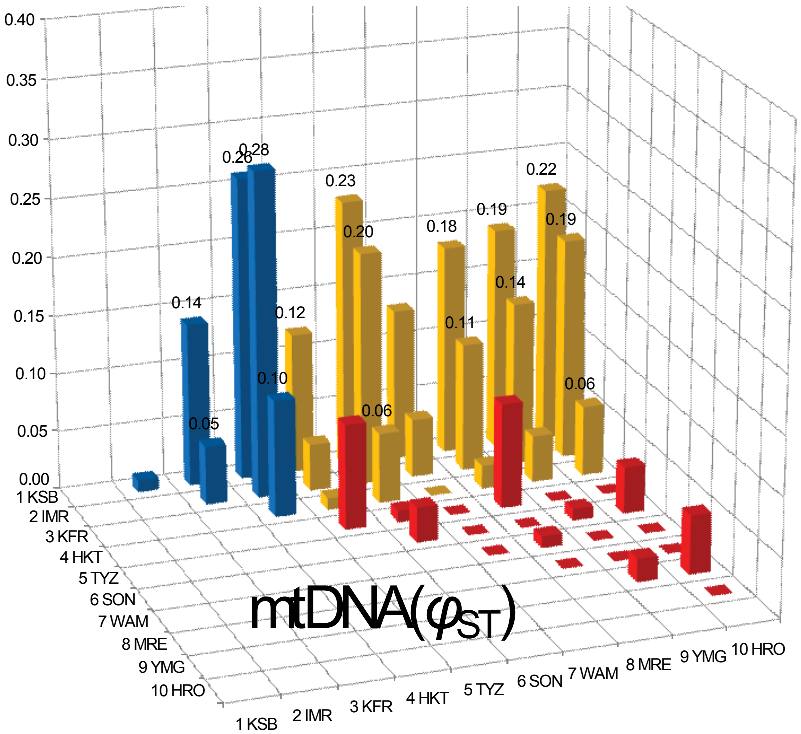


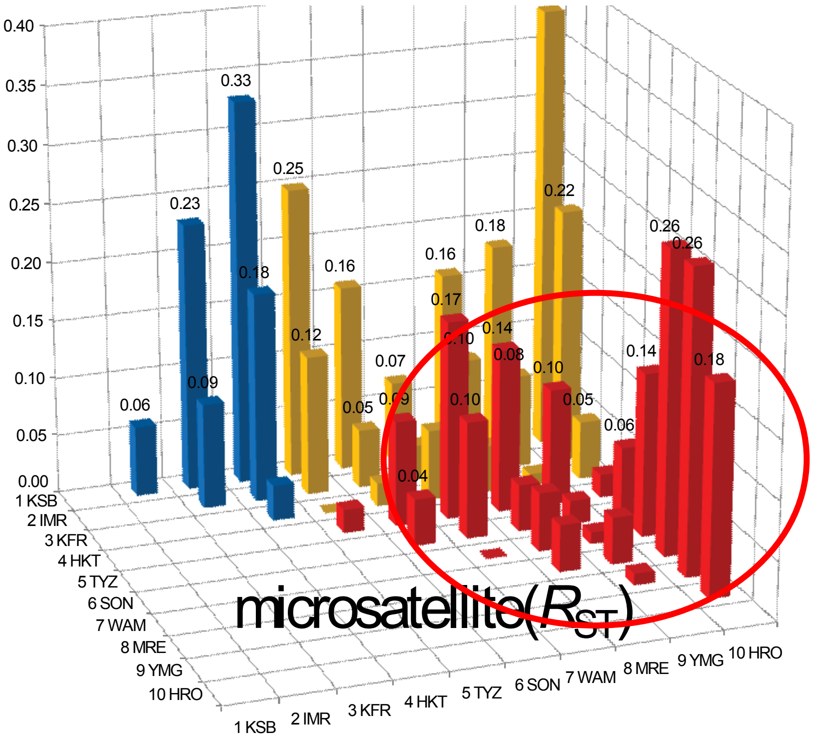


Blue bar: among Western pops. **Yellow bar:** between Western and Eastern pops, and **Red bar:** among Eastern pops.

Compare of genetic differentiations (*F*-statictics) from different DNA markers

Both maker detected strong genetic differences between Western and Eastern group.

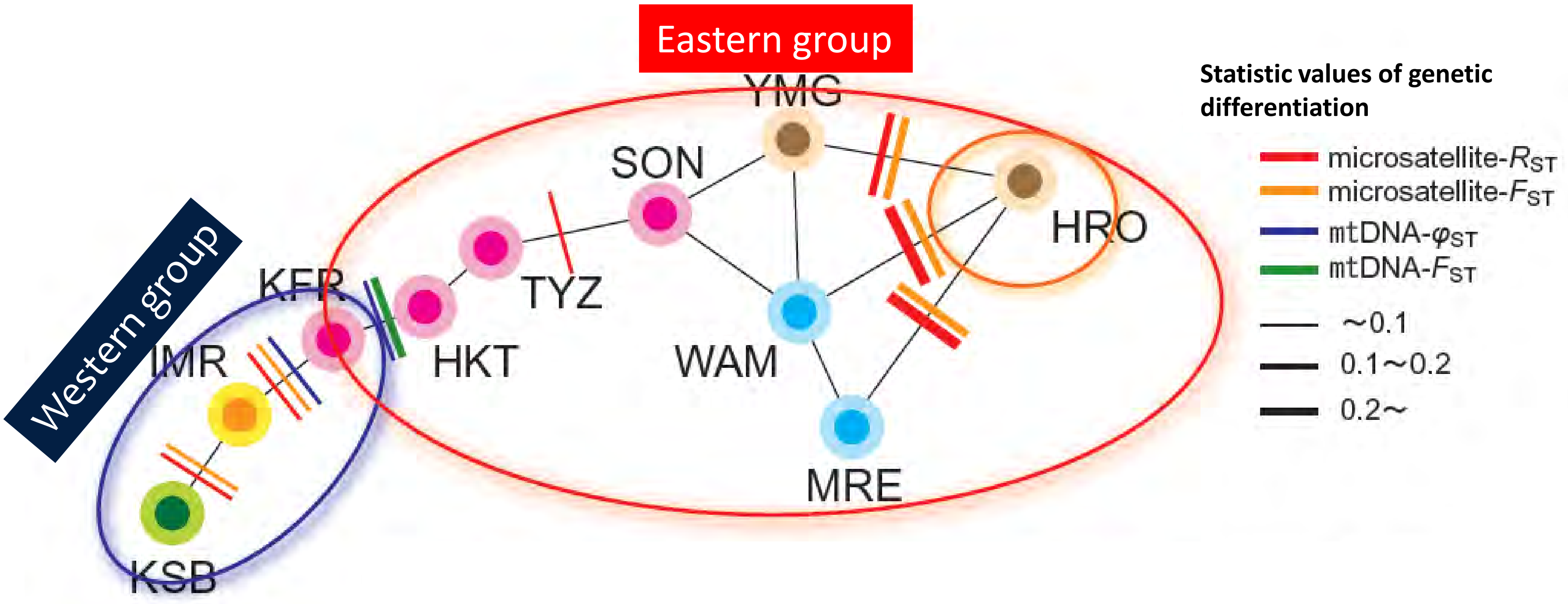




Blue bar: among Western pops. **Yellow bar:** between Western and Eastern pops, and **Red bar:** among Eastern pops.

Moreover, MS analyses was able to detect high level of genetic differences into Eastern group.

Summary of genetic structure of Japanese population for *T. tridentatus*

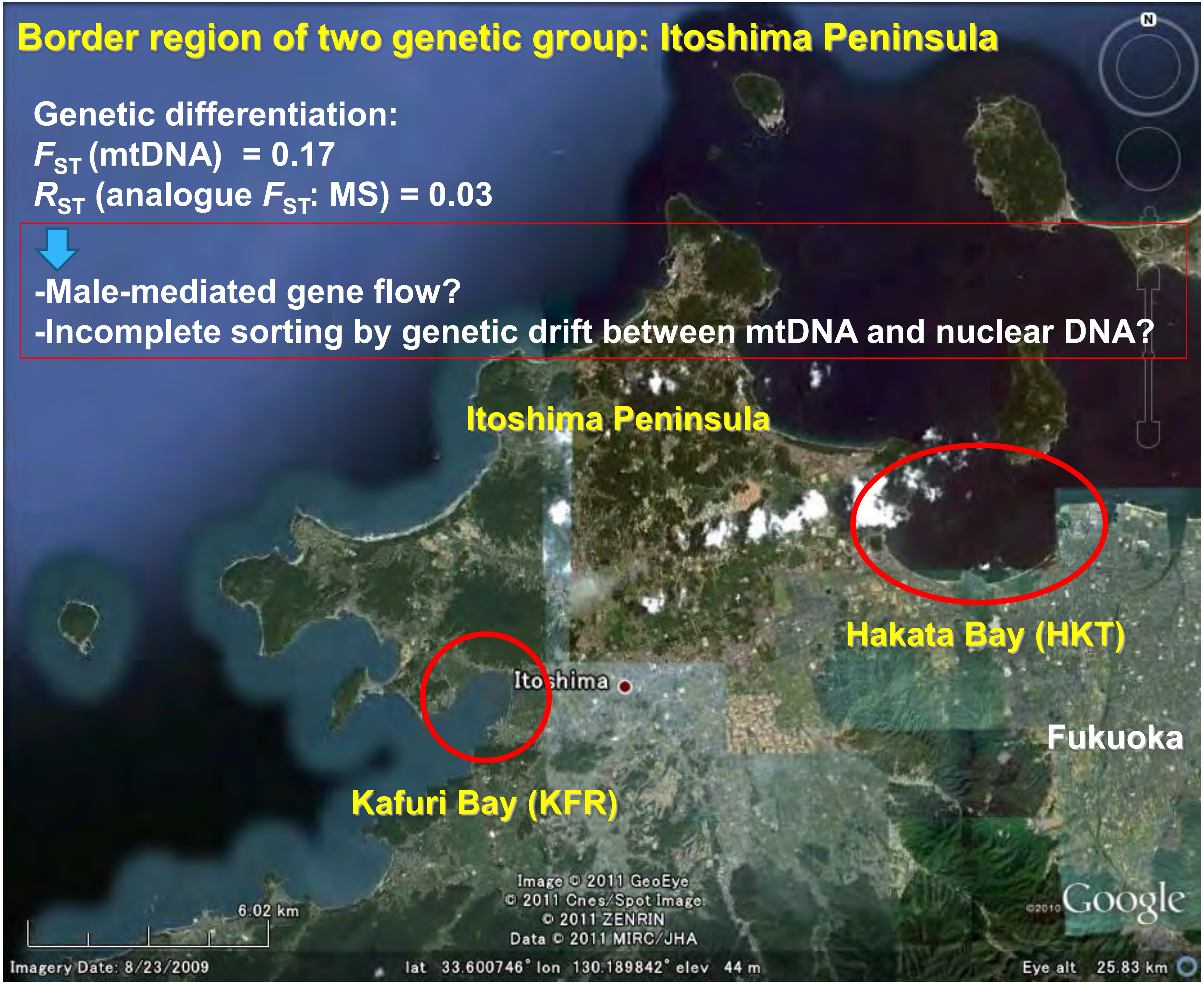


Border region of two genetic group: Itoshima Peninsula

Genetic differentiation:
F_{ST} (mtDNA) = 0.17
R_{ST} (analogue F_{ST}: MS) = 0.03

↓

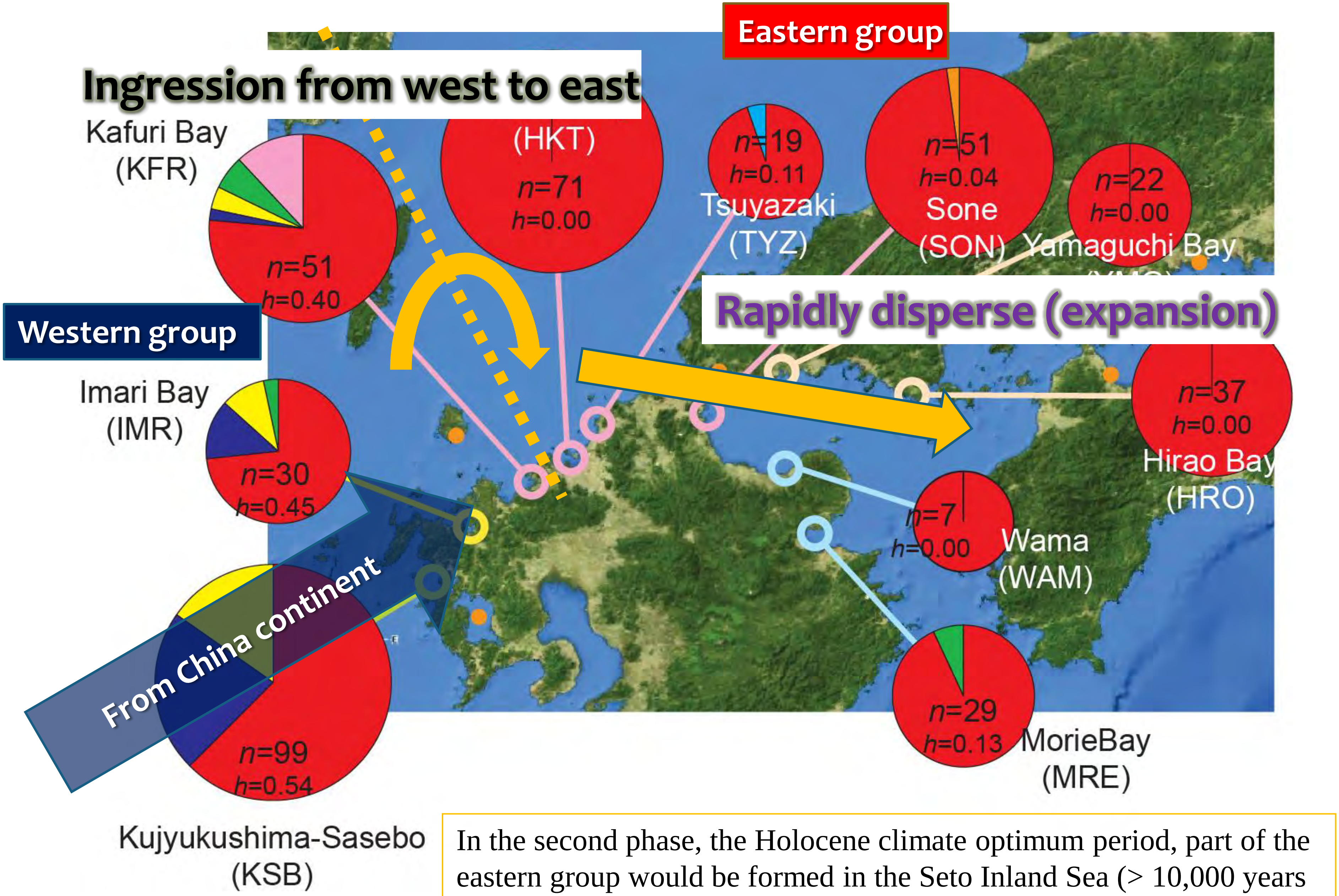
-Male-mediated gene flow?
-Incomplete sorting by genetic drift between mtDNA and nuclear DNA?



Border region of two genetic group: Itoshima Peninsula

Probably, sea level was ~5m down at around 6500 years ago.
Thus it was connected both habitats, KFR and HKT, that may be allowed some individuals of western group moved in to east.

- -Male-mediated gene flow?
- -Incomplete sorting by genetic drift between mtDNA and nuclear DNA?



In the second phase, the Holocene climate optimum period, part of the eastern group would be formed in the Seto Inland Sea (> 10,000 years ago) , as this marine area emerged during this period.

Scenario estimated of migration and radiation to Japan

Summary: feature of Japanese pops

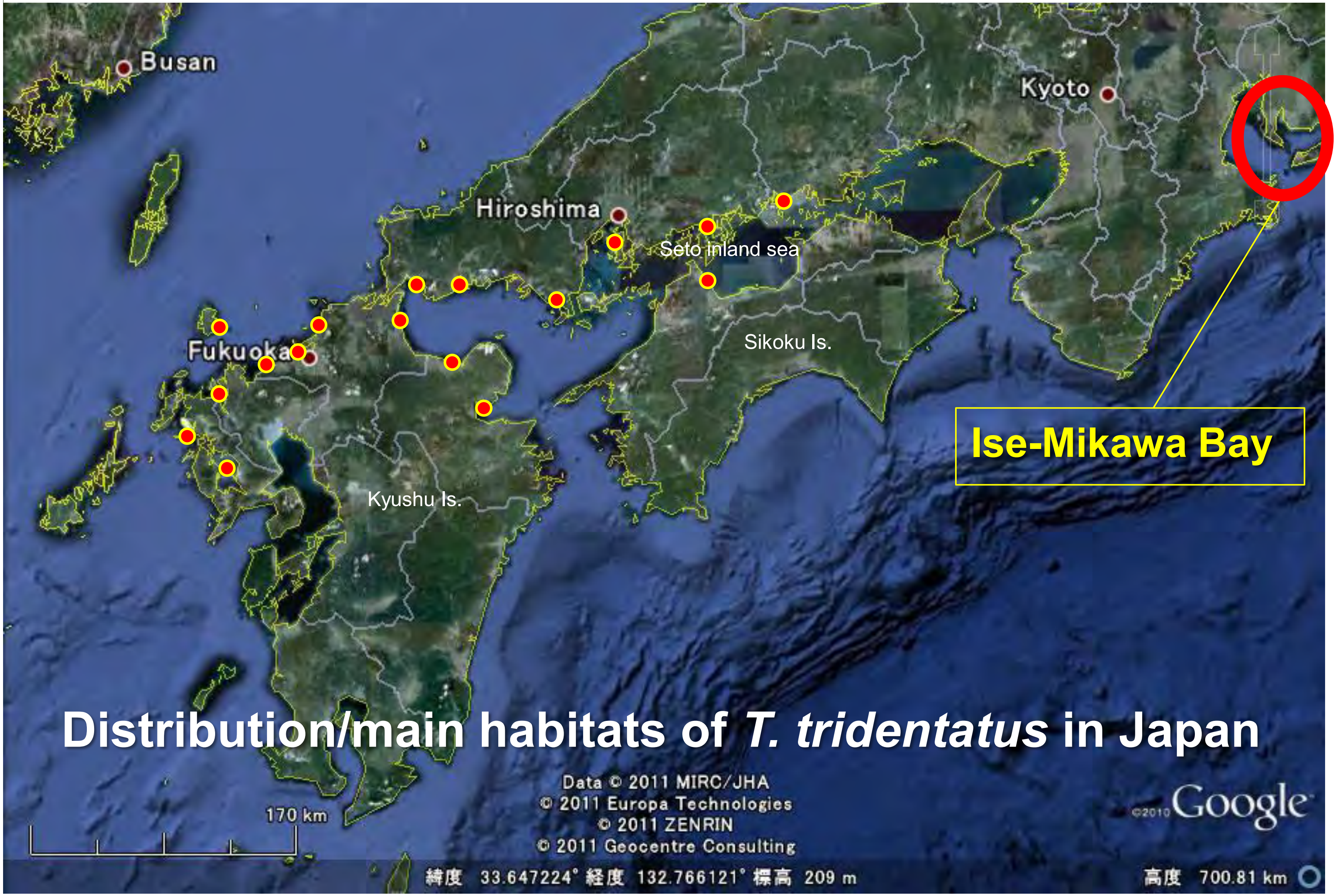
- Northernmost population(s) of *T. tridentatus*
- Relatively young population:
  - Probably, it had be formed at last glacial age
  - Especially, eastern group may be only 10,000 years old
- Two distinct genetic group were detected that occupy a small geographic area
- Low genetic diversity affected by founder on the mtDNA analyses. (Note that the western group had higher diversity with ancestral polymorphisms)
- Genetic subdivision was detected in the eastern group by MS analyses
- Such a genetic structures were caused by history of them, geographic features and structure, and fragmentation of their habitat …

Sub-topic

A case of HSC as invader species in Japan

(Collaborative research with Mr. Kenji Kuroyanagi at the Minamichita beach-land aquarium)

Many *T. tridentatus* were found from Ise-Mikawa Bay where is out of their native habitat from 2001 until 2009 (reported by Minamichita Beach-land Aquarium)



Distribution/main habitats of *T. tridentatus* in Japan

伊勢湾・三河湾におけるカブトガニ採集記録（2009/3/1 現在）

No.	採集年月日	採集場所	漁法	cm 全長	cm 前体幅	cm 尾剣長
1	2001/ 8/ 8	沖ノ瀬（水深 32m）	底引き網	27.80	14.20	14.00
2	2002/ 6/ 5	日間賀島～篠島間（水深 19～48m）	底引き網	30.72	15.03	15.79
3	2002/ 6/12	片名（チッタ・ナポリ沖100m）（水深5～6m）	定置網（角建て）	25.25	12.65	12.35
4	2002/ 7/ 1	師崎漁港沖 50m（水深 21m）	刺し網（三枚網）	32.70	16.04	16.58
5				27.58	13.89	14.23
6	2002/ 7/13	片名漁港沖 200～300m（水深20m）	アイナメ籠（メジロ籠）	26.79	13.73	13.41
7	2002/7/25	師崎～日間賀間（水深 30m）	底引き網	26.41	12.99	12.74
8	2002/9/16	片名漁港北堤防内	刺し網（三枚網）	18.20	10.46	7.86
9	2002/9/18	片名漁港北堤防内	刺し網（三枚網）	30.98	14.96	15.69
殻のみ	2002/9/24	大井漁港沖 1.15マイル	小型底引き網	31.85	15.04	14.87
10	2002/10/6	日間賀島～篠島間（水深 19～48m）	底引き網	31.20	16.00	15.69
11	2002/10/24	空港島西1マイル（水深 27m）	底引き網	35.95	17.96	17.99
12	2002/10/27	片名漁港北堤防内	刺し網（三枚網）	34.30	18.06	15.60
13	2003/6/22	篠島西～師崎沖	底引き網	39.80	19.70	19.30
14	2003/6/30	師崎～大井灯台前（水深 57.8m）	底引き網	32.80	16.50	17.00
15	2003/8/5	沖ノ瀬南（水深 48～96m）	底引き網	39.00	18.40	19.60
16	2003/8/20	大井沖（水深 32m）	底引き網	42.30	19.80	19.00
17	2003/8/19	師崎沖	底引き網？	26.40	13.50	13.90
18	2003/8/23	師崎		26.40	13.50	13.90
19	2003/9/30	？	？	32.50	20.50	9.00
20	2003/10/2	篠島～1km～明神（水深19.2m）	底引き網	31.20	16.00	14.80
21	2003/10/26	片名漁港沖6ヒロ	刺し網（三枚網）	37.70	17.50	19.60
22	2004/4/12	豊浜より6.5マイル　沖ノ瀬西1マイル（水深33.6m）	底引き網	39.30	19.70	18.70
23	2004/5/27	伊良湖岬北2マイル（水深32～48m）	底引き網	33.40	17.00	14.60
24	2004/6/17	師崎～篠島　師崎寄り	底引き網	35.40	16.70	18.70
25	2004/6/23	大井漁港前（水深30m）	底曳き網	30.80	16.40	
26	2004/9/23	片名漁港沖	刺網	39.00	20.00	18.60
27	2004/9/23	片名漁港沖	刺網	48.00	22.70	22.90
28	2004/11/19	片名漁港沖	網	50.50	25.70	23.30
29	2005/6/13	沖ノ島ブイ西	底曳き網	37.50	21.10	16.60
30	2005/6/29	ビーチランド沖	底曳き網	42.20	21.10	18.80
31	2005/7/30	？	？	43.20	20.50	21.50
32	2005/10/21	？	？	45.20	21.70	22.30
33	2005/10/26	神島北2マイル（水深64～72m）	底曳き網	40.70	19.10	20.20
34	2006/6/15	大井．南側8　山田海岸沖～大井漁港沖（水深16～29）	底曳き網	54.60	26.20	27.20
35	2006/6/21	山海海水浴場沖	刺し網（三枚網）	69.30	31.30	36.50
36	2006/8/31	日間賀島と篠島の間　（水深20～30m）	底曳き網	31.60	15.50	15.50
37	2007/6/21	師崎漁港沖 師崎水道 タドイシ灯台（水深30m）	刺し網	59.30	28.30	28.70
38	2007/8/10	師崎漁港（新師崎）前	刺し網（三枚網）	56.60	27.10	27.70
39	2008/5/1	日間賀島西2km（水深8m）	刺し網（三枚網）	47.60	24.70	21.90
40	2008/7/6	武豊火力発電所南 堤防から10mほど	刺し網	57.90	25.60	29.40
41				52.30	26.20	24.60
42	2008/7/12	河和漁港前	角建て（小型定置網）	46.70	23.90	22.10
43	2008/7/12	師崎漁港前	刺し網	64.50	32.30	29.30
44	2008/8/5	時志観音前	刺し網	51.50	28.20	22.00
45	2008/9/21	渥美火力発電所沖500m　（水深22～24m）	底引き網	49.20	25.70	25.20

査・研究・報告 > 伊勢湾・三河湾におけるカブトガニ採集記録 '2009

調査・研究・報告
Investigation, Research & Report

伊勢湾・三河湾におけるカブトガニ採集記録　～てん末記 その3～

DNA鑑定により中国産の可能性が高いことがわかった、伊勢湾・三河湾産カブトガニですが、2009年は新たに2個体（いずれも最終脱皮を終えたオス成体）が見つかり、2001年より合計47個体となりました（表-1）。

一方2008年夏、当園水槽内で繁殖し成長している幼生たちは、冬季に加温した水槽で飼育しているため順調に脱皮を繰り返し、既に7齢まで大きくなりました。
小さくても姿かたちはりっぱなカブトガニです（写真-1）。

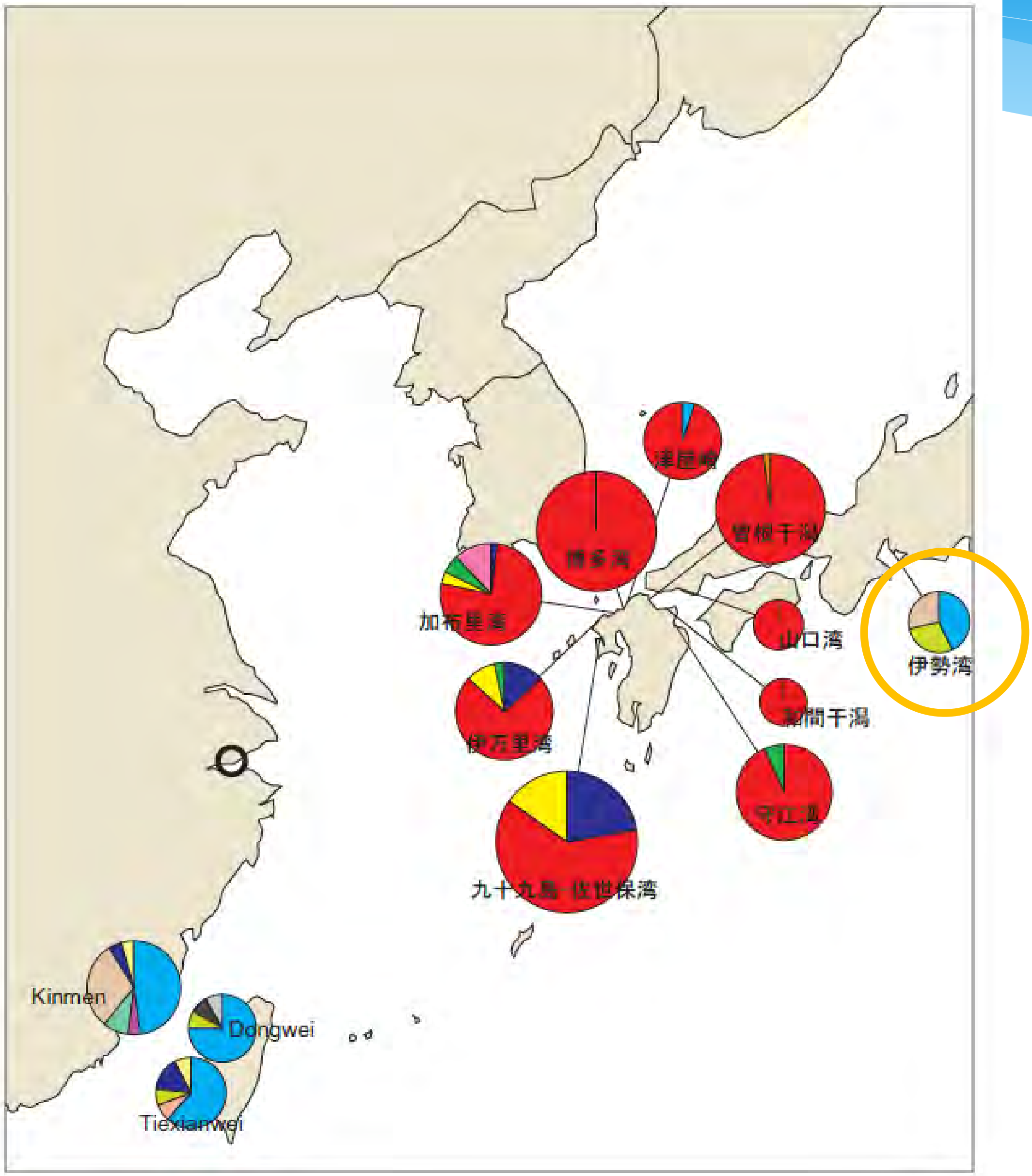
［写真-1：左：6齢脱皮ガラ・右：7齢幼生］

幸い採取される数も減り、自然界での繁殖も確認されていません。このまま終息に向かってくれる事を願っていますが、当園では引き続き調査を継続しています。
皆さんも潮干狩りなどで干潟に出かけた際、小さな小さなカブトガニ幼生（写真-2）を発見されましたら是非、南知多ビーチランドまでご連絡下さい。

［写真-2：1～3齢幼生］

From web page of minamichita beach land

Identification of source for introducing? individuals



Many individuals of *T. tridentatus* are found from outside of their native habitat (orange circle)in these years.

Result of mtDNA analysis shows that origin of these introducing individuals should be from Taiwan strait or a habitats on mainland China.

According to newspaper, actually, a group brought/imported over 200 individuals of juveniles for a pet, and accidentally /intentionally had be set free after 1996.

Attempt to comparative phylogenetic study with parasitic planarian species on Asian horseshoe crabs

“Collaborative research with Mr. Ming Che Yang, Dr. Chang-Po Chen, Dr. Chaolun Allen Chen (Taiwan group), Dr. Yusli Wardianto, Dr. Handko Adi Susant, Mr. Irzal Azhar, Mr. Ali Mashar, Mr. Irfan Yulianto (Indonesian group), and Dr. Ame Garong, Ms. Marivene Manuel-Santos (Philippines group: in progress)”

Comparative phylogeographic study on horseshoe crabs and their symbiotic planarian species.

Planarian parasite on horseshoe crabs

Have you ever seen them?

Interestingly, ecto-parasite(s) (planarian species: *Ectoplana* spp.) were reported from horseshoe crabs, and they may be in symbiosis (Kawakatsu and Sekiguchi 1988).

How have these parasites been (co-)evolved with horseshoe crabs as their hosts?

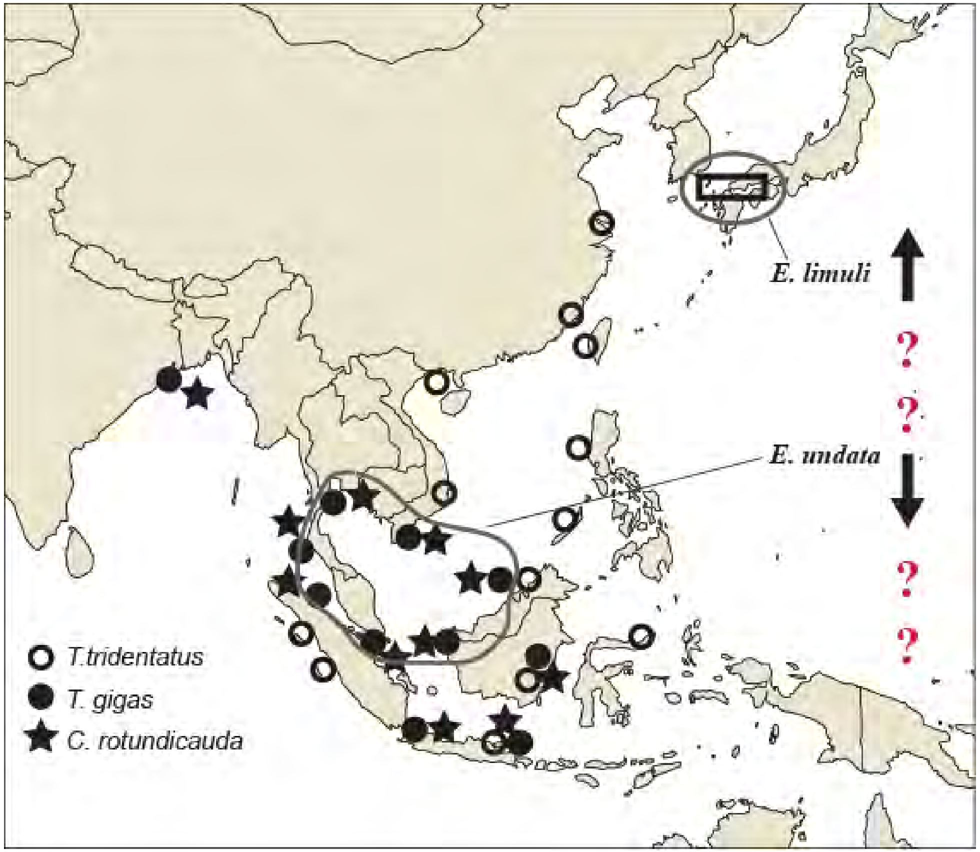
Ectoplana limli on *T. t.* in Japan

What are *Ectoplana* spp.?

Metazoa; Platyhelminthes; Turbellaria;
Tricladida; Maricola;
Bdellouridae Bdelloura
Bdelloura candida
Uteriporidae Ectoplaninae; **Ectoplana**
Ectoplana limuli
Ectoplana undata

- Ectoplana spp are ectosymbiotic on the Horseshoe Crabs *Tachypleus tridentatus, T. gigas,* and *Carcinoscorpius rotundicauda.*

- Kaburaki (1922) assumed cocoon-laying to take place in July, since he found half-grown animals in early August. Because these young triclads were attached to the gills, Kaburaki also assumed that cocoons are deposited on the gill lamellae.



Phylogeography of *Ectoplana* spp will reveal population history of horseshoe crabs in detail.

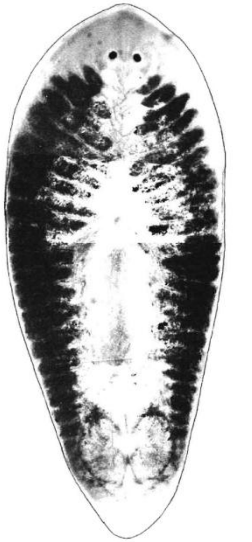
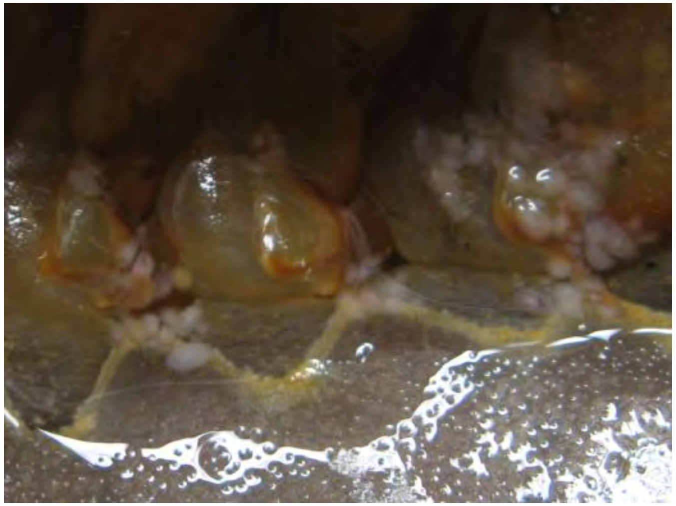


Fig. 2. *Ectoplana limuli*, photomicrograph of the preserved whole-mount specimen from Hakata Bay (No. 1267-a).

Ectoplana limli

(6-7mm long, 0.8-0.9mm width)

Ectoplana limli on *T. t*. in Japan



Ectoplana sp. on *T. t*. in East Kalimantan

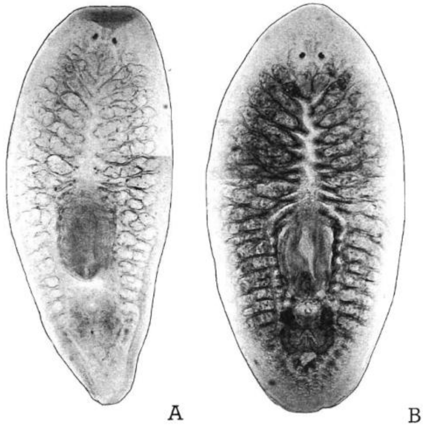


Fig. 8. *Ectoplana undata*, photomicrographs of the preserved whole-mount specimens. A: No. 1790-a (host: *Tachypleus gigas*). B: No. 1792-a (host : *Carcinoscorpius rotundicauda*).

Ectoplana undata

(4-5mm long, 0.4-0.6mm width)

(Kawakatsu and Sekiguchi 1988)

Ectoplana undata? on *C. r*. in Southeast Sumatra

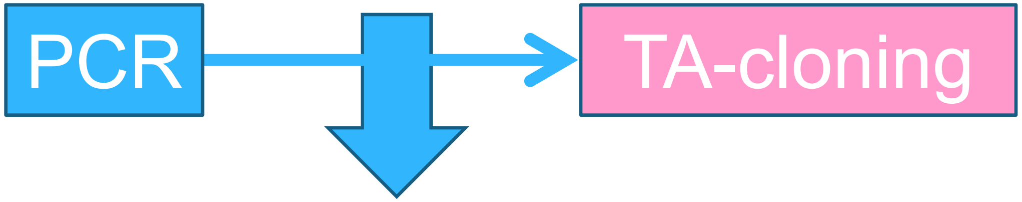
Ectoplana undata? on *T. g*. in Eastern Java

Attempt to DNA analyses

Try to amplify **COI** on mtDNA
(which is target region for DNA barcoding and usable for phylogeographic study)

Primer set:
~universal primers~
1. LCO1490 × HCO2198 (Folmer 1994, famous primer set)

~for turbellarians (basically specific) ~
2. COIs: COIF × COIR (Lázaro et al 2009)
3. COIbc: Bar2(f) × COIbar-plat-R (Lázaro et al 2009)
4. Pr-a2 × Pr-b2 (Bessho et al 1992)


PCR
TA-cloning

Of course, could amplify fragment that almost same length as target region

But, unfortunately, there were no *Ectoplana* sequence….why?

Didn't work well so far…

Possibility of estimation of food source for *Ectoplana* spp.

Although target "Ectoplana" sequences didn't read yet…

Read sequences of some clones
for amplicon from universal primer "LCO1490-HCO2198"

⇩

Sequence similarity search by BLAST with GenBank

⇩

Results are…**squids and polychaete species**

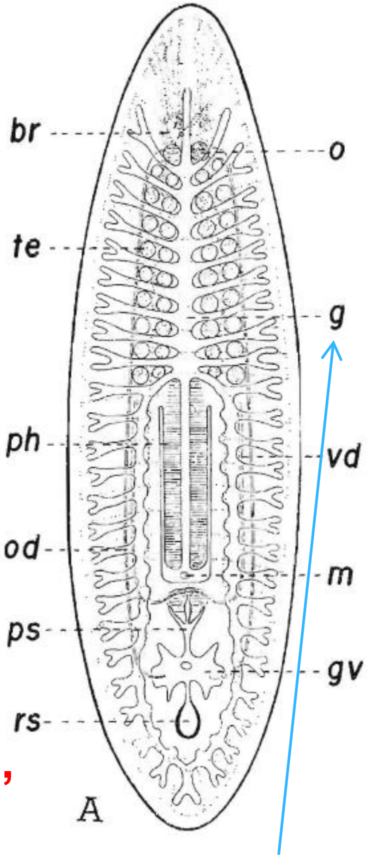
⇧

One of the individual of *Ectoplana* sp. analyzed from Aquarium and squid was food for HSC.

⇩

Intestine contents were amplified by univ. primer set

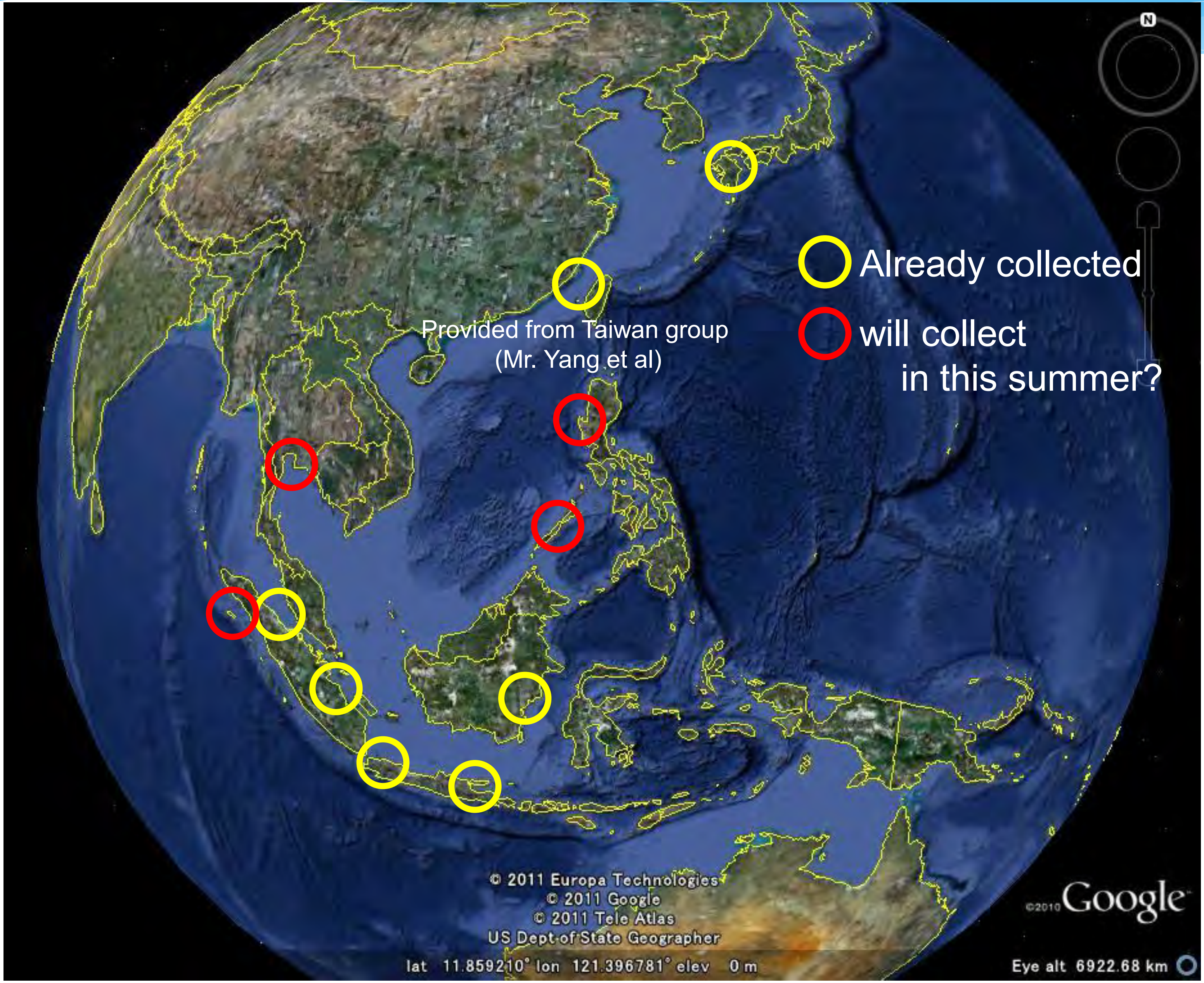
In fact, *Ectoplana* spp. may feed debris and leftovers of HSCs, they don't (rarely) eat HSC as their host?



Intestine

Probably, we can estimate food sources of HSC from food analysis of their ecto-parasites.

Sampling locations and plans



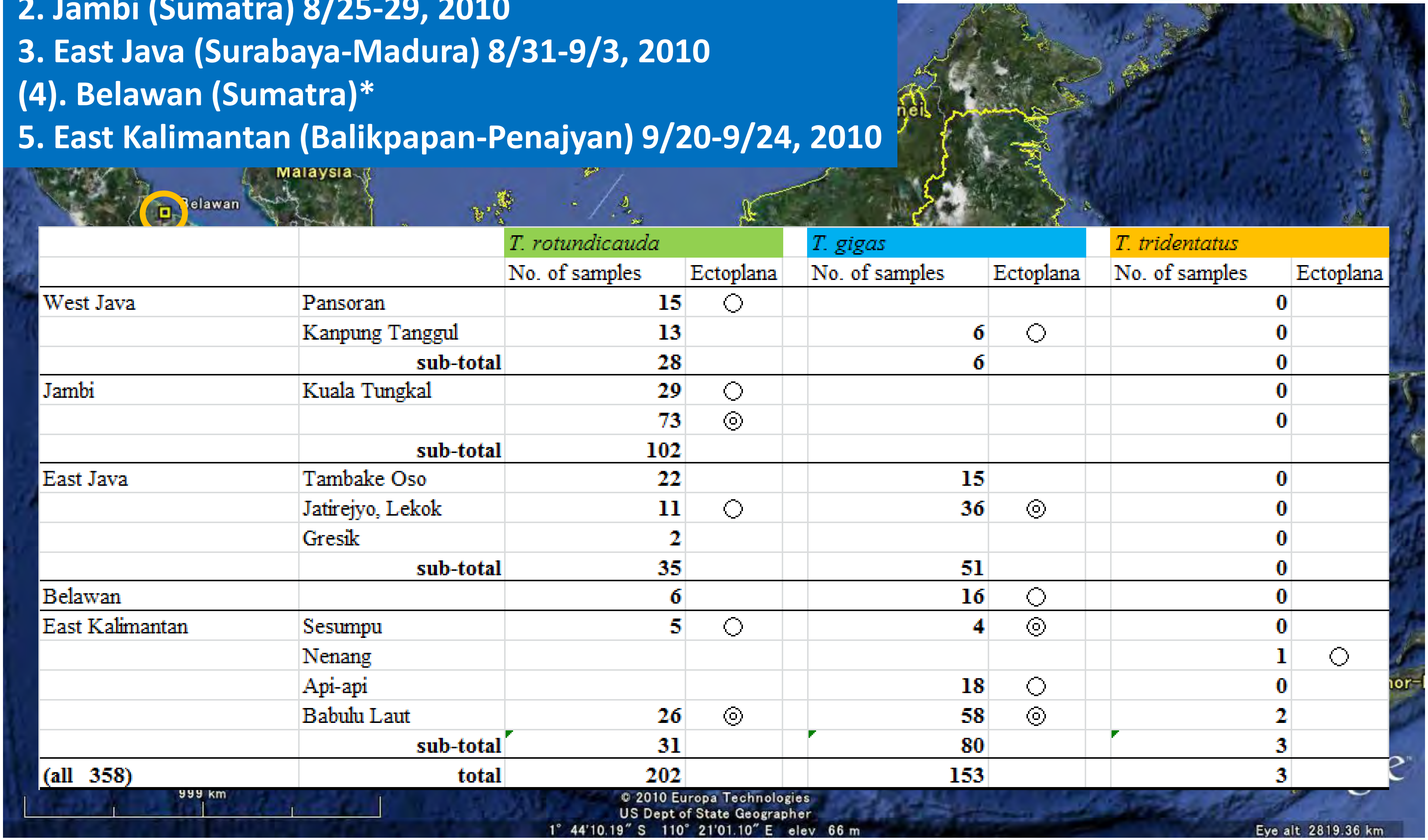
Provided from Taiwan group
(Mr. Yang et al)

🟡 Already collected

🔴 will collect
in this summer?

Field trip at Indonesia at last summer

1. West Java (Serang) 8/15-8/18, 2010
2. Jambi (Sumatra) 8/25-29, 2010
3. East Java (Surabaya-Madura) 8/31-9/3, 2010
(4). Belawan (Sumatra)*
5. East Kalimantan (Balikpapan-Penajyan) 9/20-9/24, 2010



		T. rotundicauda		*T. gigas*		*T. tridentatus*	
		No. of samples	Ectoplana	No. of samples	Ectoplana	No. of samples	Ectoplana
West Java	Pansoran	**15**	○			**0**	
	Kanpung Tanggul	**13**		**6**	○	**0**	
	sub-total	**28**		**6**		**0**	
Jambi	Kuala Tungkal	**29**	○			**0**	
		73	◎			**0**	
	sub-total	**102**					
East Java	Tambake Oso	**22**		**15**		**0**	
	Jatirejyo, Lekok	**11**	○	**36**	◎	**0**	
	Gresik	**2**				**0**	
	sub-total	**35**		**51**		**0**	
Belawan		**6**		**16**	○	**0**	
East Kalimantan	Sesumpu	**5**	○	**4**	◎	**0**	
	Nenang					**1**	○
	Api-api			**18**	○	**0**	
	Babulu Laut	**26**	◎	**58**	◎	**2**	
	sub-total	**31**		**80**		**3**	
(all 358)	**total**	**202**		**153**		**3**	

Acknowledgments

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Thank you for your attention !