MORPHOLOGICAL DIFFERENTIATION IN *ANOPHELES MACULATUS* OF THAILAND ACCOMPANIED WITH GENETICAL DIVERGENCE ASSESSED BY HYBRIDIZATION

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ABSTRACT. *Anopheles maculatus* of Thailand were distinguished morphologically into densely and sparsely scaled types and cross-mating experiments between these types revealed a substantial amount of genetic divergence. Progeny of wild females of *An. maculatus* collected throughout Thailand from north to south were examined morphologically with respect to adult tergal pale scales. Northern progeny broods could be separated into densely scaled (*willmori*) and sparsely scaled (*maculatus*) types, or W and M types, respectively. Using these two types of *An. maculatus* from Chiang Rai and Nakhon Phanom, cross-mating experiments were conducted, which resulted in sterile F_1 male hybrids in one direction of cross and very low survival rate of F_1 male hybrids in the reciprocal cross. However, southern broods could not be separated clearly because of intergradation of scaling. These results indicate that Thai *An. maculatus* involves at least 2 forms differentiated morphologically, as well as by incomplete reproductive isolation.

INTRODUCTION

Anopheles maculatus Theobald is one of the important malaria vectors in southern Thailand, Peninsular Malaysia and Sumatra (Baimai et al. 1984) and a vector of filariasis in Peninsular Malaysia (Cheong and Omar 1965). Baimai et al. (1984) and Green et al. (1985) made surveys of this species throughout Thailand from north to south and divided it into 4 species based on cytogenetic examination. However, any clear morphological differentiation associated with the separation had not been detected.

In this paper, we provide evidence that there is morphological differentiation in this species from Thailand and also that it is associated with genetic divergence assessed by laboratory hybridization.

According to Reid et al. (1966) and Reid (1968), *An. maculatus* is a species in which the morphological variation exists mainly in the pale scaling on the adult abdominal terga. In this study the assessment of these pale scales was the primary focus. We could separate at least 2 types, which were further tested for post-mating reproductive isolation by hybridization experiments. The 2 types were differentiated also in this respect.

MATERIALS AND METHODS

Collection of Anopheles maculatus. Figure 1 shows the collection localities for *An. maculatus* chosen from 1983 to 1985 from near the north border of Thailand to the south. Wild blood-fed females of *An. maculatus* were collected from water buffalo, cow or human volunteer. Each female was allowed to oviposit and the F_1 progeny were supplied for morphological identification.

Morphological identification. Several individuals of the F_1 progeny of each wild female were morphologically examined with special reference to pale scales on the adult terga. Variation in the scaling was examined to see whether families of wild females could be characterized and categorized according to the scaling. It became clear that each northern family could be distinguished into at least either densely or sparsely scaled type; families of the same type were mixed to establish a strain for further investigation. Progeny of some southern families contained individuals with a few broad pale scales on abdominal terga II and III.

Cross-mating experiments. Strains from northern localities, Chiang Rai and Nakhon Phanom provinces, were utilized for cross-mating experiments. Details of the method for mating were as those described in Kanda et al. (1981a) and Takai et al. (1984). Briefly, intra- and interstrain crosses and also backcrosses were made to assure fertility of F_1 hybrids. When required, the F_1 hybrids were dissected to examine the internal reproductive organs.

RESULTS

Morphological characterization of Anopheles maculatus. Examination of adult tergal pale-scaling in the F_1 progeny of northern wild female families revealed that there was a distinction between densely and sparsely scaled types (Fig. 2). After referring to Reid et al. (1966) and Reid (1968) we called the 2 types *willmori* (W) and *maculatus* (M) types, respectively, though the

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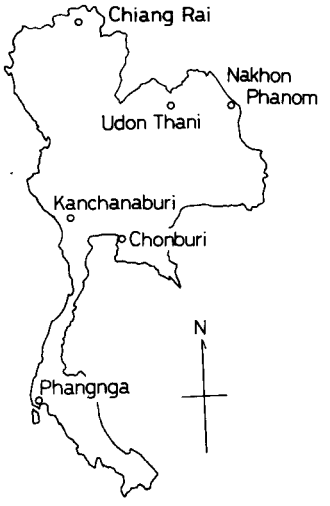

Fig. 1. Collection localities of *Anopheles maculatus* from Thailand.

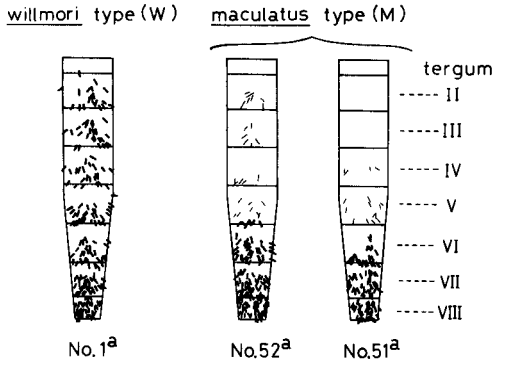

Fig. 2. Schematic presentation of pale scaling on the adult terga of *Anopheles maculatus* from Thailand. Left, a densely scaled *willmori* type; right, a sparsely scaled *maculatus* type. The *maculatus* type had more variation in scaling on terga II and III than the *willmori* type; figures show 2 extreme cases (see text). Numbers of scales on terga VII and VIII of both types were too large to follow exactly, but scales on the other terga were depicted accurately.

[a] Refers to specimen no. among F_1 progeny.

amount of pale scales in our specimens of the W type was not as much as var. "*willmori*" in Figure 175.2 of Reid (1968) but as "*willmori*-like" form in the Figure 174.5a. All individuals of the W type family had broad pale scales on abdominal terga II to VIII, and sometimes 1 or 2 broad scales on tergum I. Some individuals of the M type family had narrow pale scales on abdominal terga II to V and broad pale scales on terga V to VIII, but others had no pale scales on terga II and III. Of major importance, almost all pale scales of the W type were broader than those on terga II to IV of the M type; the pale scales of the M type on terga VI to VIII and some posterior pale scales on tergum V were as broad as those of the W type broad scales. The M type families varied in pale-scaling on terga II and III; ranging from some which had scales on both terga to others which did not have any on either. Figure 2 shows the 2 extreme examples of the M type. Note that scales of terga II to IV of the M type are less broad than those on terga VI to VIII. The left specimen of the M type (no. 52) had 6 broad scales on the posterior edge of tergum V. The broad scales on tergum V were also variable in the M type. Using these criteria, the families of *An. maculatus* collected from northern Thailand (Chiang Rai, Nakhon Phanom and Udon Thani) could be divided into the above 2 types. However, in Kanchanaburi and Phangnga, another type which had only a few broad pale scales on terga II and III was found. Table 1 summarizes the result of classification of wild-female families of *An. maculatus* into these types.

Hybridization between W and M type strains and fertility of F_1 hybrids. Because the morphological difference in the pale scaling was definite among wild-female families of *An. maculatus* from northern Thailand, families of each type were mixed to establish a colony strain. Crossmating experiments were conducted using W and M type strains of Chiang Rai (designated as W_C and M_C) and of Nakhon Phanom (W_N and M_N). When F_1 hybrids were obtained from interstrain crosses, they were further tested for fertility. It has been known that when laboratory colonies from 2 diverging populations are crossed with each other, the following F_1 and F_2 progeny results are available as indicators of

Table 1. Classification of *Anopheles maculatus* from Thailand into densely scaled (W), sparsely scaled (M), and intermediate types.

Collection		No. wild female families		
Locality	Date	W type	Interm.	M type
Chiang Rai (C)[a]	Jun. 1983	8	0	14
Udon Thani	Jun. 1983	2	0	7
Udon Thani[b]	Jun. 1983	0	0	6
Nakhon Phanom (N)[a]	Jun. 1983	3	0	4
Kanchanaburi	Jun. 1984	13	(5)[c]	(22)[c]
Kanchanaburi	Nov. 1984	19	(0)[c]	(1)[c]
Chonburi	Feb. 1985	4	0	4
Phangnga	Feb. 1984	0	(1)[c]	(5)[c]

[a] Provided for hybridization experiments.

[b] Human-baited collections. All the others were animal-baited.

[c] Identification of the M type was difficult owing to the presence of a few broad pale scales on terga II and III of some individuals; families which had at least one such individual were sorted into an intermediate type, but there was a possibility that some M type families fell into the intermediate type if all the progeny brood had been examined.

their differentiation (Kanda and Oguma 1977a, 1977b; Oguma 1978; Kanda et al. 1981a, 1981b, 1982, 1983; Takai et al. 1984): (1) unusually high mortality at some developmental stages, (2) pupae which had abnormal or malformed genital pouches, (3) sex ratio distortion, (4) non-reciprocal yields of hybrids, (5) sterile F_1 hybrids (both sexes), and (6) sterile F_1 hybrid males. We observed all of these indicators except (5) in the results shown in Tables 2 and 3. (1): crosses 7 and 14, (2): crosses 8 and 8′ to 14, (3): crosses 8 to 10 (in adults), (4): cross 5 vs. 8 and 7 vs. 9, and (6): $(WM)F_1$ males. Both crosses 7 and 14 had very low embryonation rates (0.521 and 0.451, respectively) compared with the rates in others (ranging from 0.727 of cross 11 to 0.984 of cross 6) and had markedly reduced larval survival rates. Most of the abnormal pupae died at that stage and the remainder emerged as adult males. Four $(W_NM_N)F_1$ males were backcrossed to W_N females, which neither became gravid nor oviposited. Five $(W_NM_N)F_1$ males were dissected and their internal reproductive organs were examined. All of them had degenerated testes and 3 also had degenerated, malformed accessory glands, although the external reproductive organs appeared normal (Fig. 3). These results helped to ascertain the sterility of $(WM)F_1$

Table 2. Hybridization among strains of *Anopheles maculatus* from Thailand.[a]

Cross ♀ × ♂	No. egg batches	Egg	Pupa ♀	Pupa Abn[c]	Pupa ♂	Pupa Sum	Adult ♀	Adult Abn[d]	Adult ♂	Adult Sum	χ^2 for ♀:♂[b] Pupa	χ^2 for ♀:♂[b] Adult
Intra-strain												
1. W_CW_C	2	174	0.190	0	0.247	0.437	0.184	0	0.230	0.414	1.3	0.9
2. W_NW_N	6	566	0.346	0	0.419	0.765	0.323	0	0.380	0.703	3.9*	2.6
3. M_CM_C	2	206	0.413	0	0.432	0.845	0.408	0	0.427	0.835	0.1	0.1
4. M_NM_N	6	518	0.342	0	0.411	0.753	0.315	0	0.386	0.701	3.3	3.8
Inter-strain WM crosses												
5. W_NM_N	5	390	0.462	0	0.408	0.869	0.444	0	0.390	0.833	1.3	1.4
6. W_NM_C	2	186	0.462	0	0.489	0.952	0.457	0	0.484	0.941	0.1	0.1
7. W_CM_C	2	140	0.043	0	0.043	0.086	0.043	0	0.036	0.079	0.0	0.1
Inter-strain MW crosses												
8. M_NW_N	9	626	0.377	0.310	0.062	0.749	0.369	0.003	0.008	0.380	0.0	210***
8′. M_NW_N[e]	1	73	0.548	0.041	0.342	0.932	0.521	0.027	0.342	0.890	2.1	1.9
9. M_CW_C	1	96	0.365	0.250	0.063	0.677	0.365	0.125	0.042	0.531	0.4	7.1*

[a] For each cross figures in the columns of pupa and adult show the survival rates from egg to the stages.
[b] Abnormal pupae were sorted into the male class. Values without asterisks are nonsignificant at 5% level.
[c] Morphologically abnormal pupae the genital pouch of which was mostly black-banded at the base (normal males did not have such bands) or that of which was sometimes swollen, shortened, or malformed.
[d] This class only refers to the larval stage, i.e., adults emerged from abnormal pupae. All adults in this class had male phenotypes.
[e] This cross was distinctly different from other M_NW_N crosses in that male adults were produced with few abnormal pupae.
*: Significant at 5% level.
***: Significant at 0.1% level.

Table 3. Fertility of F_1 hybrids among strains of *Anopheles maculatus* from Thailand.[a]

Cross ♀ × ♂	No. egg batches	Egg	Pupa ♀	Pupa Abn[c]	Pupa ♂	Pupa Sum	Adult ♀	Adult Abn[d]	Adult ♂	Adult Sum	χ^2 for ♀:♂[b] Pupa	χ^2 for ♀:♂[b] Adult
(WM)F_1 backcrosses												
10. $(W_NM_N)W_N$	2	239	0.222	0.059	0.100	0.381	0.209	0	0.100	0.310	2.5	9.1**
11. $(W_NM_N)M_N$	2	183	0.290	0.022	0.257	0.568	0.284	0.005	0.224	0.514	0.0	1.1
12. $(W_NM_C)M_C$	5	400	0.208	0.033	0.205	0.445	0.185	0.005	0.168	0.358	0.8	0.2
$W_N(W_NM_N)$	Unsuccessful											
(MW)F_1 backcrosses												
13. $(M_NW_N)M_N$	4	326	0.319	0.037	0.334	0.690	0.285	0	0.291	0.577	1.3	0.0
14. $(M_CW_C)M_C$	5	377	0.082	0.019	0.074	0.175	0.080	0.013	0.069	0.162	0.2	0.0
With F_1 of cross 8												
15. $M_N(M_NW_N)$	2	113	0.274	0	0.283	0.558	0.265	0	0.265	0.531	0.0	0.0

[a,b,c,d] See the footnote of Table 2.
** Significant at 1% level.

males. Cross 8′ was distinct from cross 8 in that male adults were produced without so many abnormal pupae. Fertility of $(MW)F_1$ males from crosses 8 and 9 was not tested owing to very small numbers of adult hybrid males; $(MW)F_1$ males from cross 8′ were fully fertile in cross 15. It was rather impossible that this cross 8′ was contaminated with cross WM, because it produced abnormal pupae, which was not observed in WM crosses 5 to 7. Furthermore, $(WM)F_1$ males were sterile. A similar exceptional cross had once been observed by Takai et al. (1984) in the study of reproductive isolation of the *An. hyrcanus* species group.

In conclusion, WM crosses produced F_1 hybrids with normal survival rates but the F_1 males were sterile; MW crosses yielded normal F_1 females but had F_1 males mostly abnormal at the pupal stage or died (Table 4). One MW cross was exceptional, producing normal F_1 males. The pale scaling showed intergradation in the southern specimens, however, *An. maculatus* from northern Thailand could represent at least 2 forms, W and M types, differentiated in both morphology and by reproductive isolation.

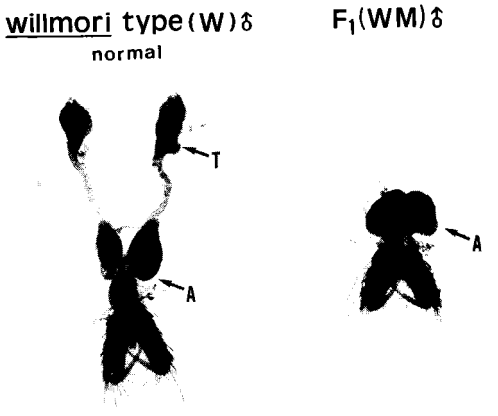

Fig. 3. Reproductive organs of a $(WM)F_1$ male. A: accessory glands, T: testes.

Table 4. Summary of the hybridization experiments for W and M type *Anopheles maculatus* from Thailand.

Cross	Development of F_1 hybrids		F_1 hybrid fertility	
	♀	♂	♀	♂
W ♀ × M ♂	Normal	Normal	Fertile[a]	Sterile
M ♀ × W ♂	Normal	Mostly unable to emerge	Fertile[a]	—[b]

[a] F_2 progeny contained abnormal male pupae.
[b] Untested due to scarcity of adults; one exceptional cross yielded fertile F_1 males.

DISCUSSION

In finding a morphological character as being diagnostic of races, semispecies or species in the species complex, it has to be considered whether the character can definitely divide wild-female families into groups. If variation of a character within a family overlaps with that of the character within another family, the character cannot be used to divide those families. The W and M type pale scaling in this study could separate wild female families of *An. maculatus*, at least for specimens from northern Thailand. Differentiation by reproductive isolation was also evident between the 2 types.

However, there was a possibility that each morphological type further involved several forms. In the maintenance of strains of each type, progeny undergoing abnormal emergence were never observed. We expected that each type would not contain forms so divergent as to produce abnormal progeny as described above.

Cross-mating was done mainly between sympatric types. One cross-mating between allopatric types was W_NM_C in cross 6. It gave similar results to the sympatric cross W_NM_N: Development of F_1 hybrids of W_NM_C was normal and female F_1 hybrids were fertile with abnormal F_2 male pupae (cross 12).

Baimai et al. (1984) and Green et al. (1985) divided *An. maculatus* from Thailand into as many as 7 forms (A to H, except D) based on examination of their polytene chromosome rearrangements. They further classified them into 4 species, A, B, C and G. Three forms, E, F and H were considered to be chromosomal races of B. Each of the 4 species had different fixed chromosome rearrangements. Baimai et al. (1984) referred to their preliminary results of cross-mating experiments involving the 4 species; in all cases F_1 male hybrids were sterile while female hybrids were fertile. Baimai et al. (1984) also presented a geographic distribution for these species and forms: Species A occurred most commonly, B the second most, and E was the only kind of *maculatus* in peninsular Thailand. Species C was rare, and G was at the north end of peninsular Thailand, not occurring at Kanchanaburi. If the relation among B, E and F as chromosomal races is correlated with morphology, we may be able to assign the W type to species A, the M type from Phangnga to form E, the M type of Chiang Rai to species B, and the M type of Udon Thani, Nakhon Phanom, and Chonburi to B, F or E.

There seemed to be indirect evidence for assignment of the W type to species A and the M type to species B. In our experience, W type strains were more difficult to maintain in the laboratory than the M type. Though similar

maintenance procedures were applied to both types, the W type happened to become extinct quite easily. According to Baimai et al. (1984), species A had no polymorphic chromosome rearrangements while species B and its races had as many as 6 polymorphic inversions. The adaptive significance of polymorphic inversions is an established concept most extensively studied by Dobzhansky's school (Dobzhansky 1970). That is, the chromosomal races of species B might have a higher adaptability to environmental changes than A. It agrees with the observation that the M type is easier to adapt to laboratory conditions than the W type if the assignment of W to A and M to B is correct.

As mentioned in Table 1, southern wild female families (from Kanchanaburi and Phangnga) which contained at least one individual having a few broad pale scales on terga II and III were sorted into an intermediate type. At Kanchanaburi and Chonburi the W type was also found. This may show that there is intergradation of this scaling in *An. maculatus*.

Among 6 and 40 wild-female families from Phangnga and Kanchanaburi (June sample), respectively, some F_1 progeny of a family from each collection (designated as M type in Phangnga and W type in Kanchanaburi) underwent abnormal pupation, that is, a reasonable number of progeny did not emerge. This was the same as had been observed in MW crosses or backcrosses in this study. There seemed to be one possibility that the wild females had mated with males of other forms in nature. Thus, there is a possibility that Phangnga natural population consists of different forms, though Baimai et al. (1984) found only one form E from peninsular Thailand. It was possible that the abnormal pupation observed in the Kanchanaburi wild female family was a result of hybridization in nature between a W female and an M male, because such crosses would yield abnormal pupae when backcrossed as in crosses 10 to 12.

According to Grant (1963), population systems can be classified on the basis of their relative phenotypic variation, relative geographical distribution, and mode of isolation. "Sympatric semispecies" in his classification refer to such population systems as to be intergrading discontinuously or partially in morphological or physiological characters, and judged to be interbreeding on a restricted scale; sympatric; and partially isolated reproductively. The data presented in this study seem inadequate to describe W and M type *An. maculatus* as sympatric semispecies. It is at least obvious that the 2 types belong to populations on the border line between geographical races and sympatric species in the process of speciation. Further investigation is needed to settle a number of questions raised above.

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