SYSTEMATIC TECHNIQUES FOR RAPID IDENTIFICATION OF SIBLING SPECIES MEMBERS IN THE TAXON *ANOPHELES* SPECIES COMPLEX

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Throughout the world: Anopheline mosquitoes < 400 species

: < 40 species vectors of malaria (*Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. knowlesi*), filaria (*Wuchereria bancrofti* *Brugia malayi*, *B. timori* & *Dirofilaris immitis*) & enchephalitis virus

28 species ~ exhibit species complex

< 107 sibling species members

~ characteristics of sibling species members

morphology: morphologically identical (isomorphic)

minimal morphological distinction

cryptic species identification

genetic isolation at pre- and/or post-mating barriers

Reid (1968); Subbarao (1998); Cox-Singh J *et al*. 2008

Significance of sibling species members:

distinct behavior ~ anthropophilic, zoophilic

~ nocturnal biting-activity

distinct vector potential ~ vector & non-vector

complication of identifying target vectors

different degrees of development for insecticide resistance

potentially misleading methods of control

Subbarao (1998)

Techniques used in the recognition of sibling species members in complexes

Old-fashioned techniques:

Markers (variants): limitation in use

Behavioral trait ~ anthropophilic & zoophilic

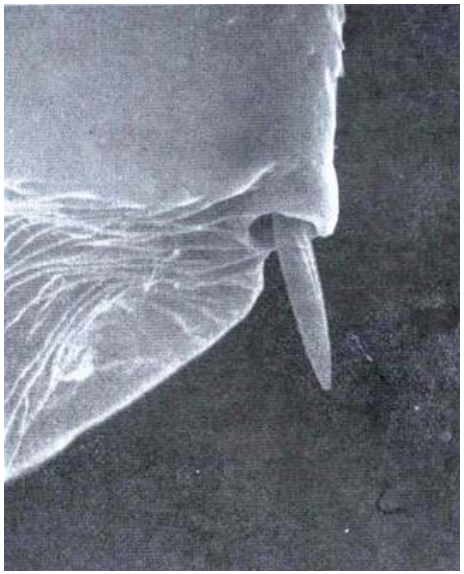
Microhabitat ~ breeding places: plane rice-paddy & forested foot-hills, etc.

Morphology ~ identical & minimal distinction

An. dirus s.s. (dirus A)

An. scanloni (dirus C)

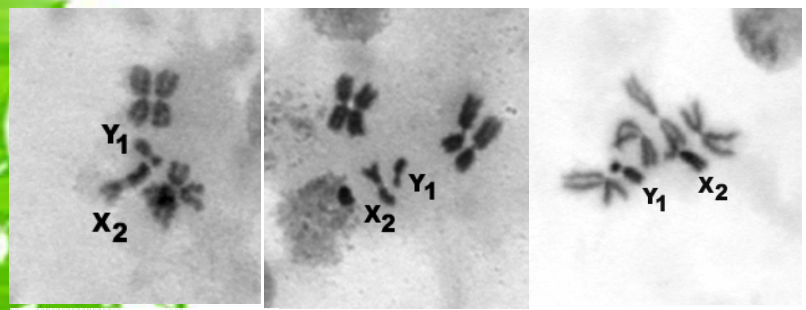
An. baimaii (dirus D)



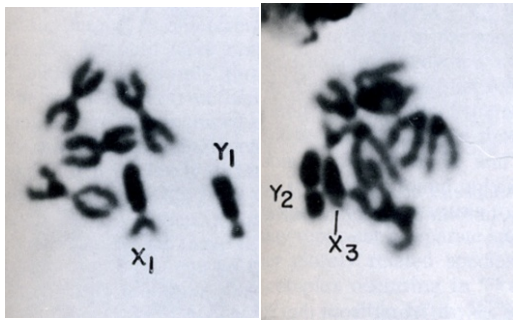
An. cracens (dirus B)

pupal seta 9-IV

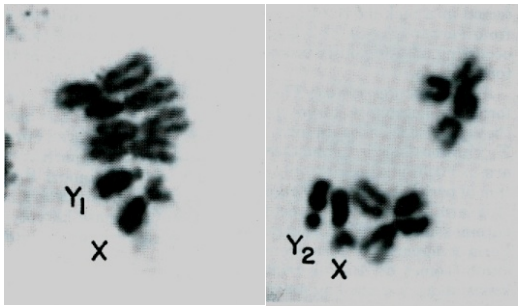
Cytology ~ metaphase karyotypes (identical, distinct & polymorphic)



species A1 species A2 species A3



species A species C



Form A Form B

Identical
An. barbirostris
species A1, A2 & A3

Distinct
An. minimus
species A & C

Polymorphism
An. sinensis
Form A & B

Old-fashioned techniques (cont.)

Post-mating barrier: post-mating reproductive isolation (inviable F_1-hybrids)

- **Crossing experiment ~ artificial mating**
  ~ iso-female line (isoline)

Pre-mating barrier: positive assortative (preferential) mating
in a natural population ~ sympatric

- **Isoenzyme divergence (band morphs)**
- **Fixed paracentric inversion of polytene chromosome**

} **degree of absence in heterozygote**

- **Disadvantage:**

isoenzyme ~ specimens must be fresh & frozen until analysis
~ requires a relatively large amount of sample materials
(rare species ≈ specific seasonal prevalence)

polytene chromosome

~ only brain of the 4th larva & ovarian nurse cell of the adult female must be used for preparation
~ needs a skillful person to prepare a perfect chromosome and identification
~ requires a relatively large amount of sample materials
(rare species ≈ specific seasonal prevalence)
~ cannot be employed in homosequential banding species

New-fashioned techniques (molecular approaches):

Robust markers ~ rDNA (IGS, ITS1, ITS2,D3)
mtDNA (COI, COII, CtyB, *ND1*, *ND5*)
~ comparative sequence analyses &
phylogenetic relationship
~ genetic distance: distinct species (0.02-0.62)
: conspecific races (<0.005)

❖ **Advantage: few nanograms of DNA from preserved specimens required for PCR**

Techniques employed after the recognition of sibling species members
~ DNA hybridization, SNPs, Microsatellite DNAs, RFLP, RAPD, AFLP & SSCP, etc.

Subbarao (1998); Beaty *et al.* (2004);
Norris (2002); Saeung *et al.* (2007, 2008)

Formation of rapid systematic procedure for the recognition of sibling species members

Old-fashioned markers:

- Behavioral trait
- Microhabitat
- Morphology
- Cytology

New-fashioned markers:

- rDNA
- mtDNA

Crossing experiment:

- isoline colony

Flow chart for rapid systematic procedure

Human-baited and/or animal-baited traps ~ different microhabitat

↓

Wild-caught fully engorged females: Species identification

↓

Individually deposited eggs: isoline colony

↓ → Molecular investigation of feral females by PCR- (rDNA: ITS2; mtDNA: COI, COII)

F_1-progeny

↓

- Morphometric & morphological studies of eggs, larvae, pupal skins & adults
- Karyotype identification of 4^{th}-instar larvae
- Molecular confirmation by PCR- (rDNA: ITS2; mtDNA: COI, COII)
- Crossing experiment

Population genetic study of *Anopheles barbirostris* species complex (Diptera: Culicidae) in Thailand

An. barbirostris/campestris **~ Morphologically cryptic**

~ seta 2-VI pupal skin: 95-97%

- **Potentially natural vector of *P. vivax* in Sa Kaeo province, Thailand**

Limrat *et al*. (2001); Apiwathnasorn *et al*. (2002)

- **Possible vector playing an important role**

↑ **cases of *P. vivax* in Thailand**

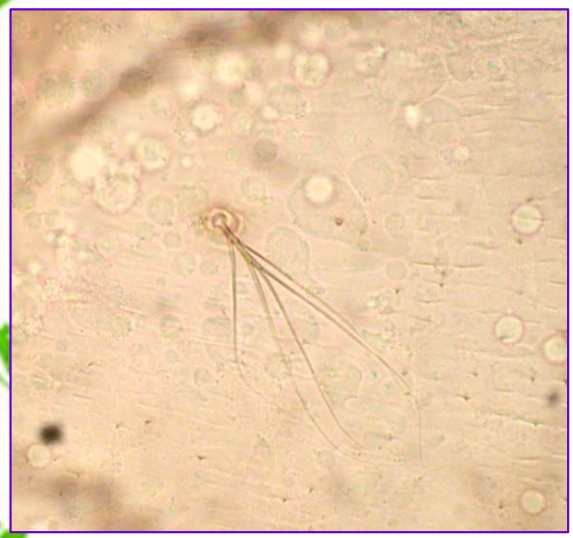
Sattabongkot *et al*. (2004)

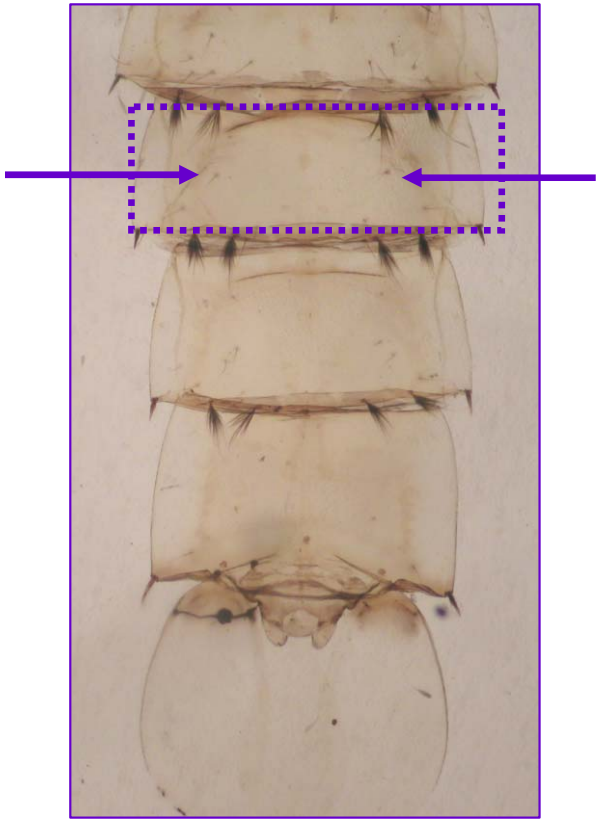
Total isolines: 133

Morphology : 2 groups of branch summation of seta 2-VI pupal skins

: 42 isolines ~ *An. barbirostris* (9-16 branches)

: 71 isolines ~ *An. campestris* (20-30 branches)





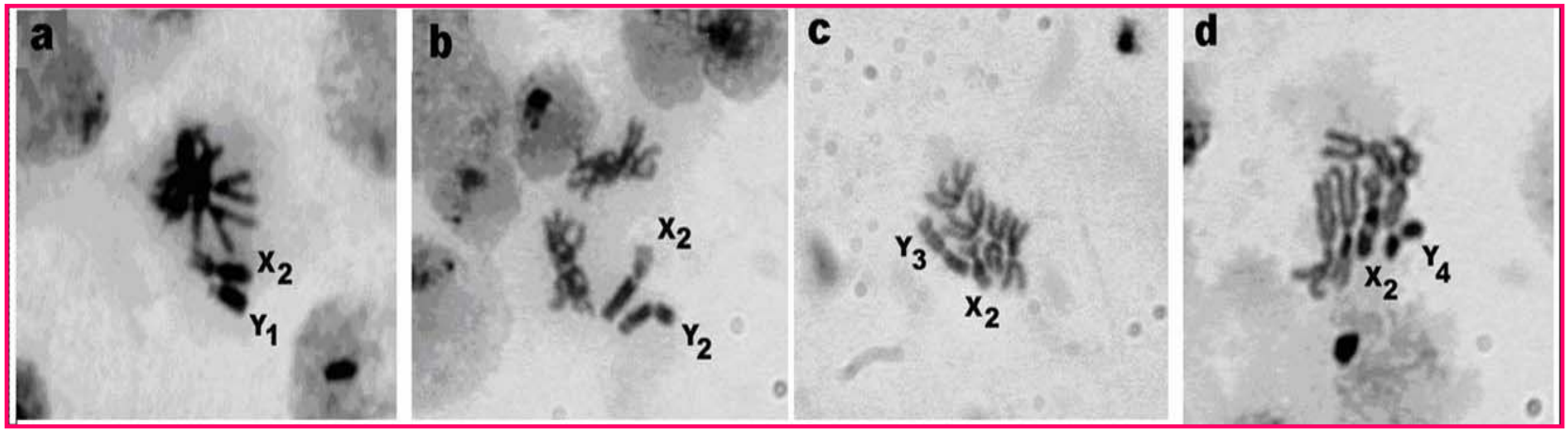
An. barbirostris

An. campestris

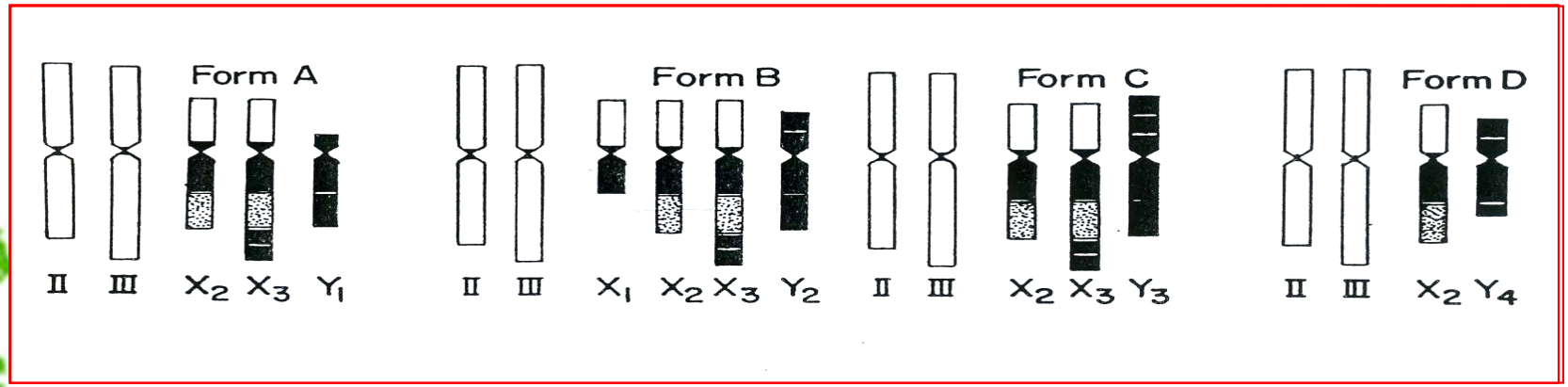
- ***An. barbirostris*** **6-18 branches**
- ***An. campestris*** **17-58 branches**

Harrison and Scanlon (1975)

Cytology: *An. barbirostris* : 4 karyotypic forms

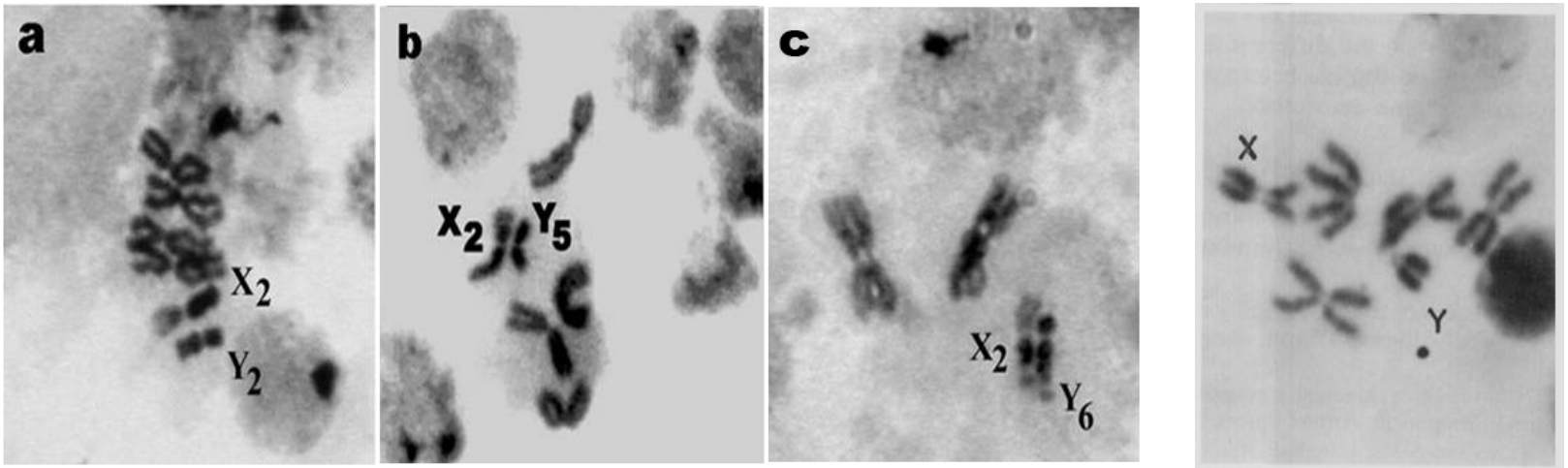


Form A **Form B** **Form C** **Form D**

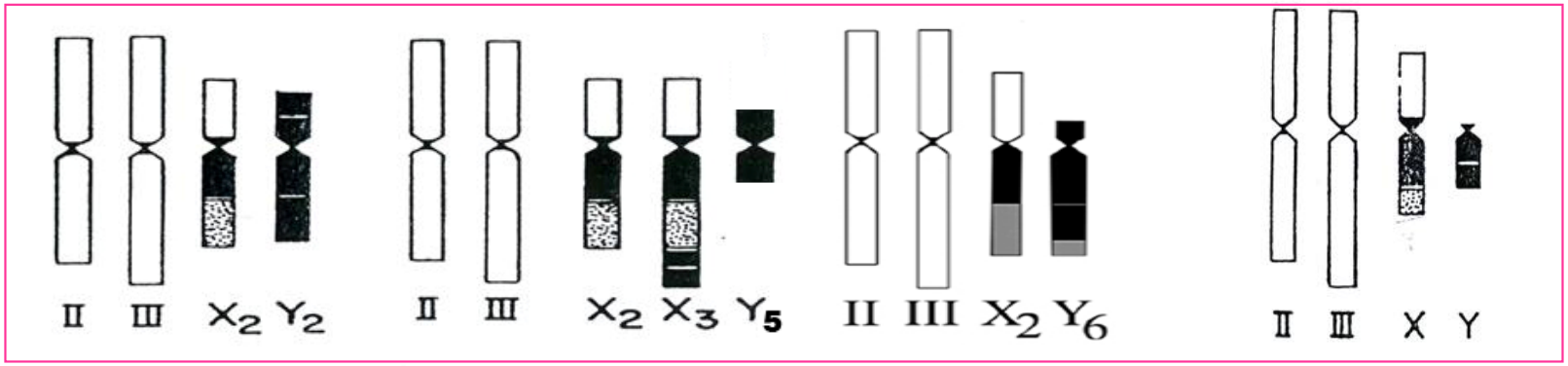


Baimai *et al*. (1995)

An. campestris: 3 karyotypic forms



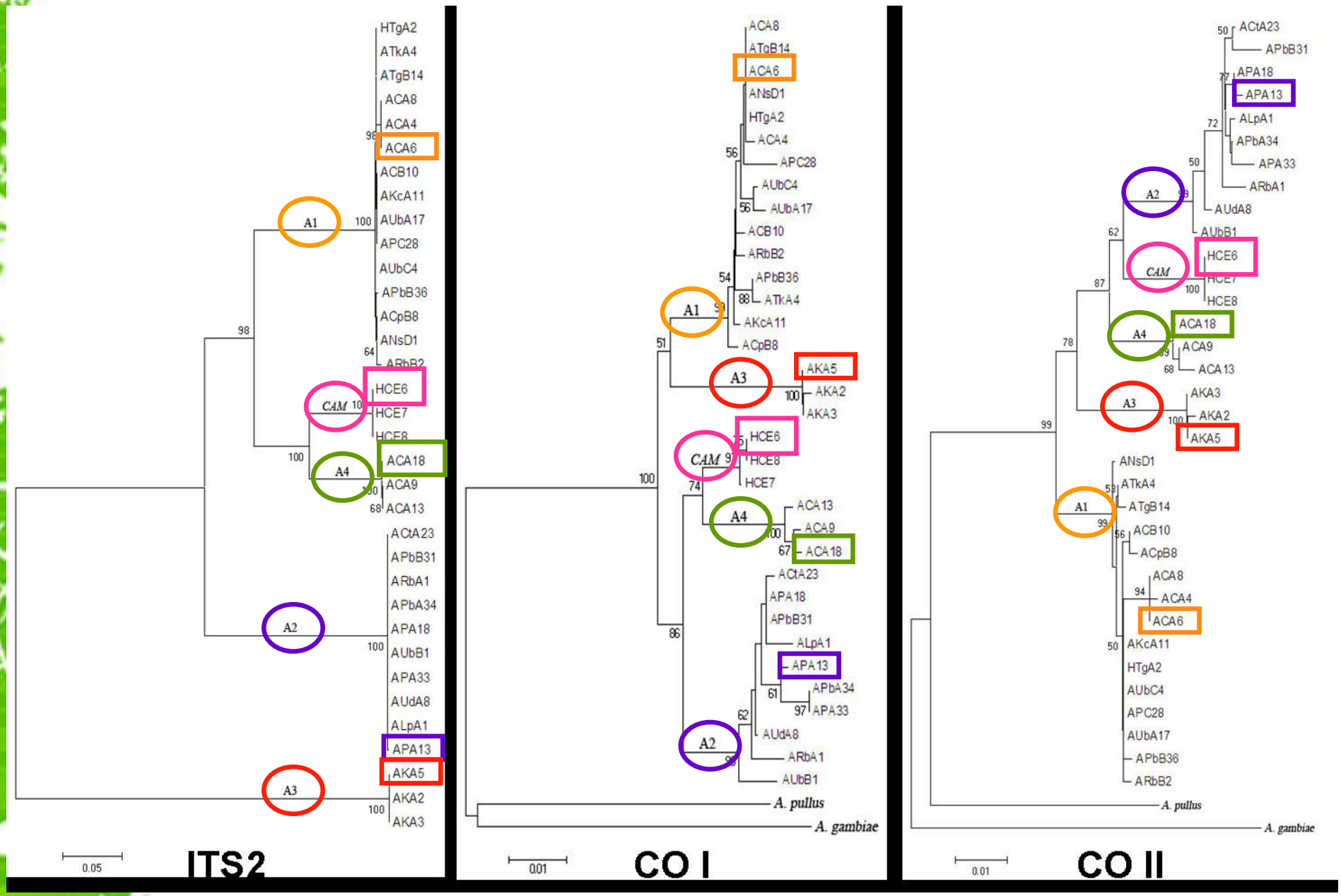
Form B | Form E | Form F | *campestris*



similarity of X_2-chromosome to *An. barbirostris*

designated as *An. campestris*-like
Form B, E and F

DNA analyses : 5 different groups ~ 4 groups of *An. barbirostris* and 1 group of *An. campestris*-like



Crossing experiment

Large sequence divergence: genetic distance (0.02-0.62)
(ITS2, COI & COII)

Group A1 (1,861 bp): Form A ~ Chiang Mai (iACA6)

Group A2 (1,717 bp): Form A ~ Phetchaburi (iAPA13)

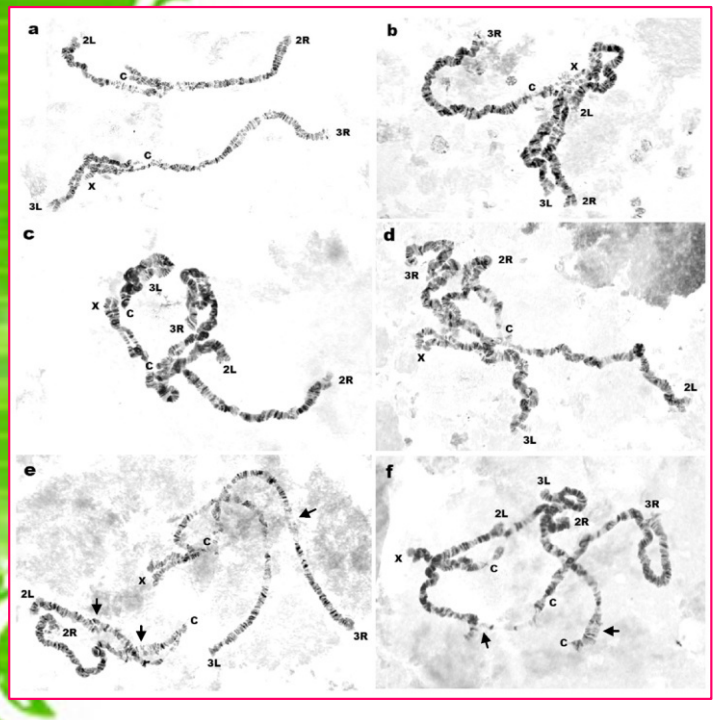
Group A3 (1,070 bp): Form A ~ Kanchanaburi (iAKA5)

Group A4 (1,676 bp): Form A ~ Chiang Mai (iACA18)

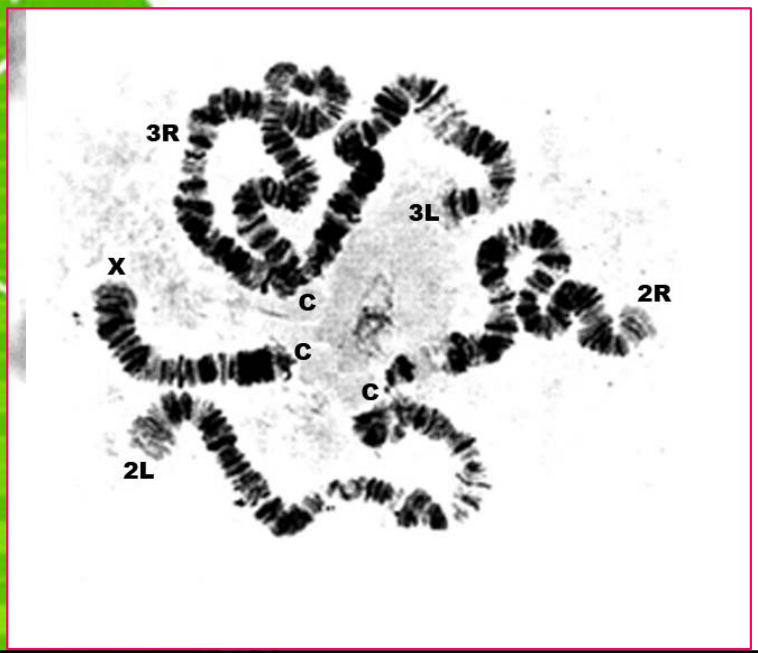
CAM (1,651 bp): Form E ~ Chiang Mai (iHCE6)

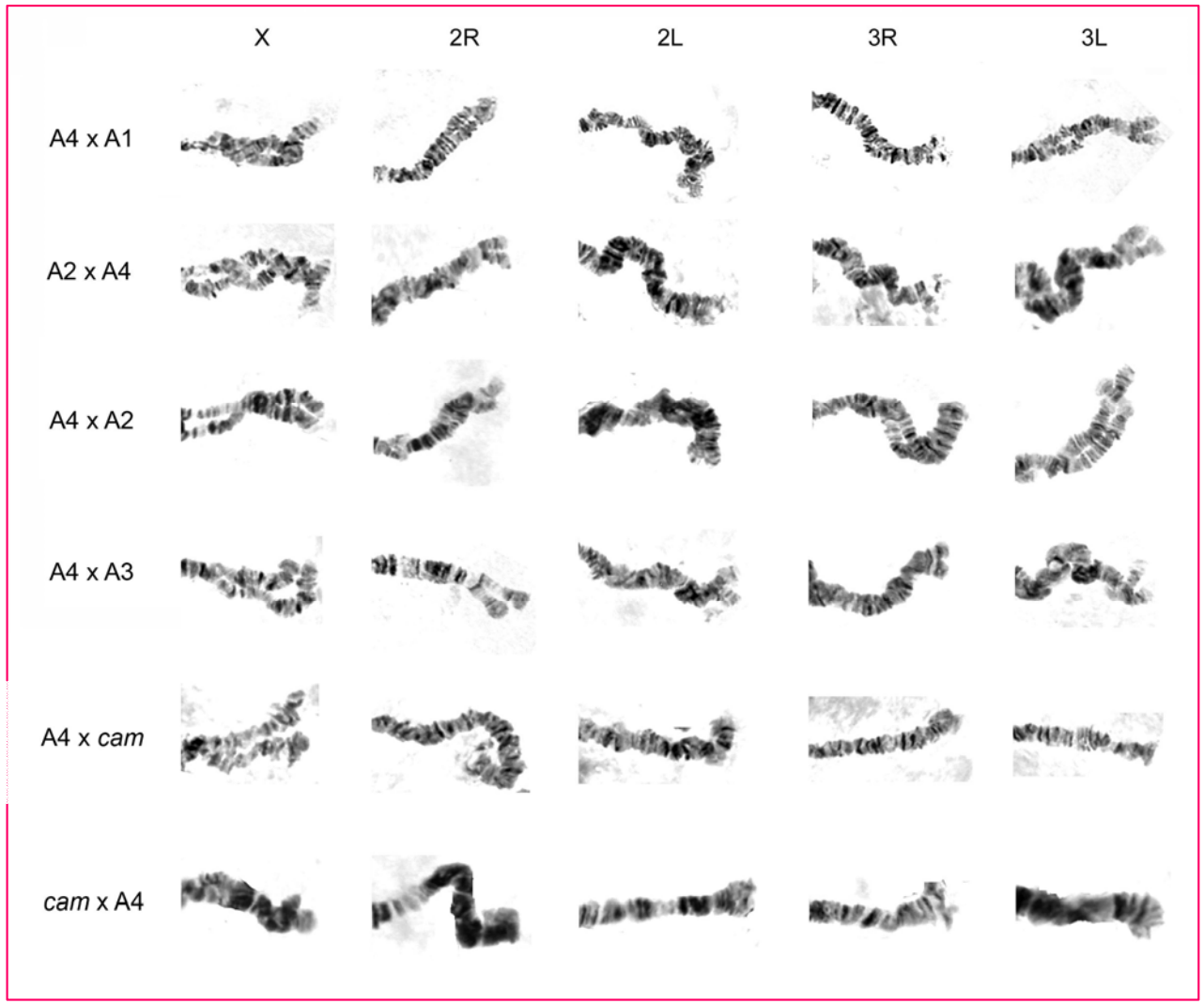
A1 X A2, A1 X A3, A1 X A4, A1 X *CAM*
A2 X A3, A2 X A4, A2 X *CAM*
A3 X A4, A3 X *CAM*
A4 X *CAM*

Post-mating reproductive isolation

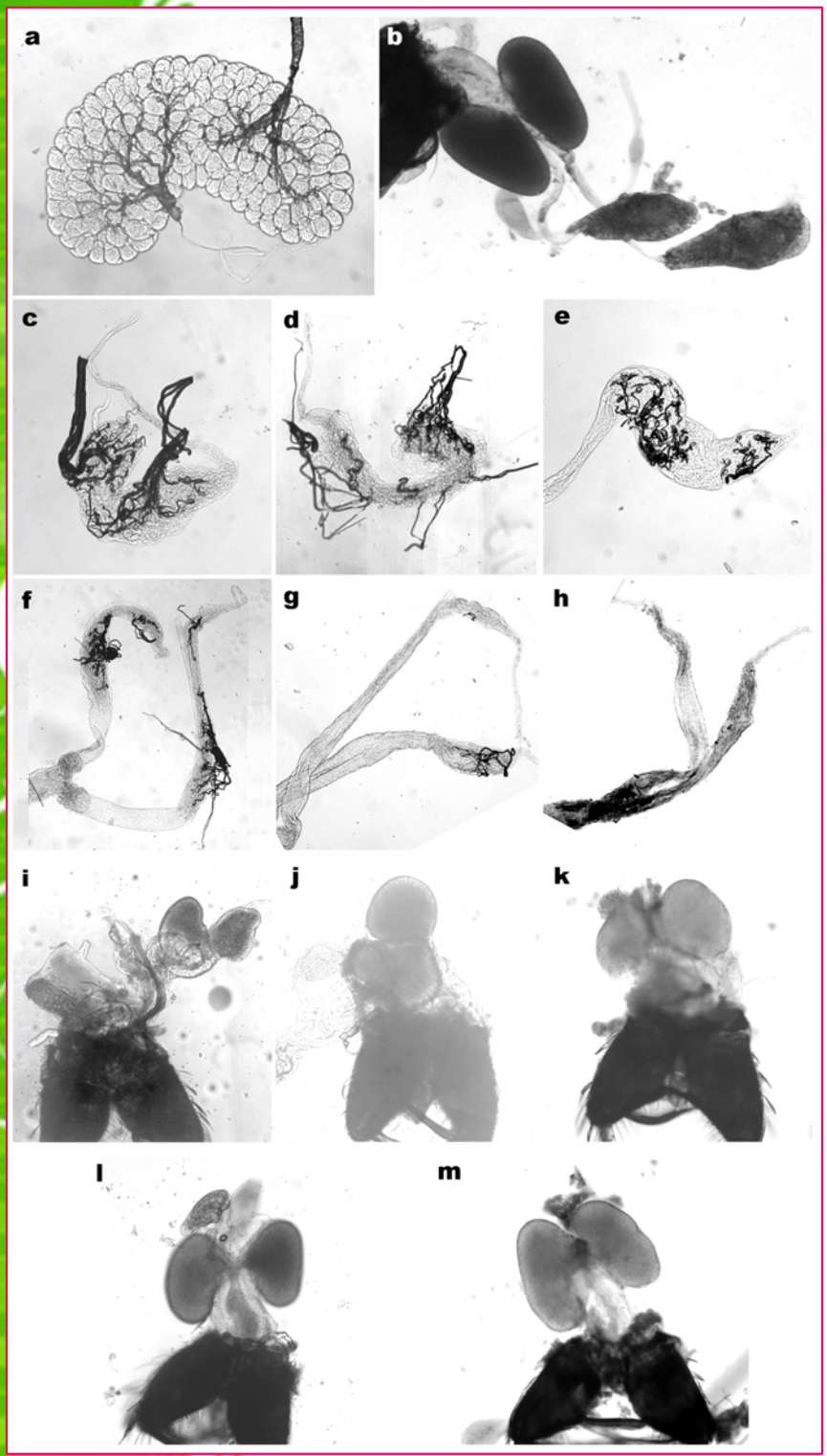


Asynapsis of X-chromosomes and autosomes





Complete synapsis in all arms



Normal reproductive systems

a: Ovarian follicles ~ *An. barbirostris* species A4

b: accessory glands & testes ~ *An. campestris*-like Form E

Atrophied ovarian follicles of F_1-hybrids

c: A4 x A1

d: A2 x A4

e: A4 x A2

f :A4 x A3

g: A4 x *An. campestris*-like Form E

h*: An. campestris*-like Form E x A4

Atrophied accessory glands & testes of F_1-hybrids

i: A4 x A1

j: A2 x A4

k: A4 x A3

l: A4 x *An. campestris*-like Form E

m: *An. campestris*-like Form E x A4

Based on comparative morphology, cytology, molecular analysis & crossing experiment

5 sibling species were discovered in the taxon *An. barbirostris*

***An. barbirostris* species A1: widely distributed throughout Thailand**

species A2: distributed and occurred in symparty with species A1 in some populations in north, northeast and central Thailand

species A3: confined to Kanchanaburi

species A4: confined to Chiang Mai

Foot-hill areas

***An. campestris*-like : widely distributed throughout Thailand**

Plane areas

Low intraspecific variation: genetic distance < 0.005

(ITS2, COI & COII)

Group A1 : Form A ~ Chiang Mai (iACA6*)

: Form A ~ Tak (iATkA4), Ubon Ratchathani (iAUbA17), Kanchanaburi (iAKcA11) & Trang (iHTgA2)

: Form B ~ Chiang Mai (iACB10), Ratchaburi (iARbB2), Phetchaburi (iAPbB36), Chumphon (iACpB8) & Trang (iATgB14)

: Form C ~ Ubon Ratchathani (iAUbC4) & Phetchaburi (iAPC28)

: Form D ~ Nakhon Si Thammarat (iANsD1)

Group A2 : Form A ~ Phetchaburi (iAPA13*)

: Form A ~ Lampang (iALpA1), Udon Thani (iAUdA8), Ratchaburi (iARbA1), Phetchaburi (iAPbA34) & Chanthaburi (iACtA23)

: Form B ~ Ubon Ratchathani (iAUbB1) & Phetchaburi (iAPbB31)

CAM : Form B ~ Chiang Mai (iHCmB18, iHCmB20), Kamphaeng Phet (iAKpB1)

: Form E ~ Chiang Mai (iHCE6*)

: Form E ~ Chiang Mai (iHCmE12, iHCmE14, iHCme15), Kamphaeng Phet (iHKpE1), Ayuttaya (iAAyE7), Khon Kaen (iAKkE4, iAKkE8), Maha Sarakham (iAMsE3, iAMsE4, iAMsE5), Mukdahan (iAMkE1), Sa Kaeo (iHSkE2, iHSkE3), Chanthaburi (iHCtE2) & Chumphon (iACpE6)

: Form F ~ Ayuttaya (iAAyF2, iAAyF6), Udonthani (iAUdF3, iAUdF4, iAUdF5), Khon Kaen (iAkkF1), Chaiyaphum (iACiF1), Sa Kaeo (iHSkF1), Chanthaburi (iHCtF4) & Prachuap Khiri Khan (iAPkF1)

Non post-mating reproductive isolation

Karyotypic variation

An. barbirostris species A1: Form A, B, C and D

species A2: Form A and B

An. campestris-like : Form B, E and F

This study confirmed: heterochromatin variation in sex chromosomes, due to the extra blocks of heterochromatin, is a general phenomenon in anopheline mosquitoes and some dipteran insects

(Baimai 1998)

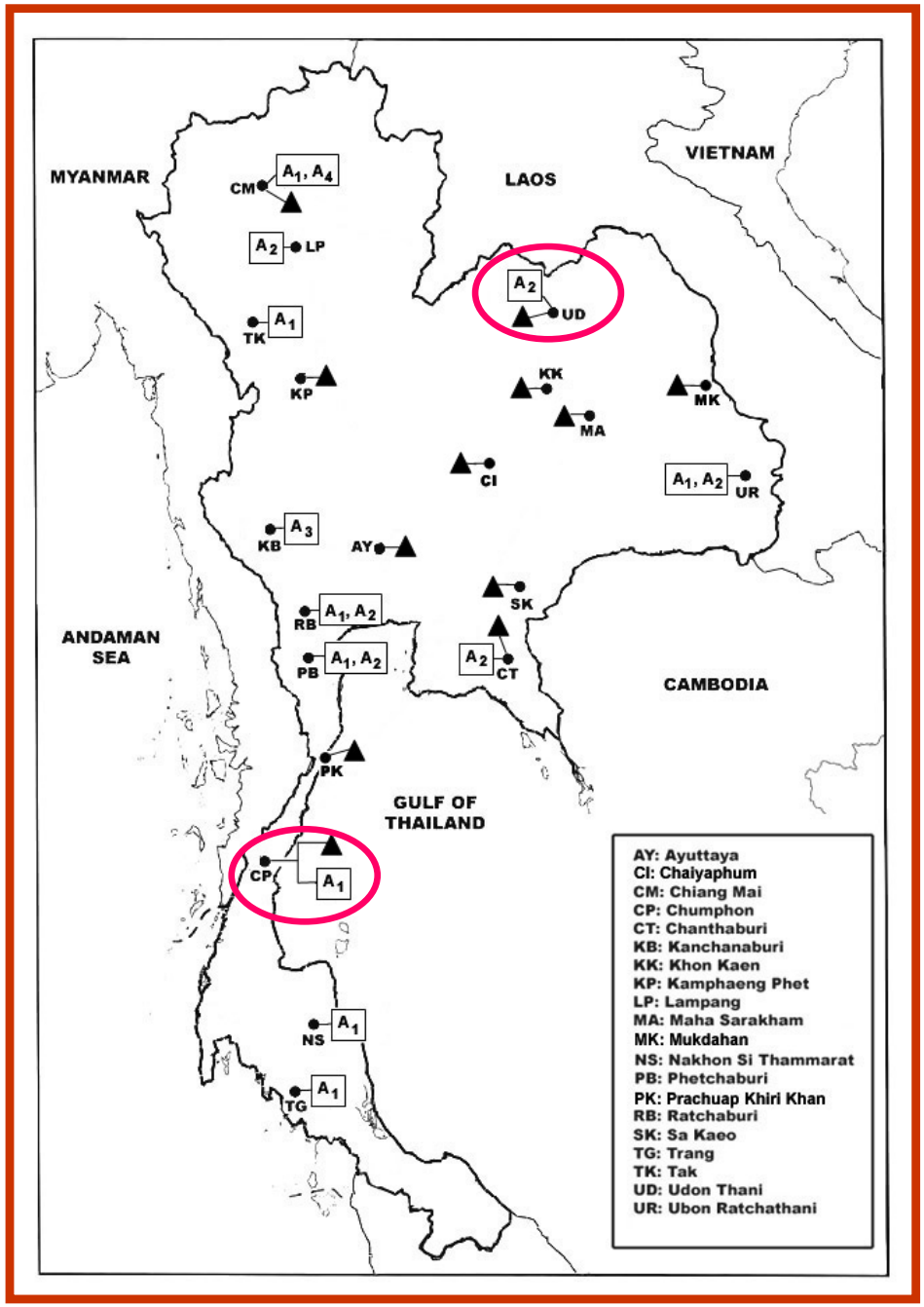


Fig 1. Map of Thailand showing distribution of *An. campestris*-like (▲) and *An. barbirostris* species A_1, A_2, A_3 and A_4 (□).

Acknowledgements

- The Biodiversity Research and Training Program:

  2549 (Grant No. BRT R_249004)

  2550 (Grant No. BRT R_250009)

- The Thailand Research Fund through the Royal Golden Jubilee Ph.D. program:

  Grant No. PHD/0052/2548

  Grant No. PHD/0082/2549

  Grant No. PHD/0031/2550

Thank you for your attention