ISOENZYME STUDY AND HYBRIDIZATION OF TWO FORMS OF *ANOPHELES SINENSIS* (DIPTERA : CULICIDAE) IN NORTHERN THAILAND

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Abstract. The screening of ten isoenzymes of two forms of *Anopheles sinensis,* Form A and B, using electrophoretic gels revealed that *Est-5*^{96} allele was the marker in both 4th larva and adult female of *An. sinensis* Form B, whereas it was lacking in Form A. Hybridization tests of the two *sinensis* forms were done by induced copulation. The results of crosses indicated that they were genetically compatible, providing viable progeny and completely synaptic polytene chromosoms.

INTRODUCTION

Anopheles (Anopheles) sinensis (Wiedemann, 1828) is a member of the *Anopheles (Anopheles) hyrcanus* species group belonging to the *Myzorhynchus* series. It was one of the first anopheline species reported from Thailand (Reid, 1968). It was incriminated as the natural vector of *Plasmodium vivax* in other countries (Otsuru and Ohmori, 1960; Ho *et al,* 1962; Chow, 1970; O'Connor, 1980), whereas it has little role in malaria transmission in Thailand (Harrison and Scanlon, 1975). However, an *An. sinensis* strain from northern Thailand, has been recently incriminated as an experimentally efficient vector of *P. vivax* by Somboon *et al* (1994). Nevertheless, the laboratory feeding of two cytological *sinensis* forms of *P. vivax* revealed that they were poor vectors, although the same indigenous mosquito species and malaria parasite were used (Rongsriyam *et al,* 1998).

Great progress has been made in the evolutionary genetics of anopheline mosquitos which are not easily identified morphologically; *eg An. gambiae* complex (Davidson, 1967), *An. maculatus* complex (Takai *et al,* 1987), *An. barbirostris* complex (Choochote *et al,* 1983), *An. dirus* complex (Baimai *et al,* 1988), etc. *An. sinensis* was firstly reported as a possible species complex by Oguma (1976, 1978). Evidence of cross-mating among six strains of *An. sinensis* from Japan revealed that the hybrid males between Engaru strain and either Kanoya or Yakumo strains were completely sterile, hybrid females were of low fertility and hybrid polytene chromosomes were completely asynapsed.

Based on metaphase karyotype studies, at least two forms of *An. sinensis* have been recently reported from Thailand (Baimai *et al,* 1993). They are *An. sinensis* Form A in which the Y_1 chromosome was subtelocentric or acrocentric with only a small portion of the short arm presented and Form B in which the Y_2 chromosome was clearly submetacentric with the short arm approximately one-half the length of the long arm. In view of the evidence of a possible species complex and the existence of at least two forms of *An. sinensis* in northern Thailand, therefore, investigations on intra-taxon of *An. sinensis,* particularly the strains from northern Thailand need to be defined more thoroughly. Hybridization experiments and isoenzyme investigations of two forms of *An. sinensis* strains from northern Thailand are presented.

MATERIALS AND METHODS

Laboratory-raised *An. sinensis*

Five laboratory-colony strains of *An. sinensis,* the same strains as studied by Rongsriyam *et al* (1998), were established based on the cytological forms (Baimai *et al,* 1993) and the geographical differences. The laboratory colonies of *An. sinensis* were sorted into two categories, the isolined and mixed colonies. For isolined colony : Form

A(XY_1)-colony was established by a using single wild-caught female from Amphoe Mae Sariang, Changwat Mae Hong Son (Ai), whereas Form B(XY_2)-colony was established from a single wild-caught female from Amphoe Muang, Changwat Mae Hong Son (Bi). For mixed colony : Form A(XY_1)-colony was established by using 13 wild-caught females from Amphoe Mae Sariang, Changwat Mae Hong Son (Am); Form B(XY_2)-colonies were established from 27 wild-caught females from Amphoe Muang, Changwat Mae Hong Son (Bm_1) and 26 wild-caught females from Amphoe San Sai, Changwat Chiang Mai (Bm_2). Metaphase karyotypes of all above five colonies, Ai, Bi, Am, Bm_1 and Bm_2, were consecutively confirmed up till the 10th generation.

Isoenzyme study

The methods of electrophoresis were followed Komalamisra (1989). Ten isoenzymes of 4thlarvae and adult females of five strains of laboratory-raised *An. sinensis* were investigated. The enzymes were esterase (EST), malic (ME), hexokinase (HK), fumerase (FUM), lactate dehydrogenase (LDH), phosphoglucose isomerase (PGI), (α-glycerophosphate dehydrogenase (α-GPDH), aldehyde oxidase (ALDOX), xanthine dehydrogenase (XDH) and amylase (AMY). Isoenzymes were numbered with respect to increasing anodal migration. Allozymes were named numerically according to their mobility relative to the commonest allele (=100).

Hybridization study

Crossing experiments were conducted between *An. sinensis* Form A and Form B in both isolined and mixed colonies. Hybridization was followed the method reported by Choochote *et al* (1983). Reciprocal crossmating, F_1-hybrid and back-cross were also carried out. Adult females and males of both species emerged from pupae that had been placed individually in test tubes were used for the experiments. The crosses were performed by induced mating soon after mosquitos were fed on blood. The gravid females were allowed to oviposit in the single colony, eggs were counted and placed in hatching pans. Spermathecae of mated females were examined for evidence of insemination. The hatching rates, survival rates, pupation rates, emergence rates, sex ratios and abnormal morphology were observed and recorded. The remained F_1-hybrids were conducted to reciprocal crosses and back crosses with their parental strains in order to observe genetic relationships. The F_2- progeny failed to survive was the criterion for reproductive isolation. The salivary gland polytene chromosome of 4th larvae from the crosses were also investigated using the standard techniques as described by Kanda (1979).

RESULTS

Isoenzyme study

The banding patterns of 4th larvae and adult females of *Est-5* are exhibited in Fig 1. Details of allele frequencies are demonstrated in Table 1 and 2. The results of investigations on allelic frequencies of 4th larvae and adult females of four laboratory-raised *An. sinensis* strains revealed that only esterase showed some degrees of differences in electromorph patterns. From the screening test, ME, HK and FUM showed a monomorphic bandmorph of the allele 100 in both adults and larvae. LDH and PGI demonstrated allele 100 in high frequency and additional allele 102 was found in low frequency only in Bm_2 *sinensis* larval strain. α-GPDH was found in only larval stage and showed a single locus with 2 alleles, 100 and 102. ALDOX showed 3 alleles, 98, 100 and 102, and allele 100 showed high frequency in both larval and adult stages among them. There were 3 alleles in a single locus of XDH, 99, 100 and 101, and showed polymorphic bandmorphs. *Amy-1* and *Amy-2* showed

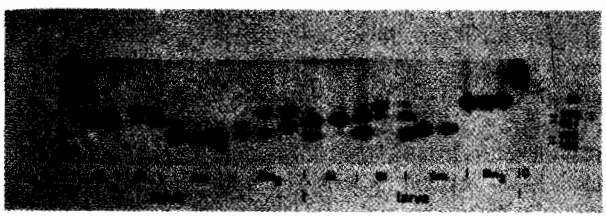

Fig 1–Band morph patterns of *Est-5* of four strains of laboratory-raised *An. sinensis*. Note a single mosquito is run on each lane and *An. dirus* A used as the control (C).

Table 1

Allele frequency and allele number of various enzymes of four strains of laboratory-raised *An. sinensis* larvae.

Isoenzyme	allele	*An. sinensis* strains			
		Ai A.freq (A. No.)	Bi A.freq (A. No.)	Am A.freq (A. No.)	Bm_2 A.freq (A. No.)
Est-5	96	0.00 (0)	0.36 (15)	0.00 (0)	0.43 (25)
	100	0.81 (26)	0.31 (13)	0.25 (12)	0.16 (9)
	101	0.00 (0)	0.00 (0)	0.04 (2)	0.22 (13)
	105	0.19 (6)	0.33 (14)	0.48 (23)	0.19 (11)
	106	0.00 (0)	0.00 (0)	0.23 (11)	0.00 (0)
Me	100	1.00 (8)	1.00 (8)	1.00 (8)	1.00 (8)
Hk	100	1.00 (4)	1.00 (4)	1.00 (6)	1.00 (6)
Fum	100	1.00 (4)	1.00 (4)	1.00 (6)	1.00 (6)
Ldh	100	1.00 (6)	1.00 (8)	1.00 (12)	0.75 (9)
	102	0.00 (0)	0.00 (0)	0.00 (0)	0.25 (3)
Pgi	100	1.00 (8)	1.00 (8)	1.00 (8)	0.75 (6)
	102	0.00 (0)	0.00 (0)	0.00 (0)	0.25 (2)
α-Gpdh	100	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
	102	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
Aldox	98	0.00 (0)	0.00 (0)	0.00 (0)	0.08 (1)
	100	1.00 (8)	1.00 (8)	1.00 (12)	0.67 (8)
	102	0.00 (0)	0.00 (0)	0.00 (0)	0.25 (3)
Xdh	99	0.20 (2)	0.83 (10)	0.00 (0)	0.14 (2)
	100	0.20 (2)	0.17 (2)	0.29 (4)	0.43 (6)
	101	0.60 (6)	0.00 (0)	0.71 (10)	0.43 (6)
Amy-1	100	1.00 (4)	1.00 (4)	1.00 (6)	1.00 (6)
Amy-2	100	1.00 (4)	1.00 (4)	1.00 (6)	1.00 (6)

A. freq = Allele frequency, A. No. = Allele number

monomorphism in larval stage whereas adult demonstrated only *Amy-1* with low density than larva.

Apart from esterase, there were 5 loci of EST in both larva and adult but only *Est-5* was intensive studied since it showed sharper band of polymorphic alleles. It was demonstrated that 5 alleles were presented in *Est-5, ie* 96, 100, 101, 105 and 106. In isolined colonies , Form A showed 2 alleles, allele 100 with high frequency and 105 in low frequency whereas Form B demonstrated 3 alleles with an additional allele 96 in moderate frequency. In the same way, alleles 100, 105, 106 were proved in larva and adult of mixed colony of Form A and an increased allele 101 was found in low frequency of larval stage. In mixed colony of Form B, alleles 96, 100, 101 and 105 were found in both stages. The different points between Form A and B were that $Est\text{-}5^{96}$ was found with moderate frequency in either larva or adult of Form B but not found in Form A. On the other hand, $Est\text{-}5^{106}$ was demonstrated only in Form A with low frequency.

Hybridization study

Details of hatchability, pupation and emergence of parental, reciprocal, F1-hybrid and back-crosses among five strains of laboratory-raised *An. sinensis* are shown in Table 3. Observations on the hatchability, pupation, emergence and adult sex-ratio of parental, reciprocal, F1-hybrid and back-crosses between two isolined and among three mixed colonies of laboratory-raised *An. sinensis* revealed

Table 2

Allele frequency and allele number of various enzymes of four strains of laboratory-raised *An. sinensis* adult females.

Isoenzyme	allele	*An. sinensis* strains			
		Ai A.freq (A.No.)	Bi A.freq (A.No.)	Am A.freq (A. No.)	Bm_2 A.freq (A.No.)
Est-5	96	0.00 (0)	0.22 (8)	0.00 (0)	0.42 (25)
	100	0.75 (27)	0.64 (23)	0.22 (13)	0.10 (6)
	101	0.00 (0)	0.00 (0)	0.00 (0)	0.25 (15)
	105	0.25 (9)	0.14 (5)	0.60 (36)	0.23 (14)
	106	0.00 (0)	0.00 (0)	0.18 (11)	0.00 (0)
Me	100	1.00 (12)	1.00 (12)	1.00 (12)	1.00 (12)
Hk	100	1.00 (4)	1.00 (4)	1.00 (6)	1.00 (6)
Fum	100	1.00 (4)	1.00 (4)	1.00 (6)	1.00 (6)
Ldh	100	1.00 (8)	1.00 (6)	1.00 (6)	1.00 (6)
Pgi	100	1.00 (12)	1.00 (12)	1.00 (12)	1.00 (12)
α-*Gpdh*	100	0.17 (2)	0.33 (4)	0.67 (8)	0.00 (0)
	102	0.83 (10)	0.67 (8)	0.33 (4)	1.00 (12)
Aldox	98	0.12 (1)	0.25 (2)	0.00 (0)	0.14 (2)
	100	0.63 (5)	0.50 (4)	0.67 (8)	0.86 (12)
	102	0.25 (2)	0.25 (2)	0.33 (4)	0.00 (0)
Xdh	99	0.25 (4)	0.50 (8)	0.11 (2)	0.33 (6)
	100	0.50 (8)	0.50 (8)	0.89 (16)	0.67 (12)
	101	0.25 (4)	0.00 (0)	0.00 (0)	0.00 (0)
Amy-1	100	1.00 (4)	1.00 (4)	1.00 (6)	1.00 (6)
Amy-2	100	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)

A. freq = Allele frequency, A. No. = Allele number

Table 3

The hatchability, pupation and emergence of parental, reciprocal, F_1-hybrid and back-crosses among five strains of laboratory-raised *An. sinensis*.

Cross*	Total eggs	No. hatch	No. pupation	No. emergence	No. females and males from total emergence (%)	
Female x Male	(range)	(%)	(%)	(%)	Female	Male
Parental crosses						
Ai x Ai	430	365	254	243	105	138
	(46-138)	(84.88)	(69.59)	(95.67)	(43.21)	(56.79)
Bi x Bi	447	359	245	245	121	124
	(53-118)	(80.31)	(68.25)	(100)	(49.39)	(50.61)
Am x Am	504	457	345	322	162	160
	(55-149)	(90.67)	(75.49)	(93.33)	(50.31)	(49.69)

Bm_1 x Bm_1	425	337	246	220	105	115
	(57-114)	(79.29)	(73.00)	(89.43)	(47.73)	(52.27)
Bm_2 x Bm_2	438	317	239	223	104	119
	(53-130)	(72.37)	(75.39)	(93.31)	(46.64)	(53.36)
Reciprocal crosses						
Ai x Bi	351	316	257	250	110	140
	(50-95)	(90.03)	(81.33)	(97.28)	(44.00)	(56.00)
Bi x Ai	475	440	272	253	119	135
	(44-131)	(92.63)	(61.82)	(93.38)	(46.85)	(53.15)
Am x Bm_1	277	210	140	138	65	73
	(35-76)	(75.81)	(66.67)	(98.57)	(47.10)	(52.90)
Bm_1 x Am	486	362	245	240	107	133
	(55-133)	(74.49)	(67.68)	(97.96)	(44.58)	(55.42)
Am x Bm_2	423	283	170	169	87	82
	(58-107)	(66.90)	(60.07)	(99.41)	(51.48)	(48.52)
Bm_2 x Am	372	324	187	182	97	85
	(51-124)	(87.10)	(57.72)	(97.33)	(53.30)	(46.70)
Bm_2 x Bm_1	422	328	225	215	111	104
	(44-127)	(77.73)	(68.60)	(95.56)	(51.63)	(48.37)
F_1-hybrid crosses						
(Ai x Bi)F_1 x (Ai x Bi)F_1	488	436	344	340	155	185
	(54-160)	(89.34)	(78.90)	(98.84)	(45.59)	(54.41)
(Bi x Ai)F_1 x (Bi x Ai)F_1	513	483	296	275	125	150
	(49-137)	(94.15)	(61.28)	(92.91)	(45.45)	(54.55)
(Am x Bm_1)F_1 x (Am x Bm_1)F_1	383	315	191	187	89	98
	(34-126)	(84.86)	(58.77)	(97.91)	(47.59)	(52.41)
(Bm_1 x Am)F_1 x (Bm_1 x Am)F_1	440	286	198	194	92	102
	(61-124)	(65.00)	(69.23)	(97.98)	(47.42)	(52.58)
(Am x Bm_2)F_1 x (Am x Bm_2)F_1	338	300	165	157	73	84
	(41-93)	(88.76)	(55.00)	(95.15)	(46.50)	(53.50)
(Bm_2 x Am)F_1 x (Bm_2 x Am)F_1	359	281	163	159	80	79
	(36-93)	(78.27)	(58.01)	(97.55)	(50.31)	(49.69)
(Bm_2 x Bm_1)F_1 x (Bm_2 x Bm_1)F_1	424	347	243	229	100	129
	(50-145)	(81.84)	(70.03)	(94.24)	(43.67)	(56.33)
Back-crosses						
Ai x (Ai x Bi)F_1	315	276	193	186	92	94
	(37-96)	(87.62)	(69.93)	(96.37)	(49.46)	(50.54)
Ai x (Bi x Ai)F_1	374	303	199	185	82	103
	(35-117)	(81.02)	(65.68)	(92.96)	(44.32)	(55.68)
Am x (Am x Bm_1)F_1	368	292	188	178	86	92
	(51-113)	(79.35)	(64.38)	(94.68)	(48.31)	(51.69)
Bm_1 x (Bm_1 x Am)F_1	498	410	262	247	115	132
	(60-159)	(82.33)	(63.90)	(94.28)	(46.56)	(53.44)
Am x (Am x Bm_2)F_1	420	368	208	202	100	102
	(54-116)	(87.62)	(56.52)	(97.12)	(49.50)	(50.50)
Bm_2 x (Bm_2 x Am)F_1	396	344	205	195	90	105
	(43-136)	(86.87)	(59.59)	(95.12)	(46.15)	(53.85)
(Bm_2 x Bm_1)F_1 x Bm_1	406	327	255	248	117	131
	(49-137)	(80.54)	(79.98)	(97.25)	(47.18)	(52.82)

* Five egg-batches of inseminated females for each cross

that all crosses yielded viable progenies and no evidence of genetical incompatibility was observed between *An. sinensis* Form A and Form B. The hatchability, pupation, emergence rates and ratio of adult female/male of parental; reciprocal; F_1-hybrid; back-crosses were 72.37-90.67 %, 68.25-75.49 %, 89.43-100 % and 0.76-1.01 ; 66.90-92.63 %, 57.72-81.33 %, 93.38-99.41 % and 0.79-1.14; 65.00-94.15 %, 55.00-78.90 %, 92.91-98.84 %, and 0.78-1.01; 79.35-87.62 % , 56.52-79.98 %, 92.96-97.25 % and 0.80-0.98, respectively. The salivary gland polytene chromosomes of the 4th larvae from all crosses showed complete synapsis along the whole length of all autosomes and X-chromosome when being compared to the parental chromosomes (Fig. 2).

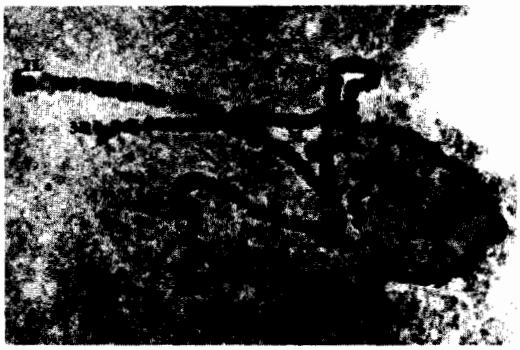

Fig 2–Salivary gland polytene chromosome of 4th stage larva of F_1-hybrid between two isolined colonies of *An.sinensis* FormA (Ai : XY_1): and Form (Bi : XY_2). Note complete synapsis in all arms.

DISCUSSION

Electrophoresis has been widely used as a tool to characterize and/or diagnose sibling species or subspecies of anopheline vectors *eg* the members of sibling species in *An. gambiae* complex (Miles, 1979), *An. dirus* complex (Green *et al,* 1992), *An. maculatus* complex (Yong *et al,* 1988) and *An. minimus* complex (Komalamisra, 1989).

In the present study ten isoenzymes, *ie* esterase (EST), malic (ME), hexokinase (HK), fumerase (FUM), lactate dehydrogenase (LDH), phosphoglucose isomerase (PGI), α-glycerophosphate dehydrogenase (α-GPDH), aldehyde oxidase (ALDOX), xanthine dehydrogenase (XDH) and amylase (AMY) were used to examine the genetic divergences among four strains of laboratory-raised *An. sinensis, ie* isolined colonies (Ai : Form A; Bi : Form B) and mixed colonies (Am : Form A; Bm_1 and Bm_2 : Form B). The results of investigations on allelic frequencies of 4th larvae and adult females revealed that both larvae and adult females of four strains yielded similar results of the nine isoenzymes except *Est-5*^{96} which was likely to be used as a marker to differentiate *An. sinensis* Form B from Form A. The lack of *Est-5*^{96} allele in the latter form might be suggested some degrees of separation; this difference, however, was not so great enough to incriminate them as a sibling species.

Hybridization experiment was one of the most efficiently and reliably diagnostic tool to definitely differentiate intra-taxon of anopheline species to a sibling species and/or subspecies. The hybrid inviability, sterility or breakdown were the criteria of genetic incompatibilities, these included insemination, egg deposition, embryonation, hatchability, larva survival, pupation, emergence, adult sex ratio, abnormal morphology and reproductive system, and asynapsis of polytene chromosomes (Kitzmiller, 1976; Kanda *et al,* 1981).

Several intra-taxa of anopheline species which were primarily detected of morphological, biological, cytological and biochemical differences, led to the doubtful status of sibling species and/or subspecies. Subsequently, it was clearly confirmed by hybridization experiments, such as *An. gambiae* complex (Davidson *et al,* 1967), *An. dirus* complex (Sawadipanich *et al,* 1990), *An. maculatus* complex (Chabpunnarat, 1988), *An. barbirostris* complex (Choochote *et al,* 1983) and *An. subpictus* complex (Suguna *et al,* 1994).

The search for sibling species and/or subspecies in *An. sinensis* has been the subject of investigation for more than 20 years. Kanda and Oguma (1976) reported the morphological variation of humeral pale spot and the different frequency of clasper movements of male terminalia during induced copulation of *An. sinensis* strains from Tomakomai and Engaru, Japan. These two *sinensis* strains have 23.30 % and 23.70 % of humeral pale spot; 14.30 and 14.60 frequency of clasper movements, respectively, while the other strains have never been found the humeral pale spot and the frequency of clasper movements were about 8. Oguma (1976, 1978) carried on the crossing among six strains of *An. sinensis* in Japan, the results indicated that the Engaru strain was reproductive isolation, the steriled hybrid males and incompletely asynaptic polytene

chromosome were recovered. He concluded the Engaru strain was a sibling species of *An. sinensis*. Base on morphologically variant, asynapsis of polytene chromosome and behavioral distinctness, Kanda and Oguma (1978) declared *An. sinensis* Engaru strain as a new species, *An. engarensis*. Since the differences of metaphase karyotypes and the presence of *Est-5*^{96} allele only in *An. sinensis* Form B were obtained. The hybridization between Form A and Form B was done to determine whether these two *sinensis* forms were reproductive isolation. The crossing experiments between two isolined colonies (Ai : Form A, XY_1; Bi : Form B, XY_2) and among three mixed colonies (Am : Form A, XY_1; Bm_1 and Bm_2: Form B, XY_2) were carried out in the present studies. The viable progenies and complete synapsis of salivary gland polytene chromosomes recovered from reciprocal, F_1-hybrid and backcrosses between two isolined and among three mixed colonies which were representative on the forms have indicated that *An. sinensis* Form A and B were the same species comprising at least two cytological races. Similar result was found in two forms of *An. maculatus* complex (Form B and E) which were karyotypic differences (Chabpunnarat, 1988). It is pertinent to note that, primarily definite classification of mosquito members or forms within the complex to be sibling species and/or subspecies, besides morphological, biological, cytological and biochemical differences, it must be intensively confirmed the reproductive isolation both from pre- and postmating, particularly the hybridization experiment.

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