

HYBRIDIZATION EVIDENCE SUPPORTING SEPARATE SPECIES STATUS FOR *ANOPHELES NIVIPES* AND *ANOPHELES PHILIPPINENSIS*¹

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ABSTRACT. Evidence of genetic incompatibility observed in F₁ hybrids from reciprocal crossing experiments between *Anopheles nivipes* and *An. philippinensis* strongly suggests that these 2 nominal taxa are distinct species. Hybrid inviability of the F₁ crosses was very obvious in egg eclosion rates and larval and pupal mortality. These data also suggest sex ratio distortion. Male sterility was noted in F₁ hybrids from crosses in both directions, and was expressed by abnormal external genitalia, abnormal internal reproductive organs and absence of sperm.

INTRODUCTION

The roles of *Anopheles nivipes* (Ludlow) and *An. philippinensis* Theobald in the transmission of human malaria parasites in Southeast Asia are very poorly defined. *Anopheles nivipes* was not recognized as a valid species until Reid (1967), and no data have been published regarding its medical importance. *Anopheles philippinensis* was incriminated as a vector of human malaria parasites in Bengal, India (Covell 1944, Ganguli 1947) and in the district of Mymensingh, Bangladesh (Quraishi et al. 1951), where it appeared to be a domestic and anthropophilic species. In other areas it is considered primarily zoophilic and of little importance in malaria transmission (Covell 1944, Reid 1970). In Thailand the situation is very confused because the presence of *An. nivipes* (Reid 1967) has not been acknowledged by the Thailand Malaria Division (unpublished annual status reports). Accordingly, most records of *An. philippinensis* for Thailand also probably include *An. nivipes* (Harrison, unpublished data). *Anopheles philippinensis* is reported as primarily zoophilic in Thailand, but since it is reported as common in human biting collections in certain malarious areas in the absence of the primary vector species, it is considered a suspected vector (Harinasuta et al. 1976). However, there are no published data to indicate whether it is *An. nivipes* or *An. philippinensis* biting man, or which

species is more common in Thailand. Efforts are being expended by the Thailand Malaria Division on the surveillance and control of *An. philippinensis*, although there is no good evidence that this species is a vector in the country.

Thailand strains of *An. nivipes* and *An. philippinensis* were colonized (Klein et al. 1982) to provide specimens for extensive laboratory studies. These studies, along with field efforts, were initiated to determine the taxonomic status, relative abundance, distribution and medical importance of these 2 nominal species in Thailand. The taxonomic, cytogenetic and malaria susceptibility aspects of this study will be published elsewhere. The hybridization aspects are reported below.

MATERIALS AND METHODS

Hybridization experiments were performed at the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand, using colonies of *An. nivipes* and *An. philippinensis* that originated from females collected near Korat and Rayong, Thailand, respectively. The experimental crossing program called for the standard interspecies crosses, with the parental colonies as controls. Backcrosses of F₁ hybrids of both sexes to the parental strains were planned, as were F₂ crosses, if necessary. Artificial mating techniques (Ow Yang et al. 1963) were necessary to maintain both colonies and to conduct the experimental crosses. The physical location, environmental conditions and the rearing procedures for the colonies were described in Klein et al. (1982). The experimental crosses were made in the same rooms housing the colony cages, and the test specimens were kept in these rooms under identical laboratory conditions as those of the colonies.

Virgin females were placed in screened cages and provided with a hamster as a food source. After the females took one blood meal, recip-

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recal crosses were made between the virgin females and males of both species. Following mating, each female was isolated in an oviposition vial. After oviposition, females were dissected and examined for spermatozoa. Eggs from each female were counted and placed in hatching cups for 10 days. Newly emerged larvae from each egg batch were counted daily and placed in rearing pans until they pupated. Pupae were removed daily and placed in individual rearing vials. Pupae which died prior to or during emergence were examined and either discarded or preserved. Newly emerged adults were preserved with associated pupal skins or discarded after examination. Males were dissected and examined for the condition of the reproductive organs.

RESULTS

The fecundity of cross females and viability of hybrids from the reciprocal crosses are summarized in Tables 1 and 2. Females involved in crosses were examined for spermatozoa following oviposition. Twelve of the 16 ovipositing *philippinensis* females (mated with *nivipes* males) deposited viable eggs. These 12 females contained spermatozoa in their spermathecae. Two of the 4 females ovipositing non-viable eggs did not have spermatozoa in their spermathecae, while the remaining 2 died and dried out and were not dissected. All 5 of the *nivipes* females (mated with *philippinensis*

males) which oviposited, contained spermatozoa. The average numbers of eggs oviposited in the F₁ interspecific crosses were not appreciably different from those obtained from the intraspecific crosses (Table 1). However, the egg eclosion rates for the ♀ *philippinensis* x ♂ *nivipes* (\bar{x} = 45.8%) and the ♀ *nivipes* x ♂ *philippinensis* (\bar{x} = 40.8%) crosses were significantly different by Chi square ($p < 0.001$) from those of the ♀ *philippinensis* x ♂ *philippinensis* (\bar{x} = 66.8%) and ♀ *nivipes* x ♂ *nivipes* (\bar{x} = 81.5%) crosses (Table 1). Larval mortality in the interspecific crosses, 64.8% (♀ *philippinensis* x ♂ *nivipes*) and 75.4% (♀ *nivipes* x ♂ *philippinensis*), was significantly higher (Chi square, $p < 0.001$) than that in the intraspecific crosses, 26.6% (♀ *philippinensis* x ♂ *philippinensis*) and 31.3% (♀ *nivipes* x ♂ *nivipes*) (Table 1).

Excessive mortality of pupae and emerging adults occurred in the ♀ *philippinensis* x ♂ *nivipes* F₁ hybrids (Table 2). Nearly all of the male pupae (94.2%) from these crosses died prior to (59.5%) or during emergence (34.7%), while 16.7% of the female hybrids also died. The dead male pupae had deformed genital lobes and/or portions of the paddle darkly pigmented (compare Figs. 1 and 2). The ♀ *philippinensis* x ♂ *nivipes* crosses also produced twice as many males (66.9%) as females (33.1%) (based on pupae and adults). Female F₁ hybrids from crosses of this direction appeared healthy and normal. Only 7 (5.8%) males emerged from these crosses and 2 died within 24 hours, while the other 5 had abnormal internal reproductive

Table 1. Oviposition, eclosion and larval mortality data from *Anopheles* crosses.

Crosses ♀ ♀ x ♂ ♂	Number	Females ovipositing	Number ovipositions hatching	Mean number eggs per oviposition	Percent eggs hatching	Percent larvae dead before pupation
<i>philippinensis</i> x <i>nivipes</i>	54	16	12	71.8	45.8 (526/1149)	64.8 (341/526)
<i>nivipes</i> x <i>philippinensis</i>	39	5	5	57.8	40.8 (118/289)	75.4 (89/118)
<i>philippinensis</i> x <i>philippinensis</i> *	—	43	35	59.3	66.8 (1703/2548)	26.6 (453/1703)
<i>nivipes</i> x <i>nivipes</i> *	—	48	45	65.0	81.5 (2541/3119)	31.3 (796/2541)

* Data from colonies.

Table 2. Pupal mortality and adult emergence data from *Anopheles* crosses.

Crosses ♀ ♀ x ♂ ♂	Number pupae	Female pupae (%)			Male pupae (%)		
		Number	Dying	Adults emerging	Number	Dying	Adults emerging
<i>philippinensis</i> x <i>nivipes</i>	181	60 (33.1)	10 (16.7)	50 (83.3)	121 (66.9)	114 (94.2)	7 (5.8)
<i>nivipes</i> x <i>philippinensis</i>	29	27 (93.1)	0 (0.0)	27 (100.0)	2 (6.9)	1 (50.0)	1 (50.0)

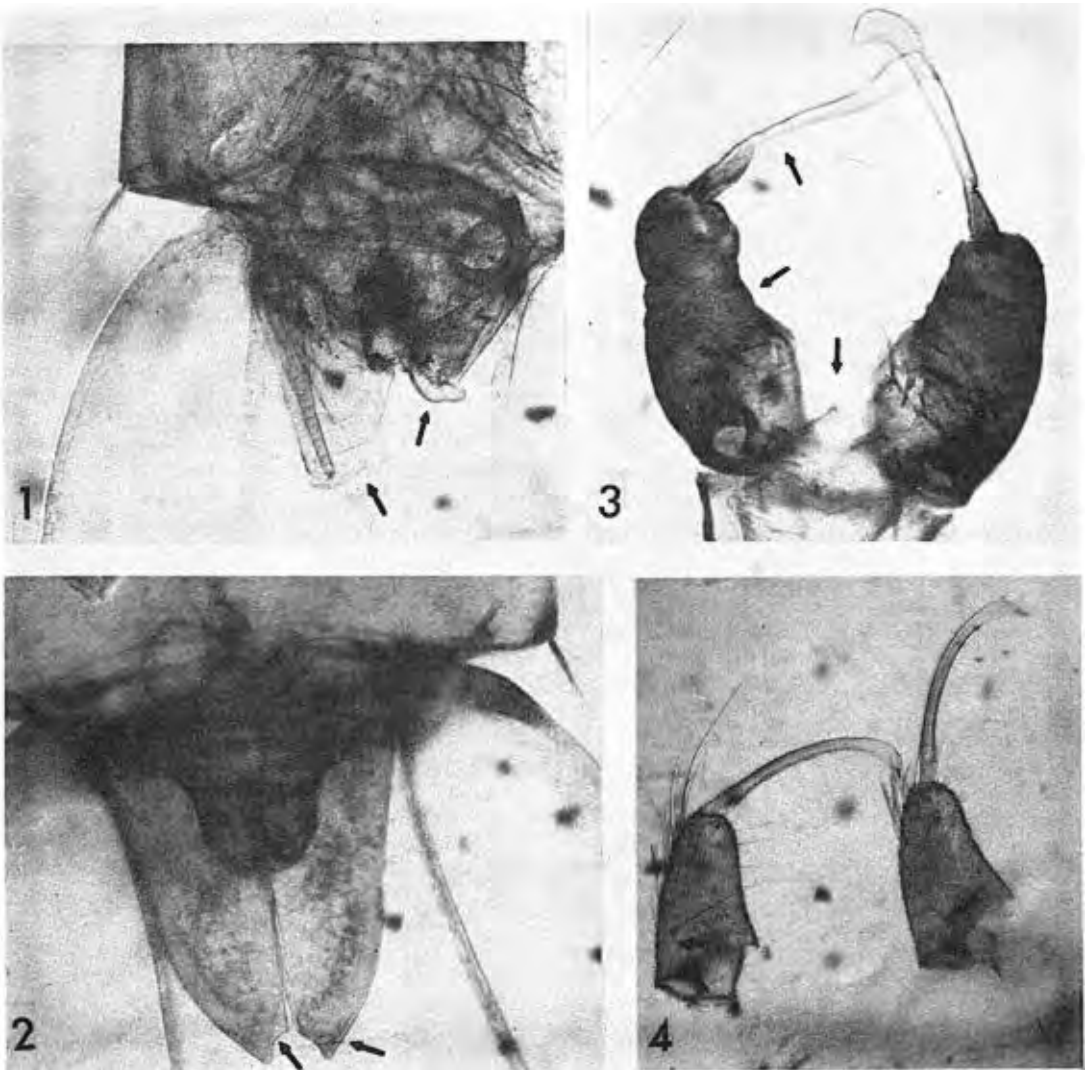


Fig. 1. Photograph of abnormal male pupal genital lobes (arrows) of F_1 hybrid from $\text{♀ Anopheles philippinensis}$ x ♂ An. nivipes cross.

Fig. 2. Photograph of normal male pupal genital lobes (arrows) of *An. nivipes*.

Fig. 3. Photograph of grossly deformed external genitalia (arrows) of F_1 hybrid male from $\text{♀ An. philippinensis}$ x ♂ An. nivipes cross.

Fig. 4. Photograph of dissected gonocoxites and gonostyli of "normal" male *An. nivipes* (magnification not same as Fig. 3).

organs or external genitalia (compare Figs. 3 and 4).

Of 29 pupae resulting from the ♀ nivipes x ♂ philippinensis crosses, 27 (93.1%) were females which emerged into normal appearing adults (Table 2). These females contained normal appearing reproductive organs when dissected. Only 1 of the 2 male pupae emerged; it possessed abnormal external male genitalia. The low number of pupae in these crosses is a direct reflection of the 75.4% larval mortality.

Although backcrosses were planned for this study, the unexpected reassignment of the senior author to the United States terminated the study before these crosses were made.

DISCUSSION

Crossing studies are a well known and practical way to separate and recognize sibling species. Such studies are very important in demonstrating isolating mechanisms such as post-

mating barriers to gene flow like hybrid inviability and sterility. The presence of these types of genetic incompatibility "may represent a more or less definite indication of specific distinctness" (Coluzzi 1970). In *Anopheles*, hybrid sterility is apparently one of the first isolating mechanisms developing during geographic isolation and probably represents a highly reliable guide to the existence of reproductive isolation (Coluzzi 1970).

Although backcross data are not available, we believe the data for the F_1 hybrids from our crosses provide strong evidence supporting the specific status of *An. nivipes*. Similar crossing data were used to identify sibling species in the Maculipennis Complex of *Anopheles* (Kitzmiller et al. 1967). Several types of genetic incompatibility that are useful in demonstrating the existence of sibling species (Coluzzi and Kitzmiller 1975), were evident in the F_1 hybrids from our crosses, i.e., reduced egg and immature viability, sex ratio distortion and hybrid sterility in the males.

There was little difference in the average number of eggs produced by the intra- and interspecific crosses; however, there was a marked reduction (21.0–40.7%) in the hatch rate of eggs from the interspecific crosses. The larval mortality in the interspecific crosses was also more than twice (64.8% and 75.4%) that of the intraspecific crosses. There was also a high pupal mortality in the ♀ *philippinensis* × ♂ *nivipes* crosses, particularly in the males. This mortality was clearly associated with a morphological abnormality which was expressed as deformed and often darkly pigmented pupal genital lobes. Several males which tried to struggle free of their pupal exuviae, completely emerged except for the terminalia which were apparently trapped in the genital lobes. A few of these had torn the abdominal integument while trying to free the terminalia, and subsequently died within 24 hours.

The low number of adult F_1 hybrids (85) emerging from the reciprocal interspecies crosses was a direct result of the reduced egg hatch rate and high larval and pupal mortality rates. Of this number, 77 were females and 8 were males. This disparity in the numbers of each sex was especially affected by the 94.2% (114/121) mortality that occurred in the F_1 male pupae of the ♀ *philippinensis* × ♂ *nivipes* crosses. Other possible evidence of sex distortion is evident in the numbers of F_1 hybrids that reached the pupal stage. In the ♀ *philippinensis* × ♂ *nivipes* crosses the pupal sex ratio was 1♀ to 2♂♂, while the ♀ *nivipes* × ♂ *philippinensis* crosses had a ratio of approximately 9♀♀ to 1♂. These ratios, however, were obviously affected by the 64.8% and 75.4% larval mor-

talities that occurred in the respective crosses.

Significant evidence of hybrid sterility in the F_1 males was expressed as morphologically abnormal external genitalia, small and abnormal testes without sperm and very fragile narrowed vasa efferentia. Two males from the ♀ *philippinensis* × ♂ *nivipes* crosses died due to injury during emergence, 3 had grossly abnormal nonfunctional external genitalia and the remaining 2, with normal appearing external genitalia, were dissected. These 2 males had small and abnormal testes without sperm, and very fragile narrowed vasa efferentia. The single male emerging from the ♀ *nivipes* × ♂ *philippinensis* crosses had grossly abnormal, nonfunctional external genitalia, and was not dissected. Dissection of males of *An. nivipes* and *An. philippinensis* from the colonies (Klein et al. 1982) did not exhibit abnormal external genitalia, and rarely exhibited abnormal internal reproductive organs.

The lack of precise morphological characters to separate the adult females of *An. nivipes* and *An. philippinensis* has made malaria workers reluctant to accept *An. nivipes* as a separate species from *An. philippinensis*. The above evidence of genetic incompatibility, biological differences detected by Klein et al. (1982) and morphological differences (Reid 1967, 1968), provide strong supporting evidence that Reid (1967) was correct to recognize *An. nivipes* as a distinct species. Accordingly, it is suggested that malaria control agencies in Southeast Asia redefine their concepts of *An. philippinensis* and evaluate the role of *An. nivipes* in malaria transmission.

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