THE SPECIFIC STATUS OF *ANOPHELES MINIMUS S.L.* COLLECTED FROM TAIWAN

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Abstract. At least three members (species A, C, and E) of the *Anopheles minimus* complex have been described in the Orient. This study investigated the specific status of *An. minimus* collected in the southern part of Taiwan by crossing experiments with species A from Thailand and species E from Japan. Crosses between Taiwan *An. minimus* and species A revealed genetic compatibilities. Post-zygotic isolation was observed in crosses between Taiwan *An. minimus* and species E. Hybrid progeny were only obtained from Taiwan female X species E male. F_2 hybrid progeny were not obtained, since the hybrid males were sterile or almost sterile, with atrophied testes or abnormal spermatozoa. The hybrid females backcrossed with either Taiwan F_1 progeny and species E males, and laid eggs with lower fertility and viability. This study supports previous published data regarding the analysis of the D3 region of the 28S gene of ribosomal DNA that *An. minimus* species A is indigenous to Taiwan. Whether other members of the *An. minimus* complex exist in Taiwan is not conclusive and needs more study.

INTRODUCTION

Anopheles minimus s.l is regarded as an important vector of human malaria in the Orient (Reid, 1968), including Taiwan where transmission was disrupted since the mid 1960s (Anonymous, 1991). In the past, it was distributed throughout the island, but after the eradication program, recent surveys have shown that the species has been found mainly in the northern and southern regions (Lien, 1991). The recognition of sibling species in the *Anopheles minimus* complex consisting at least 3 sibling species (A, C, and E) elsewhere (Sucharit *et al*, 1988; Green *et al*, 1990; Subbarao, 1998; Van Bortel *et al*, 1999; Kengene *et al*, 2001; Somboon *et al*, 2001; Chen *et al*, 2002) has prompted us to investigate the specific status of *An. minimus* in Taiwan. It is interesting because Taiwan is the island located approximately a half way between the main land China and the Iriomote/Ishigaki Islands where *An. minimus* species A and C (Chen *et al,* 2002) and species E (Somboon *et al*, 2001) are indigenous, respectively. Based on DNA analysis of the D3 region of the 28S gene of ribosomal DNA (rDNA), Chen *et al* (2002) has reported that our samples collected from Taiwan are identical to species A. However, only molecular evidence without additional information do not provide strong conclusion on the specific status. The present study reports the results of crossing experiments between *An. minimus* from Taiwan and species A and species E to further determine the specific status of this species on the island.

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MATERIALS AND METHODS

Anopheles minimus Taiwan fourth-stage larvae and pupae were collected from streams in Pingtung and Taitung (the latter was wrongly stated as Taichung in Chen *et al*, 2002) located in the southern region. They were then transferred to an insectary for rearing in the Department of Parasitology, Faculty of Medicine, Chiang Mai University. The pupae were sexed and placed in separate vials. Emerged adults were identified using the key of Harrison (1980) and used for crossing experiments. A number of them were analyzed for the D3 region of the 28S

gene of rDNA and the result of this has already been reported (Chen *et al*, 2002). A sub-population of them were maintained to produce F_1 progeny for controls in crossing experiments. A forced mating technique (Ow Yang *et al*, 1963) was applied since they rarely copulated in the cage. They were then reared by the method of Kanda (1979).

Anopheles minimus species A colony

The CM strain of An. minimus species A from Thailand (Somboon et al, 2001) was used in this study.

Anopheles minimus species E colony

A sub-colony of the ISG strain identified previously as species E by Somboon et al (2001) was taken from Japan and maintained in the insectary in Chiang Mai.

Crossing experiments

A*nopheles minimus* Taiwan was crossed with CM and ISG strains to determine genetic compatibility. Virgin females separated at the pupal stage were placed in screened cups provided with 2% sugar solution and offered a blood meal when 5 days old. Reciprocal crosses using a forced mating technique were made between the virgin females and males. Following mating, each female was isolated in an oviposition vial. Eggs were counted and left until they eclosed. Eggs from uninseminated females were excluded. The hatching larvae were counted and reared (Kanda, 1979). Egg batches with no or little hatching were examined for embryonation. Pupae were removed daily, sexed, and placed separately in cups until the adults emerged. The F_1 hybrid adults that emerged were counted. Their fertility and viability were observed by conducting further crosses among the hybrids and by backcrossing with the parental colonies. The testes and ovaries of the hybrids were also dissected to check fertility. The crosses were made in the same rooms housing the colonies, and test specimens were kept in these rooms under identical laboratory conditions to those of the colonies.

RESULTS

Crossing experiments between wild *An. minimus* from Taitung, Taiwan and species A

Table 1
Results from crosses among *Anopheles minimus* from Taiwan (Taitung) (TWtt), species A (CM strain) Thailand and species E (ISG strain) Japan.

Crosses[a]	No. of broods	Eggs		No. of eggs examined	Embryonation rate	Hatching rate[b]	Pupation rate[b]			Emergence rate[b]		
		Total	Average/brood				Female	Male	Total	Female	Male	Total
Interstrain crosses												
TWtt x CM	7	423	60.4	n.d.	n.d.	88.4	35.4	38.6	74.0	34.7	38.1	72.8
CM x TWtt	11	629	57.2	n.d.	n.d.	85.5	42.1	35.6	77.7	40.4	34.8	75.2
TWtt x ISG	5	318	63.6	n.d.	n.d.	86.5	35.2	37.1	72.3	34.3	35.2[c]	69.5
ISG x TWtt	5	235	47.0	235	0.1	0						
Control crosses												
CM x CM	3	203	67.7	n.d.	n.d.	92.1	43.3	38.0	81.3	41.9	36.9	78.8
ISG x ISG	3	221	73.7	n.d.	n.d.	88.7	38.9	38.5	77.4	38.5	34.4	72.9
TWtt x TWtt	3	191	63.7	n.d.	n.d.	94.2	40.8	43.5	84.3	39.8	41.9	81.7

[a]All the orders of the crosses are represented as female x male.
[b]Each rate was calculated by dividing the total number of individuals that reached each developmental stage by the total number of eggs multiplied by 100.
[c]Of 24 F_1 hybrid males dissected, 20 (83.3%) were completely sterile with atrophied testes. The remaining had spermatozoa but most were inactive and had an enlarged head.
n.d. = not determined.

Table 2
Results from fertility tests of backcrosses between the hybrids and the parental strains and of the (TWttx ISG)F_1 hybrids.

Crosses[a]	No. of broods	Eggs		No. of eggs examined	Embryonation rate	Hatching rate[b]	Pupation rate[b]			Emergence rate[b]		
		Total	Average/brood				Female	Male	Total	Female	Male	Total
(TWtt x ISG)F_1 x TWtt F_1	4	295	73.8	295	77.6	60.0	17.3	14.9	32.2	13.2	11.9[d]	25.1
(TWtt x ISG)F_1 x ISG	6	369	61.5	369	82.9	79.7	11.1	9.8	20.9	9.5	8.4[c]	17.9
TWtt F_1 x (TWtt x ISG)F_1	5[c]	266	53.2	266	0	0						
(TWtt x ISG)F_1 x (TWtt x ISG)F_1	5[c]	287	57.4	287	0	0						
TWtt F_1 x TWtt F_1 (control)	3	237	79.0	n.d.	n.d.	89.4	37.6	34.5	72.1	37.1	32.5	69.6
ISG x ISG (control)	3	241	80.3	n.d.	n.d.	91.7	38.2	39.8	78.0	37.4	34.0	71.4

[a]All the orders of the crosses are represented as female x male.
[b]Each rate was calculated by dividing the total number of individuals that reached each developmental stage by the total number of eggs multiplied by 100.
[c]No sperm were observed in the spermathecae of any of these females.
[d]Of 18 F_1 hybrid males dissected, 10 (55.6%) were completely sterile with atrophied testes. The remaining were fertile with active spermatozoa.
[e]Of 21 F_1 hybrid males dissected, 14 (70.0%) were completely sterile with atrophied testes. The remaining were fertile with active spermatozoa.
nd = not determined.

revealed that F_1 hybrid progeny were obtained from both directions of crosses (Table 1). The hatchability of the eggs, development of the immature stages, and the adult emergence, including sex ratios, were normal compared with those of the control crosses. The testes of the hybrid males were fully fertile with a lot of normal, active spermatozoa. The ovaries of the hybrid females looked normal. F_2 hybrids with excellent fertility were obtained (data not shown).

Crosses carried out between the Taiwan *An. minimus* and species E, by contrast, resulted in hybrid progeny from only the Taiwan female X species E males (Table 1). The hatchabilitiy of the eggs, development of larvae, pupation, and emergence rates, including sex ratios of the hybrids, appeared to be normal. Of the 24 F_1 hybrid males dissected, 20 (83.3%) were completely sterile with atrophied testes and 4 (16.7%) had spermatozoa which were inactive and had an enlarged head. These males failed to inseminate, although they succeeded in copulating with females when subjected to forced mating. Thus, no F_2 hybrids were obtained (Table 2). The ovaries of the hybrid females looked normal. When backcrossed to either Taiwan F_1 or species E males, they produced eggs with lower hatching rates compared with the control (Table 2). A high mortality was observed among 1st-stage larvae resulting in low pupation and emergence rates. Most of the hybrid males were completely sterile with atrophied testes, whereas the ovaries of the hybrid females were normal. No further crosses were attempted.

DISCUSSION

Laboratory hybridization to demonstrate post-zygotic isolation is a powerful tool to establish the species status of isomorphic populations (Subbarao 1998). Post-zygotic isolation between *An. minimus* species A and C (Choochote *et al*, 2002), species A and E (Somboon *et al*, 2001), and species C and E (Somboon *et al*, 2005) has been previously reported. In species A X C or E crosses, hybrid progeny were obtained

from one directional crosses, species A females X species C or E males. The hybrid males from species A X C crosses were fertile, but the hybrid females showed abnormal ovaries (Choochote *et al*, 2002). In species A X E crosses, in contrast, the hybrid males were sterile, whereas the hybrid females looked normal (Somboon *et al*, 2001). In species C X E crosses, both directions of crosses produced hybrid progeny, but the hybrid males were sterile or almost sterile (Somboon *et al*, 2005). In the present study, there was no post-zygotic isolation between *An. minimus* from Taitung, Taiwan and species A from Thailand. When crossed with species E, hybrid progeny were obtained from a one directional cross, similar to the phenomena of the species A X E cross reported by Somboon *et al* (2001). DNA analysis revealed that all 17 of our samples (8 from Pingtung and 9 from Taitung) reported previously were identical to species A (Chen *et al*, 2002). This evidence strongly suggests that *An. minimus* species A exists on Taiwan Island. Morphologically, most of our *An. minimus* females collected in Taiwan had the presector pale spot on the wings. One feral female collected from a cattle shed in Pingtung had the humeral pale spot but was identified as species A by DNA analysis (Somboon P, unpublished data). We did not find evidence for the existence of species C or E in Taiwan. However, it is still too early to conclude that species A is the only member of the *An. minimus* complex in Taiwan because our samples were from only 2 locations in the south. Further study is required, especially in the northern area.

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