Journal of the American Mosquito Control Association, 17(2):98–113, 2001

EVIDENCE FOR A NEW SIBLING SPECIES OF *ANOPHELES MINIMUS* FROM THE RYUKYU ARCHIPELAGO, JAPAN

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ABSTRACT. The *Anopheles minimus* complex is known to comprise at least 2 sibling species (A and C) in Thailand and Vietnam. This study investigated the specific status of *An. minimus* on Ishigaki Island, the Ryukyu Archipelago, Japan using morphological and genetic analyses. Morphological studies revealed that almost all (99.5%) of the adult mosquitoes are characterized by the humeral pale spot on the costa of their wings, a character that partially differentiates species A and C elsewhere. A high frequency (81.4%) have a pale fringe spot at the tip of vein 1A, a character rarely observed in other *An. minimus* populations. Significant seasonal variation in the size of wild *An. minimus* mosquitoes on the island was observed, with the largest size in the winter. Scanning micrographs of the cibarial armature of females from Ishigaki Island revealed that over 90% had cone filaments clearly differing in shape from those of species A or C. The Giemsa-stained metaphase karyotypes of larval brain cells were somewhat similar to those of species A, with a few exceptions, but were very different from those reported for species C. Crossing experiments between species A (CM strain) from Thailand and the progeny of *An. minimus* from Ishigaki Island (ISG strain) revealed postzygotic genetic incompatibility, although no prezygotic isolation. Hybrid progeny were only obtained from CM female × ISG male. F_2 hybrid progeny were not obtained, since the hybrid males were sterile or almost sterile with atrophied testes or abnormal spermatozoa, although the polytene chromosomes of hybrid larvae showed synapsis. The hybrid females backcrossed with either CM or ISG males laid eggs with significantly lowered fertility and viability. The sequence for the D3 region of the 28S gene of ribosomal DNA of the ISG strain differed from those of species A and C. In addition, sequence data from Vietnamese mosquitoes suggest that the *An. minimus* complex may contain additional species. The morphological, cytogenetic, molecular, and hybridization evidence together suggest the existence of another sibling species of the *An. minimus* complex on Ishigaki Island, which is provisionally designated *An. minimus* species E.

KEY WORDS *Anopheles minimus,* Japan, Thailand, morphology, DNA, hybridization, species complex

INTRODUCTION

Anopheles minimus Theobald is one of the major vectors of malaria throughout the Oriental Region (Reid 1968, Rao 1984), apart from Sri Lanka, most of Malaysia, Singapore, Brunei, the Philippines, and Indonesia, where it is considered to be absent (Harrison et al. 1990). An extensive study by Harrison (1980) provided useful information on intra- and interspecific morphological variation of *An. minimus* and its close relatives. More recent studies, which employed primarily enzyme electrophoresis, have shown that *An. minimus* is in fact a species complex consisting of at least 2 closely related species (Sucharit et al. 1988, Green et al. 1990). Other putative members of the complex, 'Form B' on Hainan Island, China (Yu Yuan 1987), and a possible species D in Thailand (Baimai 1989), have been proposed, but sufficient information to support their species status is not yet available.

Anopheles minimus species A is the predominant species of the complex in Thailand (Green et al. 1990). It has also been reported in India (see Subbarao 1998 for review) and Vietnam (Van Bortel et al. 1999). According to these reports, *An. minimus* species A is probably the most widespread species of the complex in the Oriental Region and has been suggested to be Theobald's species (Harrison et al. 1990). Species A in India, however, appears to be different from that found in Thailand with respect to host feeding preference, resting behavior, sporozoite positivity, and the response to vector control using insecticide-treated bed nets (Jana-Kara et al. 1995, Somboon et al. 1995). Species C was first reported in Thailand (Sucharit et al. 1988) and is common in Kanchanaburi Province in sympatry with species A, but absent or rare in other provinces (Green et al. 1990, Sharpe et al. 1999). Recently, based on enzyme electrophoresis, species C has been reported in Vietnam (Van Bortel et al. 1999), where it occurs in sympatry with species A in varying proportions depending on locality, host preferences, and season. The vector status of *An. minimus* species C in transmitting malaria has not been determined. Little is known about the distribution of these 2 species and any other sibling species in the *An. minimus* complex in other countries, including Japan.

Anopheles minimus was the primary vector of *falciparum* malaria in the southern islands of the Ryukyu Archipelago, Japan, particularly after World War II until 1962 when transmission was completely disrupted (WHO 1966). The density of *An. minimus* was markedly reduced because of the effect of DDT residual spraying during antimalaria

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control operations. However, recent surveys have found that the species has become widely prevalent on Ishigaki and Iriomote Islands, along with, to a much lesser extent, Miyako Island (Toma and Miyagi 1986; Toma et al. 1996a, 1996b).

The discovery of sibling species in the *An. minimus* complex elsewhere prompted us to investigate the specific status of *An. minimus* in the Ryukyu Archipelago. Previously, Kanda et al. (1984) reported cytogenetic and hybridization studies among a strain of *An. minimus* from Ishigaki Island and 2 strains (KCH-1 and KCH-2) from Kanchanaburi Province, Thailand. However, because rearing conditions caused high mortality in the immature stages (even of the control crosses), no clear conclusions could be made from the crosses. Nonetheless, the polytene chromosomes of the 3 strains were all similar in their banding patterns, and complete synapsis was observed in the hybrids of all crosses. The KCH-1 strain is most probably *An. minimus* species A (as most individuals lacked the humeral pale spot), whereas the KCH-2 strain was most probably *An. minimus* species C (as all individuals possessed the humeral pale spot). The suggestion that species A and C could be distinguished on the basis of the presence or absence of humeral pale spots was first put forward by Sucharit et al. (1988). Subsequently, however, Green et al. (1990) and Van Bortel et al. (1999) did not find this character to be completely diagnostic. This report presents the results of morphological and genetic studies of *An. minimus* on Ishigaki Island that provide evidence that it is another species in the *An. minimus* complex.

MATERIALS AND METHODS

Ishigaki Island: The island (24°26′N, 124°11′E) is about 222 km² and located approximately 400 km southwest of the main island of Okinawa, Japan. The island's climate is classified as subtropical with average minimum–maximum temperatures for the past 22 years (1968–1990) in August (summer), October (autumn), and February (winter) of 27–32, 22–27, and 16–21°C, respectively. More information regarding the island, as well as the distribution of *An. minimus,* was provided by Toma and Miyagi (1986) and Toma et al. (1996a).

Mosquitoes: Fourth-stage larvae and pupae of *An. minimus* were collected from Nishihama stream on the north side of the island in August and October 1998 and February 1999. Adults and immature stages were also collected from the same area in August 1999. The specimens were kept in the field at room temperature (about 25°C) for a few days. The larvae were then transferred to an insectary in Nagasaki and reared in stream water from the collection site with the addition of larval food until they pupated. Emerged adults with associated larval and pupal exuviae were identified using keys in Tanaka et al. (1979) and Harrison (1980). Attempts were made to find morphological characters of the various stages that might help to differentiate the specimens from the known sibling species. The size of adult mosquitoes was determined by measuring the length of wings, usually the right wing, from the axillary incision to the wing tip, excluding the fringe, using an ocular micrometer. *Anopheles minimus* from the collection made in October 1998 were retained in colony and designated as ISG strain, and their progeny were used for hybridization and other studies. An artificial mating technique (Ow Yang et al. 1963) was necessary to maintain the colony. The morphological terminology used in the report follows Harbach and Knight (1980).

A stenogamous strain of *An. minimus* (CM strain) from northern Thailand (Somboon and Suwonkerd 1997) was taken to Nagasaki for crossing and other experiments. There was no genetic incompatibility when crossed with several strains of *An. minimus* s.l. in northern Thailand. The CM strain was confirmed as species A by enzyme electrophoresis (K. Sawabe, personal communication) and DNA analysis (see results). The insectarium was maintained at 27°C and 70% relative humidity (RH) with a photoperiod of 16:8 h L:D. The rearing methods followed Kanda (1979).

Cibarial armature: Freshly killed adult females were stored in 70% ethanol until dissected. The cibarial armature was dissected from heads in a drop of distilled water under a dissecting microscope, covered with a coverslip and observed under a compound microscope (400× magnification). For scanning electron microscopy, the dissected cibarial armatures were dehydrated through a graded ethanol series and mounted on stubs. After being sputter-coated with gold, the specimens were scanned in a JEOL scanning electron microscope (JSM-840AN; JEOL Ltd., Akishima, Japan).

A few *An. minimus* species A and C females from Thailand (Green et al. 1990, Sharpe et al. 1999) and Vietnam (Van Bortel et al. 1999) identified previously by these authors using enzyme markers or a DNA analysis were obtained as dry specimens. In order to dissect these specimens, it was necessary to place the head–thoracic portions in 1 ml of distilled water and incubate them at 4°C for 1–2 days. After incubation, the supernatant was replaced with 70% ethanol. The specimens were then dissected as above.

Metaphase karyotypes: The brain ganglia of the 4th-stage larvae were examined for metaphase karyotypes, using a modification of the techniques described by French et al. (1962) and Baimai (1977). Briefly, instead of placing the dissected brain ganglia in colcemid solution, the larvae were placed in 0.1% colchicine for 2 h and transferred to a drop of 1% trisodium citrate for dissection of the brain. Fixing and staining methods then followed Baimai (1977).

Crossing experiments: The ISG strain was

crossed with the CM strain to determine genetic compatibility. Virgin females separated at the pupal stage were placed in screened cups provided with 3% sugar solution and offered a bloodmeal when 5 days old. After the females took 1 bloodmeal, reciprocal crosses using the forced mating technique noted above were made between the virgin females and males of both strains. Following mating, each female was isolated in an oviposition vial. Eggs were counted and left until they hatched. Following oviposition, females were dissected to check for spermatozoa in their spermatheca, and eggs from uninseminated females were excluded. Some of the females were checked after taking another bloodmeal and laying another batch of eggs. The larvae reared from these egg batches were used to examine polytene chromosomes of the salivary glands (Kanda 1979). Newly hatched larvae (normally 2 days after oviposition) from each egg batch were counted and placed in rearing trays until they pupated. Egg batches with no or little hatching were allowed to stand for another 3 days, after which they 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 and their morphological characteristics were noted. Their fertility and viability were observed by further crosses among the hybrids and backcrosses 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 the test specimens were kept in these rooms under identical laboratory conditions to those of the colonies.

To observe if there is preferential mating behavior in the laboratory, a crossing experiment was carried out in which virgin females or males of the CM strain were confined with the opposite sex of the ISG strain in a 30-cm cage. In each cage, 100 pairs of 1–4-day-old adults were released, and 10 days after their release, the insemination rate was determined by checking spermathecae for the presence of sperm. As the control, 100 pairs of the CM and ISG mosquitoes were released in separate cages, and their insemination rate was compared.

DNA sequencing: Genomic DNA was extracted from individual adult mosquitoes using either a phenol-chloroform method (Sambrook et al. 1989) or a salting out method (Sunnucks and Hales 1996). A product of approximately 370 base pair (bp) of the D3 region of the 28S gene of ribosomal DNA (rDNA) was amplified by PCR using primers D3a (5′ GACCCGTCTTGAAACACGGA 3′) and D3b (5′ TCGGAAGGAACCAGCTACTA 3′). Amplification was performed according to Sharpe et al. (1999), although the hot start procedure was found to be optional. The products were purified on spin columns (Promega, Madison, WI) and sequenced using the PCR primers and *Taq*FS dye-terminator fluorescent chemistry (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions, on an Applied Biosystems Model 373 automated sequencer. All products were sequenced in both directions, and no ambiguities were observed. The sequences were aligned using the PILEUP program of the Genetics Computer Group (Program Manual for the Wisconsin Package Version 8, 1994, 575 Science Drive, Madison, WI). The PHYLIP 3.4 (Felsenstein 1993) programs, DNAPARS, SEQBOOT, CONSENSE, DNADIST, and NEIGHBOR were used for phylogeny reconstruction, and TREEVIEW (Page 1996) was used to visualize phylogenetic trees.

RESULTS

Morphological characteristics: Some of the wing characters on *An. minimus* collected from Ishigaki Island and randomly selected progeny of the ISG strain are summarized in Table 1. All except a small proportion of the mosquitoes collected in the winter (February 1999) had the humeral pale spot on both wings. In addition, the pale fringe spot at the tip of vein 1A was present in high proportion.

Collections in February 1999 failed to obtain high numbers. Nonetheless, it is interesting that the wild 4th-stage larvae, as well as the emerged mosquitoes, were significantly larger than those collected in other seasons (Table 1), suggesting a seasonal effect on mosquito development. Six feral females previously collected from human bait in the same area in February 1997 were also large (mean wing length 3.13 mm, range 2.92–3.35 mm). The mean wing length of the feral females collected in August 1999 (Table 1) was not significantly different from that of *An. minimus* s.l. females collected from human bait in Chiang Mai, Thailand, in June 1999 in the early rainy season (mean 2.46 mm, range 2.17–2.70 mm; $t = 0.79$, df 155, $P = 0.43$). In the laboratory, the ISG females tended to be larger than the CM females, as investigated in a rearing experiment using similar conditions (mean wing length of ISG females 2.72 mm, range 2.49–2.97 mm; mean of CM females 2.55 mm, range 2.31–2.69 mm; $t = 5.39$, df 45, $P < 0.0001$).

The eggs of both the CM and ISG strains resembled the general descriptions of *An. minimus* s.l. (Reid 1968). Most eggs had a complete deck, but approximately half of the broods had some individuals with an incomplete deck. Such individuals were usually rare (10% of the brood), but occasionally more common (up to 89% in the CM and 65% in the ISG strains). The adults reared from eggs with an incomplete deck usually produced eggs with a complete deck. The length of 653 CM and 755 ISG eggs randomly selected from the colonies varied from 0.383 to 0.508 mm (mean 0.421 mm, SD 0.025) and 0.393 to 0.548 mm (mean 0.452 mm, SD 0.031), respectively. The 4th-stage larvae of the 2 strains also resembled the general descriptions of *An. minimus* s.l. (Reid 1968, Harrison 1980), including the anterior tergal plate on

Table 1. Frequency of selected wing characters and wing length of wild-caught *Anopheles minimus*, collected from Ishigaki Island, Japan, in different seasons, and the ISG strain colony.

Characters[1]	Female[2]				Male[2]		
	Aug.–Oct. 1998[3] (51)	Feb. 1999[3] (12)	Aug. 1999[4] (69)	ISG (123)	Aug.–Oct. 1998[3] (39)	Feb. 1999[3] (7)	ISG (97)
Costa with humeral pale spot (%) (both)	100	91.7	100	100[6]	100	85.7	100
Costa with presector pale spot (%) (one) (both)	96.1	25.0	94.2	71.5	94.9	28.6	87.6
	92.2	25.0	89.9	63.4	79.5	28.6	81.4
1A with 3 dark spots (%) (both)	3.9	0.0	0.0	2.4	0.0	0.0	0.0
1A with pale fringe spot (%) (one) (both)	72.5	66.7	76.8	80.5	84.6	71.4	91.7
	64.7	41.7	71.0	77.2	53.8	71.4	90.7
Wing length (mm)[5]	2.44 a (2.08–2.73)	2.95 b (2.75–3.05)	2.45 a (2.10–2.67)	n.d.	2.44 a (2.12–2.72)	3.04 b (2.82–3.17)	n.d.

[1] Both, character on both wings; one, character on at least one wing.
[2] Total number of specimens examined for all characters is in parentheses.
[3] Females and males reared from wild larvae and pupae.
[4] Feral females collected from a cow shed.
[5] Mean with range in parentheses. Means followed by the same letter are not significantly different ($P > 0.05$). n.d., not determined.
[6] One female had this spot on only the ventral side of both wings.

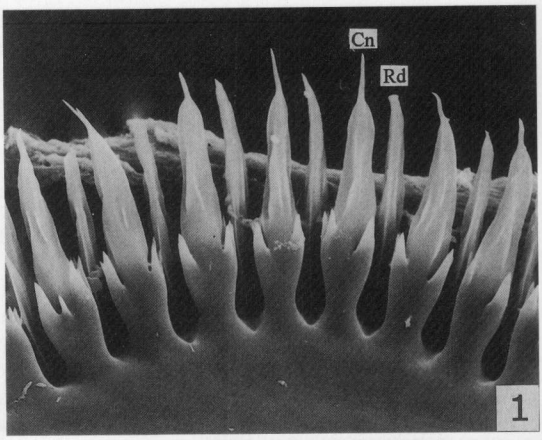
III–VII, seta 0-IV, V, and the number of teeth of the dorsomentum (usually 9, including 2 minute teeth at the base). However, seta 1-I in ISG larvae (89/100) was fully palmate with leaflets with distinct shoulders, whereas in CM larvae with distinct shoulders they were found in a much lower proportion (7/100). Over 100 pupal exuviae of the CM and ISG strains were examined, but no significant character differences were detected, including setae 7-VI, -VII, and 0-III–VII.

Cibarial armature: A total of 64 CM and 66 ISG females were examined. Figs. 1–6 show scanning micrographs of the cibarial armature. The appearance of the armature under a light microscope (400×) is shown in Figs. 7–8. The CM females have cone filaments that are wide at the base and change abruptly to form a fine needle at the apex (in about ⅓ to ⅐ of the filaments), producing a lancetlike shape (Figs. 1–2). The ISG females typically have cone filaments that are relatively narrow and gradually reduced to pointed ends, producing a thornlike shape (Figs. 4–5). None of the CM females examined had thornlike filaments. However, a few ISG females (6/66 or 9%) had the tip of filaments similar to the CM type. In addition, variation in the apex of the filaments was observed in both strains where a few filaments (up to 7, usually 1–3) had bifurcated or more rarely fimbriated ends (Figs. 3, 6). This variant is more common in the CM strain (34/64 or 53%) than in the ISG strain (19/66 or 29%). The shape of the cone filaments, including the occurrence of bifid or fimbriated ends, can also be distinguished under the light microscope (Figs. 7–8).

The armature of 14 and 12 females of *An. minimus* species A and C, respectively, that had been identified by other authors (i.e., Green et al. 1990, Sharpe et al. 1999, Van Bortel et al. 1999) were examined. Several wild-caught *An. minimus* s.l. females from Thailand and a few from Yunnan, southern China, were also examined. All exhibited lancetlike cone filaments similar to the CM type. Some, particularly those of Green et al. (1990), showed bifid or fimbriated ends, as found in several CM females.

Metaphase karyotype: More than 40 larvae of each of the CM and ISG strains were examined for metaphase karyotypes. They were uniform in chromosome number (2n = 6), consisting of 1 pair of heteromorphic sex chromosomes and 2 pairs of autosomes similar to other members in the Myzomyia Series reported by Baimai et al. (1996a). However, some variation was observed in the sex chromosomes and in the pericentric heterochromatin of autosomes as follows.

a) The CM strain (Figs. 9–16). Female larvae of this strain exhibited short and/or long submetacentric X chromosomes. The shorter X chromosome had a ratio of short to long arms of about 1:2 and the longer chromosome 1:2.5 to 1:3 (Fig. 9), which are comparable to X_1 and X_2, respectively, de-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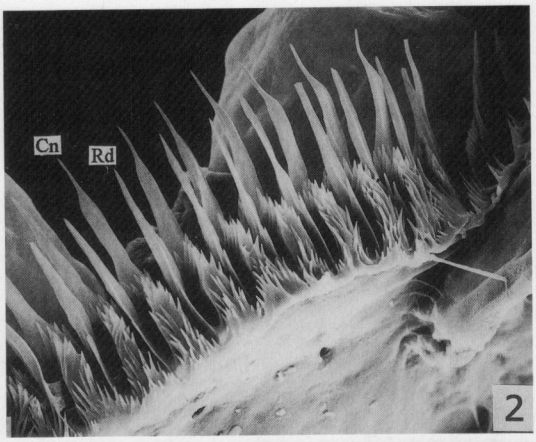
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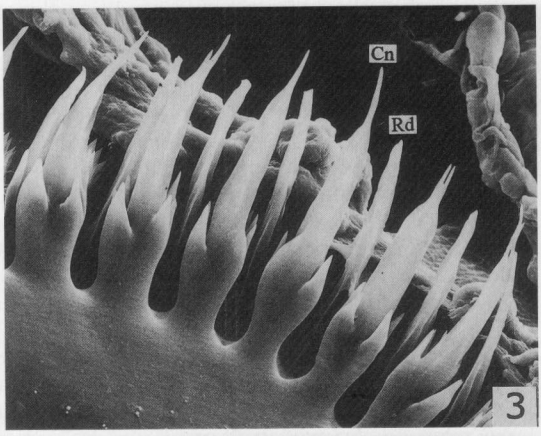
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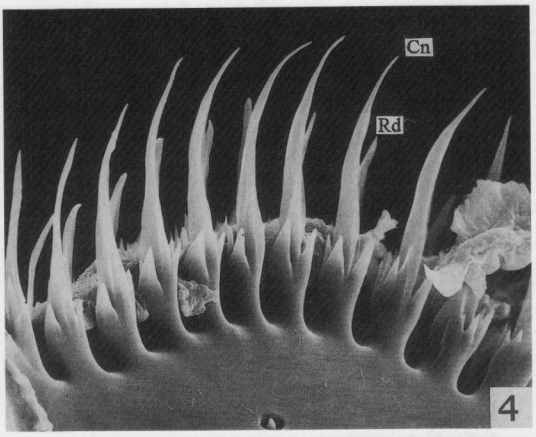
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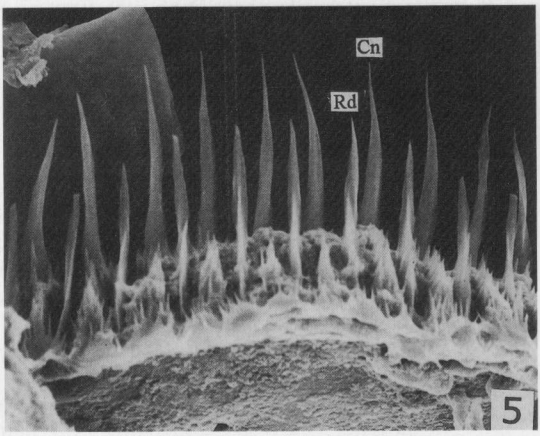
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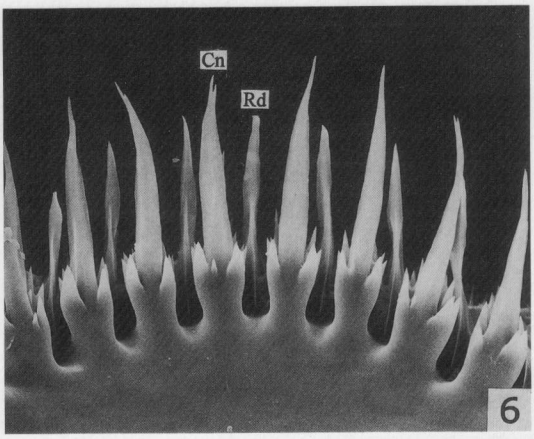
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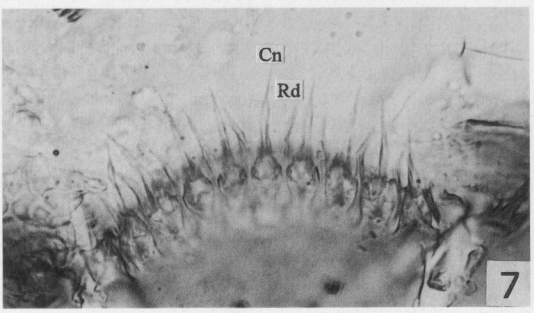
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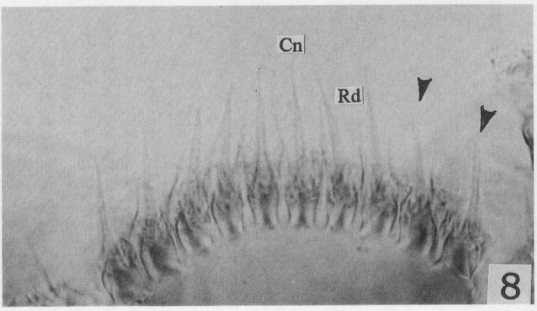
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scribed by Baimai et al. (1996a). In addition, some larvae exhibited X chromosomes having a smaller heterochromatic area at approximately the middle of the long arm (Figs. 10–12). This variation was common in the short X chromosome, but rare in the long. Because the meaning of this variation is not yet known, it is preliminarily called X_{1a} or X_{2a}, depending on the length of the chromosomes. In male larvae, the X_1, X_2, or X_{1a} chromosomes were observed. There were short and long Y chromosomes (Figs. 13–16). The short chromosomes have a ratio of short to long arms of about 1:3, comparable to the submetacentric Y_1 chromosome previously reported by Baimai et al. (1996a). The long chromosome has a ratio of 1:4, being somewhat subtelocentric (acrocentric) in appearance. Because this variation has not been reported previously, it is designated as Y_3 (Y_2 is described in *An. minimus* species C). In good preparations, the submetacentric autosomes usually exhibit a conspicuous block of pericentric heterochromatin in the short arm, whereas the metacentric autosomes usually have a small amount of pericentric heterochromatin in both arms.

b) The ISG strain (Figs. 17–24). Short and long submetacentric X chromosomes were observed in female larvae. Based on the ratio of short and long arms, they are comparable to the X_1 and X_2 chromosomes mentioned above. In addition, some larvae exhibited short X chromosomes with a lesser amount of heterochromatin or a small euchromatin block near the distal end of the long arm (Figs. 17–18, 22). This type of chromosome is preliminarily called X_{1b}. In male larvae, X_1, X_2, or X_{1b} chromosomes were observed. There were 2 forms of Y chromosome comparable to the Y_1 or Y_3 as found in the CM strain (Figs. 20–24). The autosomes look similar to those of the CM strain.

Figure 25 shows a diagrammatic representation of Giemsa-stained mitotic karyotypes of the CM and ISG strains observed in this study.

Crossing experiments: Crosses carried out between the CM and ISG strains resulted in hybrid progeny from only the CM females × ISG males (Table 2). There was a high rate of adult emergence, apart from 1 cross that showed a rate of only 11.3% (11/97). The sex ratio of each cross was 1:1 (all $P > 0.05$). Morphologically, hybrids having a humeral pale spot on at least 1 wing were present in all crosses, but in varying proportions ranging from 10–70% (overall 43%) in the females and 10–63% (overall 32%) in the males. Dissection of 82 F_1 hybrid males (5–7 days old) revealed that 85.4% were completely sterile with atrophied testes (Fig. 26). The remaining had spermatozoa, but most were inactive and had an enlarged head (Fig. 27). Their accessory glands looked normal, but the vas efferens were very fragile, often causing the testes to be detached during dissection. These males failed to inseminate, although they succeeded in copulating with females when subjected to force mating. Thus, no F_2 hybrids were obtained. The ovaries of the hybrid females looked normal. When backcrossed to either CM or ISG males, they produced eggs with low embryonation and hatching rates (Table 3). A high mortality was observed among 1st-stage larvae. A total of 10 females and 15 males were obtained from the backcrosses, but half of the females died within 5 days. Four of 8 males dissected had spermatozoa, some of which had an enlarged head. Further crosses among the backcross progeny yielded a total of 146 eggs from 2 females, but the overall embryonation rate was 32.9% and the hatching rate was 6.8%. Further crosses were not attempted.

More than 20 F_1 hybrid larvae from 5 of 7 crosses (CM × ISG F_1, Table 2) were examined for polytene chromosomes. No asynapsis was observed (Fig. 28).

Study of mating behavior showed that the adults of the 2 strains mate readily in 30-cm cages. The insemination rates of ISG female (F_6) × CM male and CM female × ISG male (F_6) crosses were 81.9% (50/61) and 80.3% (53/66), respectively. These were as high as the insemination rate observed in the CM control cage (86.0%, 74/86), but higher than was observed in the ISG control cage (56.1%, 23/41).

DNA sequence analysis: The D3 region of the rDNA has been used as the basis for a species identification method for *An. minimus* species A and C from Thailand and other members of the Minimus Group (Sharpe et al. 1999) and is therefore potentially informative of the species status of the ISG strain. D3 rDNA sequence data was obtained from the ISG strain; the CM strain; *Anopheles flavirostris* (Ludlow) from Lombok Island, Indonesia; and *An. minimus* from Hoa Binh Province, Vietnam (kindly provided by Wim Van Bortel), which was included for comparison (Table 4). Figure 29 shows a phylogenetic tree of this data in the context of previous sequence data from the *An. minimus* group, using *An. flavirostris* and *An. aconitus* Doenitz as outgroups. Both parsimony and distance-based meth-

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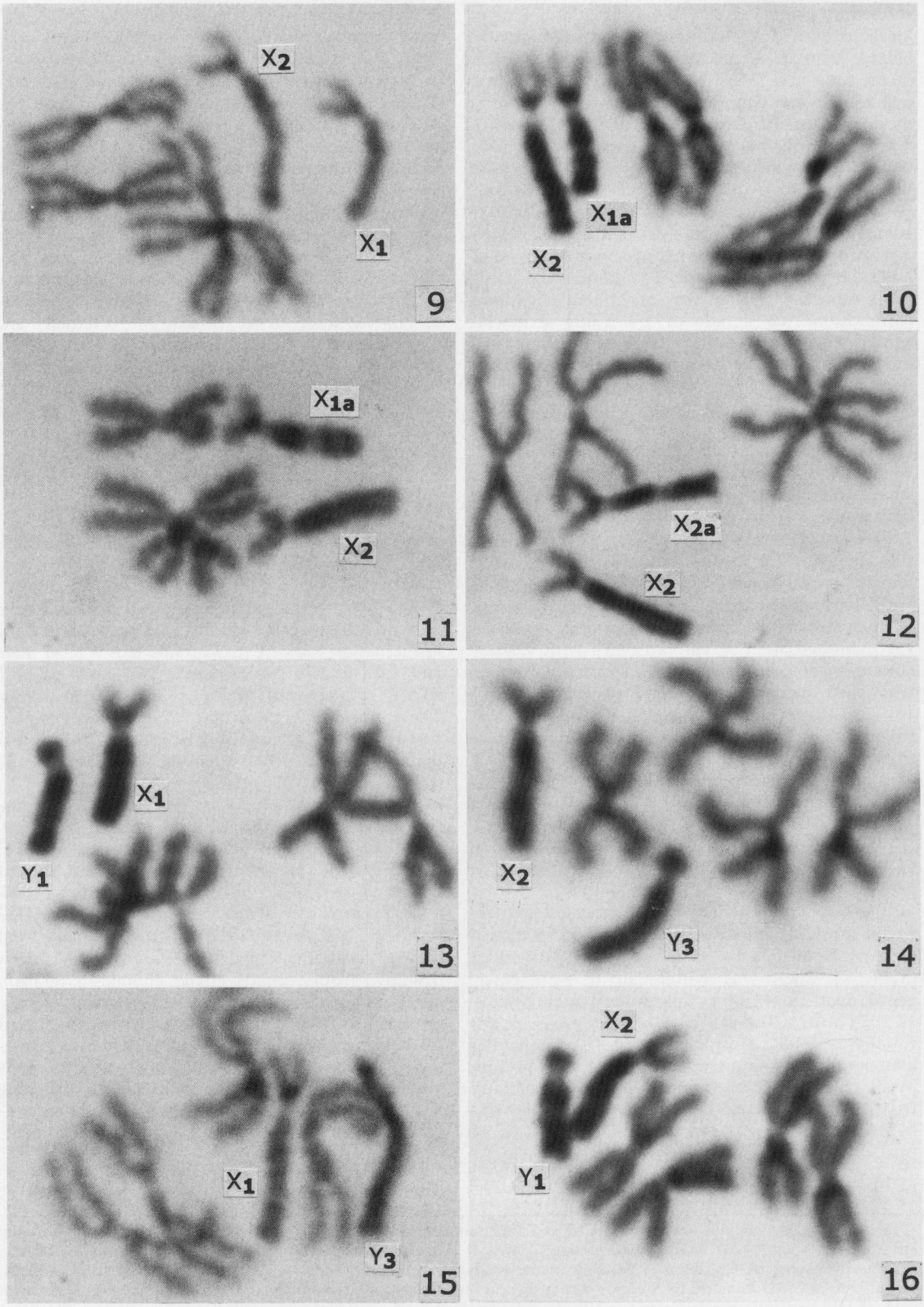
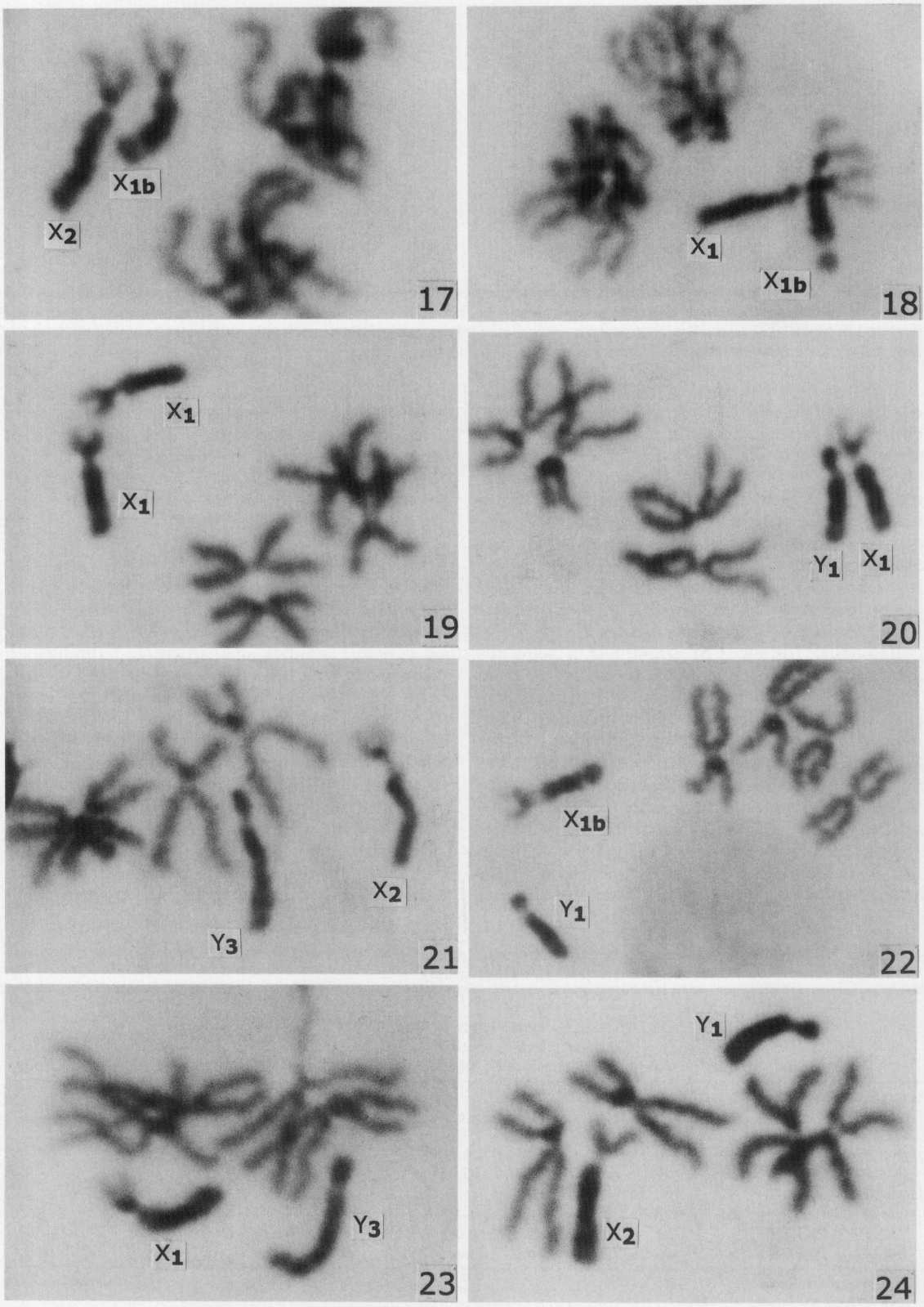
Figs. 1–8. The cibarial armature of CM and ISG females of *Anopheles minimus* examined under the scanning electron microscope (1–6) and light microscope (7–8). 1, 2. Anterior and posterior aspects, respectively, of CM females. 3. Anterior aspect of CM female showing a few filaments with bifid ends. 4, 5. Anterior and posterior aspects, respectively, of ISG females. 6. Anterior aspect of ISG female showing cone filaments with bifid ends. 7–8. Anterior view of CM and ISG females, respectively; the bifurcated ends of cone filaments are indicated by arrows. Note that the scanning micrographs are present in different magnifications. Cn, cone; Rd, rod.



Figs. 9–16. Metaphase karyotypes from larval neuroblast cells of *Anopheles minimus,* CM strain. 9–12. Female. 13–16. Male.



Figs. 17–24. Metaphase karyotypes from larval neuroblast cells of *Anopheles minimus,* ISG strain. 17–19. Female. 20–24. Male.

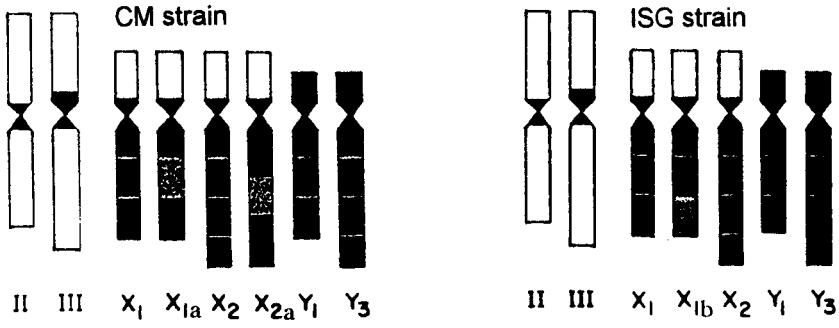


Fig. 25. Diagrammatic representation and comparison of Giemsa-stained metaphase karyotypes of CM and ISG strains of *Anopheles minimus*. Only 1 set of autosomes II and III is presented. Variable heterochromatic portion is depicted in black or shaded. The centromeres are indicated by constrictions of each chromosome. Chromosome lengths, arm ratios, and heterochromatic portions are shown in proportion.

ods gave a tree of consistent topology. All the *An. minimus* s.l. sequences form a well-supported clade, to which *An. flavirostris* is external. The bootstrap values within the *An. minimus* s.l. clade reflect the limited amount of phylogenetic information in the sample (because of the close relationships between *An. minimus* species) rather than indicating a conflict between characters. Within the *An. minimus* s.l. clade, the sequences fall into 2 clades, one of which contains *An. minimus* species A and the other *An. minimus* species C.

Individuals of the CM strain had a sequence identical to that of *An. minimus* species A from Thailand and Vietnam reported previously (Sharpe et al. 1999), confirming that this strain was *An. minimus* species A (Fig. 29). Both individuals of the ISG strain had the same sequence as each other, but this differed from all other sequences. One of the Vietnamese samples had the same sequence as *An. minimus* species C from Thailand. The other 2 Vietnamese specimens fell within the clade containing *An. minimus* species A. These sequences not only differed from each other, but also from *An. minimus* species A from Thailand and prior samples from Vietnam (Sharpe et al. 1999). The implications of the 3 novel sequences observed (1 from the Japanese ISG strain and 2 from Vietnamese mosquitoes) are considered further in the discussion.

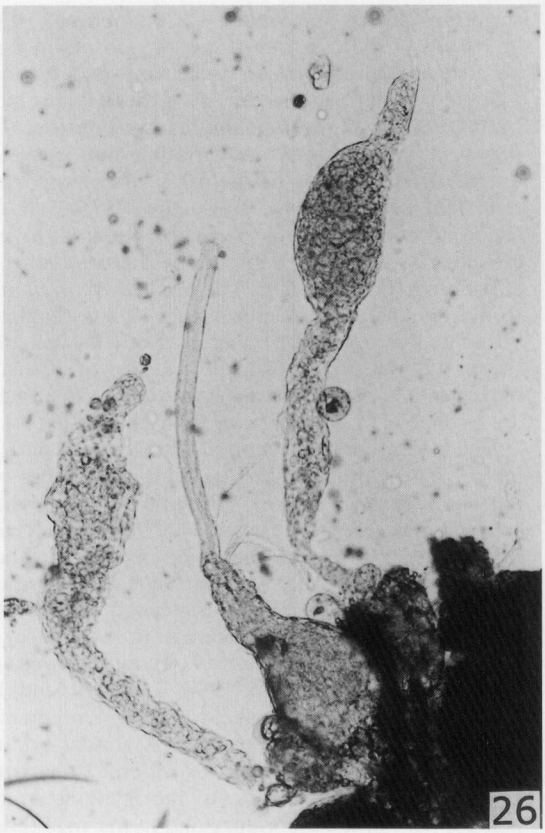
DISCUSSION

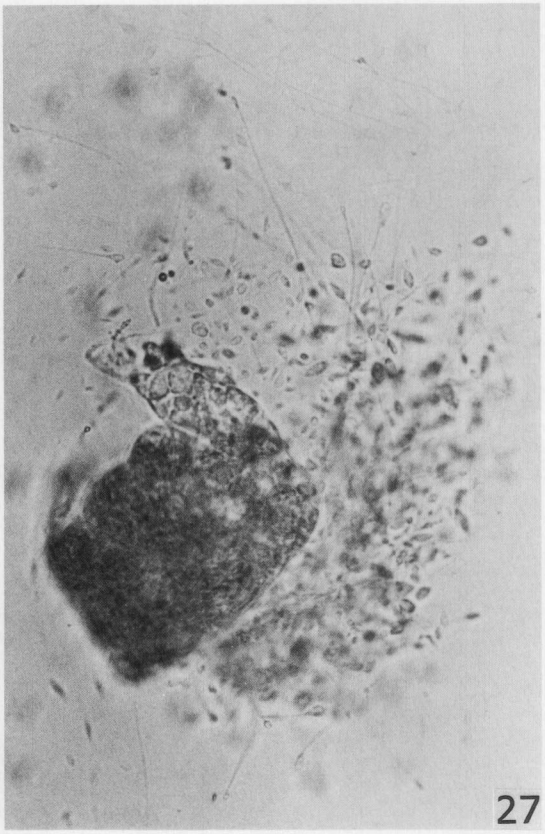
In an attempt to determine the specific status of *An. minimus* ISG, we have made extensive genetic and morphological comparisons with species of the *An. minimus* complex and carried out hybridization experiments with *An. minimus* species A. In agreement with previous observations (Tanaka et al. 1979), we found that *An. minimus* mosquitoes in Ishigaki Island and its neighboring islands are often characterized by the presence of both a humeral pale spot (86–100% of individuals) and a pale fringe spot at the tip of vein 1A (67–92% of individuals) on the wing of the adult. The humeral pale spot is also common in *An. minimus* species C and in the closely related species *Anopheles aconitus*, *Anopheles jeyporiensis* James, and *Anopheles pam-*

Table 2. Results from interstrain crosses between *Anopheles minimus* from Thailand (CM strain) and Japan (ISG strain).[1]

Crosses[2]	No. of broods	Eggs		No. of eggs examined	Embryonation rate	Hatching rate[3]	Pupation rate[3]			Emergence rate[3]		
		Total	Average/brood				Female	Male	Total	Female	Male	Total
Interstrain crosses												
CM × ISG F_1	7	729	104.1	n.d.	n.d.	94.8	44.7	45.0	89.7	35.6	35.8	71.4
ISG F_1 × CM	7	530	75.7	276	27.2	0						
ISG F_3 × CM	3	330	110.0	301	26.2	0						
ISG F_5 × CM	12	757	63.1	757	15.9	0.4[4]						
Control crosses												
CM × CM	5	458	91.6	n.d.	n.d.	97.8	39.9	41.5	81.4	38.6	37.1	75.7
ISG F_1 × ISG F_1	3	251	83.7	n.d.	n.d.	92.8	45.8	43.8	89.6	40.2	38.2	78.4
ISG F_5 × ISG F_5	5	342	68.4	n.d.	n.d.	90.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[1] n.d., not determined.
[2] All crosses are female × male.
[3] Each rate was calculated by (total number of individuals that reached each developmental stage)/(total number of eggs) × 100.
[4] All larvae died shortly after hatching.





Figs. 26–27. Testes of F_1 hybrid males from CM female × ISG male, showing atrophied testes without spermatozoa (26) and a ruptured testis with enlarged-head spermatozoa (27).

panai Buttiker and Beales. The pale fringe spot is absent or very rare (less than 2%) in *An. minimus* from Hong Kong and Thailand, but common in the related species *An. aconitus* and *Anopheles filipinae* Manalang (Harrison 1980). We therefore consider both the humeral pale spot and the pale fringe spot to be prominent characteristics of *An. minimus* on Ishigaki Island.

The eggs and immature stages of the ISG as well as Thai Chiang Mai (CM) *An. minimus* resemble the general descriptions of *An. minimus* s.l. (Reid 1968, Harrison 1980). Sucharit et al. (1995) reported that *An. minimus* species A and C could be simply differentiated by egg morphology, with the former having a complete deck and the latter an incomplete deck. However, although most eggs of species A (CM) had a complete deck in our study, some did not. Therefore, we did not find this character to be diagnostic for species A. Similarly, the ISG strain of *An. minimus* was found to be variable for the complete deck egg form. It is known that some species such as *Anopheles sinensis* Wiedemann exhibit various forms of eggs (Beales 1984). The validity of this egg characteristic for identifying members of the *An. minimus* complex should be reinvestigated.

Scanning micrographs of female cibarial armature were applied in this study to differentiate sibling species in the *An. minimus* complex. The shape of the cone filaments of *An. minimus* from Ishigaki Island, with a few exceptions, clearly differs from those of specimens of *An. minimus* species A and C examined. Structures of the cibarial armature have been found to be useful for reconstructing the phylogeny of *Anopheles* mosquitoes (Christophers 1933, Anthony et al. 1999) and also for subgeneric or specific diagnosis of *Culex* species (Sirivanakarn 1978). We recently used this character to support the specific status of *An. flavirostris* (Somboon et al. 2000), a related species that was considered to be a subspecies of *An. minimus* Theobald until it was elevated to species status by Baisas (1957). Christophers (1933) and Reid (1968) described the character of cone filaments of *An. minimus* observed under the light microscope. However, there is confusion because in the texts of both books the filaments are clearly characterized as "thorn-like without fimbriated or bifurcated ends," but in the figures of Christophers (1933, p. 213), reproduced by Reid (1968, p. 315), the filament is bifurcated at the apex. The filaments of the ISG females, but not the CM strain, appear comparable to the de-

Table 3. Results from fertility tests of backcrosses between the hybrids and the parental strains and of the (CM × ISG)F_1 hybrids.[1]

Crosses[2]	No. of broods	Eggs		No. of eggs examined	Embryonation rate	Hatching rate[3]	Pupation rate[3]			Emergence rate[3]		
		Total	Average/brood				Female	Male	Total	Female	Male	Total
(CM × ISG)F_1 × CM	15	1479	98.6	1327	21.8	1.0[4]	0.2	0.3	0.5	0.1	0.2	0.3
(CM × ISG)F_1 × ISG F_2	5	505	101.0	487	49.7	10.3[5]	2.2	3.6	5.8	1.8	2.4	4.2
CM × (CM × ISG)F_1	7[6]	669	95.6	609	0	0						
(CM × ISG)F_1 × (CM × ISG)F_1	5[6]	435	87.0	411	0	0						
CM × CM (control)	3	241	80.3	n.d.	n.d.	96.7	39.8	38.2	78.0	37.3	35.7	73.0
ISG F_2 × ISG F_2 (control)	2	172	86.0	n.d.	n.d.	88.4	37.8	43.0	80.8	32.0	33.7	65.7

[1] n.d., not determined.
[2] All crosses are female × male.
[3] Each rate was calculated by (total number of individuals that reached each developmental stage)/(total number of eggs) × 100.
[4] Hatching observed in 6 of 15 broods; 40.0% of 1st-stage larvae died within 1–2 days after hatching.
[5] Hatching observed in 4 of 5 broods.
[6] No sperm were observed in the spermathecae of any of these females.

scriptions of *An. minimus* by Christophers (1933) and Reid (1968). In our study, we observed both pointed and bifurcated or fimbriated ends of cone filaments in both strains. Because these forms have been detected in several families, we consider that they exhibit intraspecific variation and that the overall shape of the filaments is more important than that of the apex. Yu Yuan (1987) used the shape of cone filaments observed under light microscopy as one of the important characteristics in describing 'Form A' and 'Form B' of *An. minimus* from Hainan Island, China. Form A has filaments bifurcated at the apex, whereas Form B has filaments without the fimbriated end. However, insufficient details of the cibarial armature of the 2 forms are available for comparison with our results.

The size of wild *An. minimus* mosquitoes on Ishigaki Island varies seasonally, with mosquitoes being significantly larger in the winter than in other seasons. No seasonal variation in the size of wild *An. minimus* mosquitoes has been reported in Thailand or other tropical countries. Such physiological changes seem likely to be associated with hibernating behavior, which is common in subtropical or temperate anophelines such as *Anopheles quadrimaculatus* Say (Lanciani 1992, Lanciani and Le 1995) because of the effects of low temperature coupled with short photoperiods. A similar finding has been observed in *Anopheles saperoi* Bohart and Ingram, a species endemic to the Ryukyu Archipelago, both in the laboratory and in the wild (Higa et al. 1998). In the laboratory and under similar rearing conditions, a significant difference in the size of female mosquitoes between the ISG and CM strains was found. This is evidence for a significant biological difference between *An. minimus* species A and mosquitoes from Ishigaki Island, with the latter being adapted to subtropical conditions.

From morphology alone, *An. minimus* ISG appears to be distinct from the available descriptions of species A and C, although there is no unique character or set of characters that are peculiar to the ISG strain. Polytene chromosomes are often a very powerful means to identify mosquito species (e.g., Coluzzi et al. 1979, Baimai et al. 1988). However, we confirmed the findings of Kanda et al. (1984) that there are no differences in banding pattern between *An. minimus* A and C or the ISG strain and no asynapsis between any of the hybrids. The metaphase karyotypes of the ISG *An. minimus* are far different from those of species C, but somewhat resemble those of the CM strain and species A reported by Baimai et al. (1996a), with a few exceptions. Minor differences of the karyotypes have been observed between the CM strain and *An. minimus* species A reported by Baimai et al. (1996a), probably, in part, because of differences in geographical origins. It is known that differences in metaphase karyotypes do not necessarily reflect interspecies difference, mainly because of chromosome polymorphism that is evident in many *Anoph-*

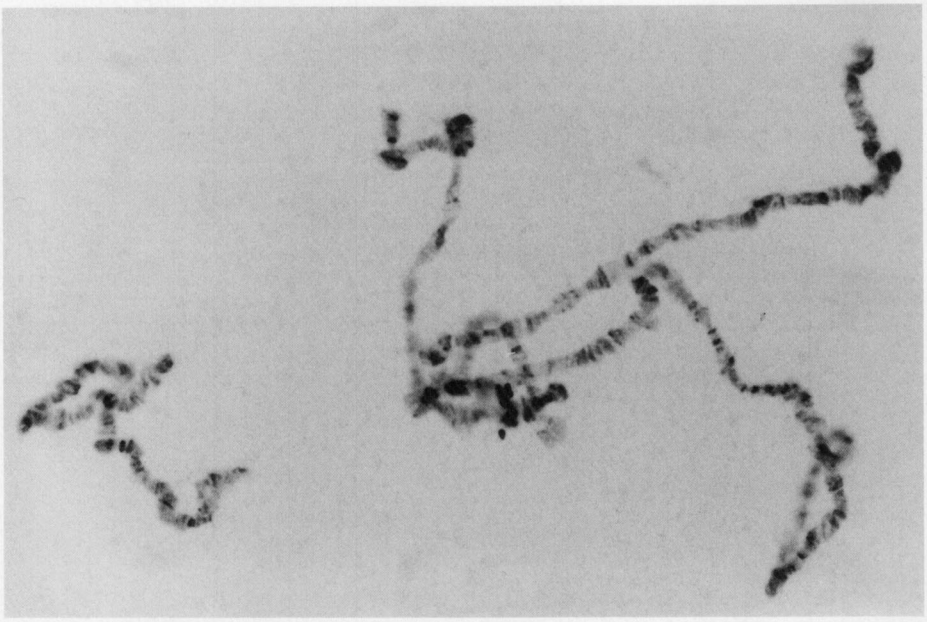


Fig. 28. The salivary gland polytene chromosome of F_1 hybrid larvae from CM female × ISG male.

eles species (Baimai et al. 1995, 1996a, 1996b; Adak et al. 1997). Therefore, although the metaphase karyotypes provide further evidence to support the specific status of *An. minimus* from Ishigaki Island, they do not provide conclusive proof.

The evidence of reproductive isolation from our hybridization experiments between *An. minimus* from Ishigaki Island (ISG strain) and *An. minimus* species A (CM strain) strongly indicates that they are distinct species. Although we found strong postzygotic isolation between the CM and ISG strains, the mating behavior experiments indicated no prezygotic isolation. However, the lack of mating barriers in the cages could simply be because these 2 species do not naturally encounter each other. Further crossing studies with other species/strains are clearly essential to establish species status. In addition, our crossing experiments revealed no postmating barriers between *An. minimus* from Ishigaki and Iriomote Islands (Somboon et al., unpublished data). This suggests that *An. minimus* populations on the 3 islands of the Ryukyu Archipelago are the same species.

The evolutionary relationships inferred from the rDNA sequences (Fig. 29) indicate that crossing studies between ISG and *An. minimus* species C would be of particular interest. The clustering of ISG within the same clade as *An. minimus* species

Table 4. Alignment of all available D3 rDNA sequences of *Anopheles minimus, An. flavirostris,* and *An. aconitus,* showing site substitutions numbered from the start of the sequence. The new sequences have been deposited in the EMBL Nucleotide Sequence Database with the accession numbers AJ401277–81. A dot indicates identity with the reference sequence of *An. minimus* species A; a dash indicates a deletion; Y is the ambiguity code for C and T. An asterisk indicates that sequences were taken from Sharpe et al. (2000).

	Site number
	11111111111222
	29334445556677788024555557793 49
Species/strain (country)	89247890172902757188245674755 86
An. minimus A (Thailand*/Vietnam*)	AAGTCCATGACGAGAGGAAC-CC--TAGTAG
An. minimus CM (Thailand)	·····
An. minimus Form I (Vietnam)	·····A·····
An. minimus Form II (Vietnam)	·····GA
An. minimus ISG (Japan)	·····TA···T·····C·····
An. minimus #157 (Thailand*)	·····T·····C·····
An. minimus C (Thailand*)	·····AC··T·····T···C·····
An. minimus Form II (Vietnam)	·····AC··T·····T···C·····
An. flavirostris (Indonesia/Malaysia*)	·····T·····CTATG·G··AT·G····
An. aconitus (Thailand*)	--TGYTCGAT·····T·TGTT--AC··TC··

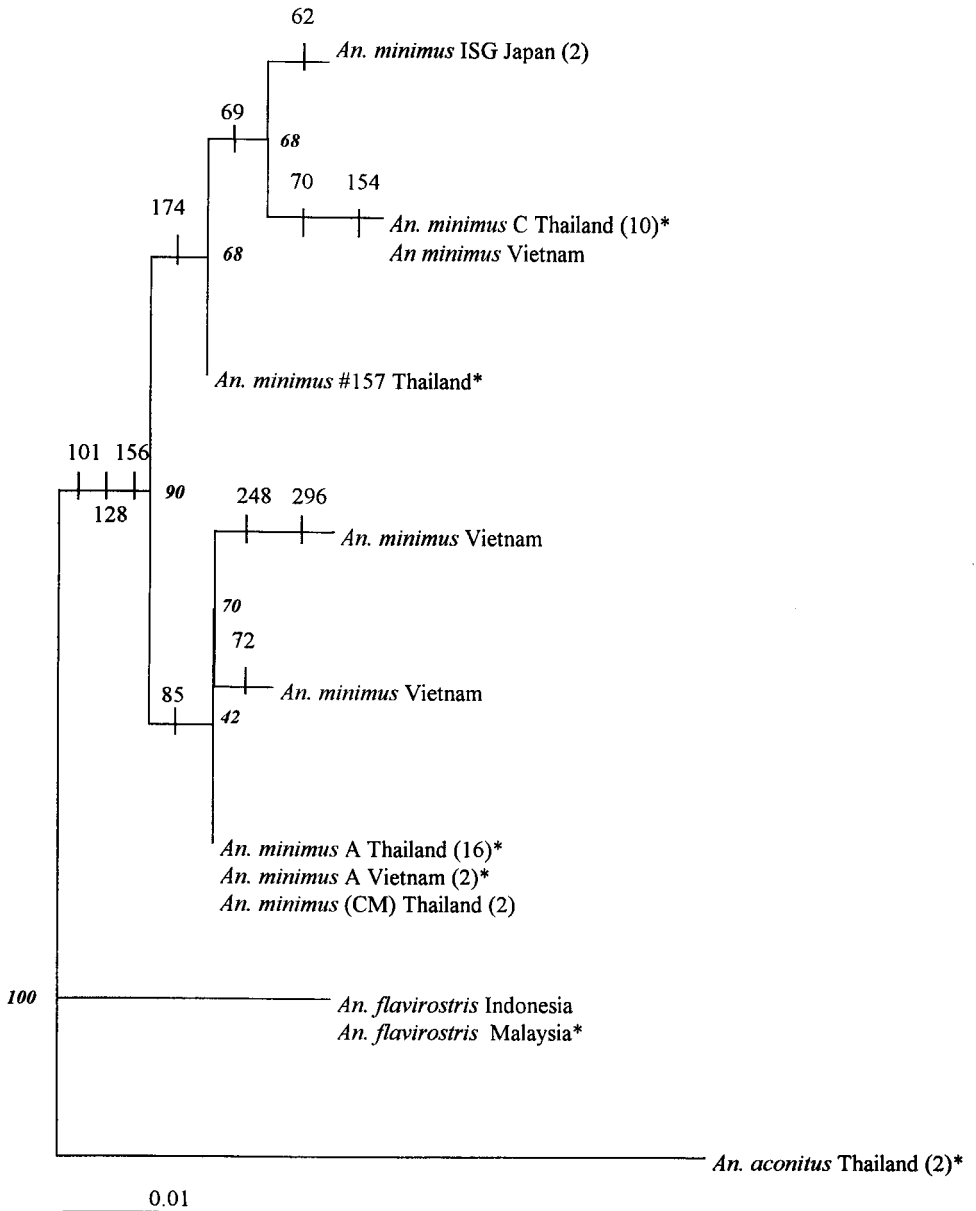


Fig. 29. A neighbor-joining tree of the D3 region of rDNA for all available *Anopheles minimus* sequences using *An. aconitus* and *An. flavirostris* as the outgroup. When greater than 1, the number of individuals with the same sequence is given in brackets. An asterisk indicates the sequences taken from Sharpe et al. (2000). Numbers in bold italic at nodes are percentage values of 1,000 bootstrap pseudoreplicates with parsimony analysis. For the *An. minimus* s.l. clade, the character changes inferred by parsimony analysis are indicated by the number of the corresponding site (Table 4) along the branches. The scale bar indicates the estimated genetic distance (percent).

C indicates that *An. minimus* ISG is more closely related to species C than to species A. Differentiation between the 2 clades is based on only 2 characters, so further DNA sequence data are required to confirm this inference. Although rDNA can be very informative of species status (Walton et al. 1999), geographically isolated populations of the same species could also become fixed for different sequence variants. Consequently, although the sequence data concur with morphological and mitotic karyotype data, which suggest that ISG is a separate species, the possibility of being a geographically isolated population of *An. minimus* species C cannot be excluded on the basis of sequence data alone. However, ongoing isozyme studies of *An. minimus* larvae from Ishigaki Island have revealed

the absence of *Odh*[134] alleles (Green et al. 1990) or *Odh*[133,142] alleles (Van Bortel et al. 1999), which are regarded as the markers of *An. minimus* species C (K. Sawabe, personal communication), suggesting that ISG is distinct from *An. minimus* species C. In addition, *Odh*[100] alleles, which are regarded as the markers of *An. minimus* species A (Green et al. 1990), are common in *An. minimus* on Ishigaki Island.

The D3 sequence data obtained from specimens from Vietnam that were included for comparison yielded very surprising results. Because the 2 novel sequences from the Vietnamese specimens fall within the clade of the *An. minimus* complex, they must be from individuals of *An. minimus* s.l. and not from other closely related species. *Anopheles flavirostris,* for instance, which is considered to be the most closely related species to *An. minimus* s.l., is positioned externally at some distance from the *An. minimus* sequences. Unfortunately, the small number of samples from Vietnam makes these results difficult to interpret. There appear to be 2 possible explanations. The first of these is that *An. minimus* species A and/or *An. minimus* species C in Vietnam could have a large amount of intraspecific variation in rDNA. Alternatively, each sequence could come from an individual of a different species.

Although the first explanation cannot be discounted at this stage, the second hypothesis seems to us more likely, particularly when a comparison is made with the situation in Thailand. In Vietnam, there would have to be 4 distinct variants of the D3 rDNA sequence in a geographically restricted area associated with only 2 species of *An. minimus.* In Thailand, however, all samples had either the sequence of species A or the sequence of species C over a greater geographical range (Sharpe et al. 1999). The only exception to this was individual #157 from Thailand (Fig. 29), which it was suggested may be from another species. Although the sample size is too small to be conclusive, the 4 individuals from Vietnam are all homozygous, consistent with each sample originating from a reproductively discrete population. Furthermore, the sequences that appear to be definitive for *An. minimus* species A and C in Thailand are also present in Vietnam, suggesting intraspecies continuity between Thai and Vietnamese populations of *An. minimus* species A and C. In an extensive allozyme study of Vietnamese *An. minimus* in Hoa Binh Province, Vietnam (Van Bortel et al. 1999), mosquitoes were classified as form I or form II according to their *Odh* type. Form I was equated with *An. minimus* species A and form II with *An. minimus* species C. However, even after separation into form II, a significant deficit of heterozygotes within populations (F_{IS}) was still observed at 2 loci (*Ldh* and *Gpi*). By contrast, at the *Ldh* locus (*Gpi* not studied) in Thailand, no such deficiency was found within *An. minimus* species A or C (Green et al. 1990). We interpret this as suggesting that the form II classification in Vietnam contains another species in addition to *An. minimus* species C.

This study presents evidence for the existence of another sibling species within the *An. minimus* complex in addition to the 2 known species (A and C). The Japanese species more closely resembles Thai species A in terms of egg morphology and metaphase karyotype but Thai species C in terms of wing morphology and rDNA sequence. Based on the evidence presented in this paper, the Ryukyu species is provisionally designated *An. minimus* species E to avoid confusion with Form B from China (Yu Yuan 1987) and unpublished evidence for species D from Thailand (Baimai 1989). Although Form B of Yu Yuan (1987) has been elevated to the status of *An. minimus* species B by Sucharit et al. (1988), this rests primarily on some morphological characteristics of larvae and adults that are the same as those described for *An. minimus* s.l. by several authors. More work is therefore required to investigate the specific status of Form B and species D. We also tentatively suggest that there could be up to 4 species in Vietnam and that, consequently, *Anopheles minimus* is a complex of several species. It is important that these species be discriminated so that their individual roles in disease transmission can be studied. This work illustrates the importance of collaboration in order to allow analytical methods to be applied simultaneously to mosquitoes from different geographic regions or putative species, thereby avoiding some of the difficulties and dangers inherent in making between-study comparisons.

ACKNOWLEDGMENTS

We thank Rampa Rattanarithikul and Wim Van Bortel, for providing specimens of *An. minimus* species A and C and useful information, and Kyoko Sawabe for providing the enzyme electrophoresis information. Thanks are also extended to Ralph E Harbach for comments on the manuscript. This work was supported by Grant-in-Aid for Scientific Research C(2) 60024684.

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