CHAPTER II

LITERRATURE REVIEW

1. Techniques used in the identification of species complexes

Populations within a species sometimes exhibit distinct differences with reference to resting habitats, preference to feed on a host, the rate of development of resistance to insecticides, susceptibility to acquiring infection, and so on. All these differences may indicate the presence of isomorphic species within a taxonomic species (in the traditional concept), but these differences cannot confer species status to populations. Hence, genetic techniques that can demonstrate reproductive isolation in the natural populations are needed. Unequivocal evidence for isomorphic or cryptic species comes from population genetic data generated from sympatric samples. It is for this reason that chromosomal variations and electrophoretic variations at enzyme loci, which provide excellent population genetic evidence, have been extensively used in studies to recognize species complexes. Where laboratory colonies or allopatric populations have to be tested for species status, crossing experiments are used. Sibling species by definition are species with obscure morphological differences. However, a careful examination may sometimes reveal morphological differences that are minute and may be restricted to a particular stage in the life cycle. White (1977) says that morphological studies should not come too early in the process of detecting anopheline sibling species, since it might be

misleading to characterize taxa which have not been identified by trustworthy techniques like cross-breeding experiments, cyto or biochemical characterizations.

1.1. Morphological variation

Morphological characters that are often used to identify adults of anopheline species are largely confined to elements of scale, pattern and colour, and their distribution. Characters that are used in the description of immature stages are sculpture of eggs, setation and pigmentation of larvae, and the forms of paddles and trumpets, as well as chaetotaxy of pupae. Spermatheca and spiracular morphology are also used in the identification of species. In addition to light microscope examination for the specific characters, scanning and transmission electron microscopes are also used to study morphological variations. Morphometric has proved useful in studying some species complexes, when used in conjunction with statistical analysis.

The morphological variations of *An. minimus* have been extensively studied in populations from Thailand (Harrison, 1980). These variations included (1) proboscis with small ventral pale patch; (2) palpus with distal third or club pale or nearly so; (3) fluviatilis-like palps: Costa with prehumeral and/or humeral pale spot (one), Costa with presector pale spot (both); (4) R sector and accessory sector pale spots separate (one); (5) R_2 and/or R_3 with median pale spot (both); (6) R_{4-5} with basal dark spot (one); (7) Cu fork pale (one) (8); Cu_1 with one long dark mark beyond m-cu crossvein (both); (9) 1A with 3 dark spot (both) and (10) 1A with pale fringe spot (both).

Sucharit *et al.* (1988b) identified three morphologically variant forms in the Thai population : Typical minimus form (M form) – wing with presector pale spot

(PSP) spot, Varuna form (V form) – wing with completely dark prehumeral and humeral bands in costa, and Pampanai form (P form) – wing with presector pale spot and humeral pale spot (HP). In addition, the differentiation of morphology could be used to identify the *An. minimus* into two groups: V form and M form wing types fixed in *An. minimus* species A and the P form wing types fixed in *An. minimus* species C. However, The use of these morphological characteristics to identify the two *An. minimus* species from Thailand led to 37% error (Green *et al.*, 1990) and to 33% error in Vietnam (Van Bortel *et al.*, 1999). In addition, Chen *et al.* (2002) found that the presence of HP spots cannot be used with any degree of confidence to differentiate species C from species A, and the PSP spot is also unreliable for species differentiation.

Yu and Li (1984) described *An. minimus* forms A and B from Hainan Island, China based on morphological characters of larvae, pupae and adults. In addition, Yu Yuan (1987) reported these forms in several provinces of China (Fujian, Guangdong, Guangxi, Yunnan) and noted differences in their esterase electromorphs. In addition Sucharit *et al.* (1988b) had elevated the status of *An. minimus* form B of Yu Yuan (1987) to species B, but the morphotypes A and B have no taxonomic significance, and these "forms" are nothing more than morphological variants of the same biological species (Chen *et al.*, 2002).

1.2 Biochemical divergence

Enzyme electrophoresis is being extensively used in the studies of species complexes. The technique involves the detection of protein bands of an enzyme system with different mobilities as a function of electric charge and molecular structure. On a gel zymogram of an enzyme system, electrophoretic variations in the form of bands with different mobilities represent proteins coded by different alleles (allozymes). These alleles being codominant behave like paracentric inversions, and the two homozygotes and heterozygotes can be differentiated. Variations at a locus, thus, enable the detection of the reproductive isolation within populations resulting due to positive assortative matings within a population. Because of the simplicity of the procedures for the processing and interpretation of data, this technique permits a large-scale sampling of natural populations, and is very useful as a diagnostic tool in the routine identification of species.

Electrophoretic variations at enzyme loci are not only useful for the identification of isomorphic species but also useful for the correct identification of morphologically unidentifiable species. For *An. minimus*, Sucharit *et al.* (1988b) studied enzyme electrophoresis of seven enzymes from several locations in Thailand (Kanchanaburi, Phrae and Chanthaburi Provinces): aldehyde oxidase (Aldox), esterase (Est), leucine aminopeptidase (Lap), lactate dehydrogenase (Ldh), malate dehydrogenase (Mdh), malic enzyme (Me) and xanthine dehydrogenase (Xdh). It was reported that alleles at esterase-2 (*Est-2*) locus were found to be identified the *An. minimus* complex, *Est*-2^{100} and *Est*-2^{102} fixed in species A and *Est*-2^{98} fixed in species C.

Green *et al.* (1990) studied enzyme electrophoresis of seven enzymes in *An. minimus* mosquitoes from several locations in Thailand (Kanchaburi, Tak, Chiangmai, Uttaradit and Nakhon Si Thammarat Provinces): phosphogluconate dehydrogenase (Pgd), mannose phosphate isomerase (Mpi), glycerol dehydrogenase (Gcd), octanol dehydrogenase (Odh), hydroxyacid dehydrogenase (Had), lactate dehydrogenase (Ldh) and malate dehydrogenase (Mdh). It was found that the octanol dehydrogenase could be used to identify the *An. minimus*: species A presented Odh^{100} allele and species C presented Odh^{134} allele. Van Bortel *et al.* (1999) used the *Odh* locus to distinguish members of the *An. minimus* complex from closely related species in northern Vietnam. Sawabe *et al.* (1996) studied enzyme electrophoresis on three populations of *An. minimus* from China and found a genetic differentiation between them.

Baimai (1989) reported that C.A. Green and colleagues had recognized a third genetic species D in sympatry with species A and C in western Thailand (Ban Phu Rat, Kanchanaburi Province), based on electrophoretic data, but the sufficient information to support this species status is not yet available.

1.3 Hybridization or Crossing experiment

The assortative mating observed between sibling species in nature due to pre-mating barrier (s) generally breaks down in the laboratory and different species mate at random and produce hybrid progeny. Genetic differences between species are expressed in the form of non-viability of hybrid progeny at immature stages, hybrid sterility, or both. Hybrid males in one or both crosses are sterile and hybrid females

are fertile. Therefore, hybrid sterility is used as the criterion in designating populations as separate species. Hybrid males exhibit partial development of reproductive organs (the extent of development ranges from atrophied testes and vas deferent to fully developed testes but without sperm; accessory glands and ejaculatory duct are generally normal) and do not produce progeny when crossed. For species which do not mate in laboratory cages, artificial mating methods (Ow-Yang *et al.*, 1963) can be adopted. Thus, laboratory crossing experiments demonstrate post-mating barriers and establish the species status of the isomorphic populations. *An. gambiae* was first recognized as a species complex from results observed between two populations which were crossed to study genetics of resistance to an insecticide (Davidson and Jackson, 1962). It may be noted that though these post-mating barriers are studied between members of the complexes, they are not required to give populations species status. Further, species which exhibit a pre-mating isolating mechanism need not necessarily have any post-mating barrier, as has been observed between species B and C of the *Anopheles culicifacies* complex (Subbarao *et al.*, 1988b). F_1 hybrid males of reciprocal crosses between species B and C are fully fertile. Dobzhansky (1970) reports that viable and fertile hybrids may be obtained in experiments between undoubtedly distinct species that maintain complete reproductive isolation in nature. Therefore, the reproductive status of hybrid males cannot be diagnostic in the identification of species complexes.

Kanda *et al.* (1984) reported cytogenetic and hybridization studies among a strain of *An. minimus* from Ishigaki Island and two 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 three strains were all similar in their banding patterns, and complete synapsis was observed in the hybrids of all crosses. Sucharit *et al.* (1988a) reported the sterile hybrids from hybridization experiment between *An. minimus* M form (species A) and P form (species C) from Thailand.

Somboon *et al.* (2001) have described another sibling species of *An. minimus* from the Ryukyu Archipelago, Japan which was provisionally designated species E, based on several techniques including crossing experiment. The sterile testes or almost sterile with atrophied testes or abnormal spermatozoa in hybrid males occurred when species E was crossed with species A. In addition, F2 hybrid progeny were not obtained. Recently, Choochote *et al.* (2002) crossed *An. minimus* species A and C. Partial development of ovarian follicles in females adult from hybrid progeny were demonstrated whereas the hybrid males were fertile.

1.4 Polytene chromosomes

Polytene chromosomes are the result of repeated replication of chromosomes at interphase without nuclear division, the process being known as endomitosis. Chromatids after division remain attached, causing thickening of chromosomes which results in the appearance of long ribbon-like structures with dark and light horizontal portions representing band and interbrand regions, respectively. The dark and light regions represent differential condensation of chromosomes. The banding pattern of each chromosome is specific in a given species; thus each species differs from others in characteristic banding pattern. Any changes in the pattern can be easily detected.

Asynapsis in polytene complements in hybrids is used as one of the criteria in determining species status. The degree of asynapsis may vary, and thus has to be used with caution.

In the polytene chromosome complement, only euchromatic regions are seen and the heterochromatic portions of the chromosomes which are under-replicated are not seen. Therefore, in anophelines, the short arm of X chromosome and Y chromosome are not seen in the polytene complement. In some species a definite chromocentre is seen. In such cases, all the chromosome arms are seen attached to the chromocentre by their centromeric ends. Generally, this is not the case with anophelines, and chromosome arms are seen separately; occasionally the two arms of a chromosome are seen attached at the centromeric ends. Homologous chromosomes exhibit high affinity for pairing, hence are seen as a single chromosome.

In anopheline cytogenetics literature, two types of designations are seen for the polytene chromosome arms: (*i*) The two arms of a chromosome are designated as right (R) and left (L) arms. This system is followed by *Drosophila* cytogenetic and is adapted by many anopheline cytogeneticists. (*ii*) The new nomenclature for arm designation is that suggested by Green and Hunt (1980). In this, each arm is given a separate number 2, 3, 4 and 5 for autosomal arms and the euchromatic arm of the X chromosome seen in the polytene complement as X. Taking *An. gambiae* belonging to Pyretophorous series as an arbitrary standard, the two arms of chromosome 2, 2R and 2L are referred as 2 and 3 respectively and those of chromosome 3, 3R and 3L as 4 and 5 respectively, in the new nomenclature.

The preparation of polytene chromosomes from adult females is generally very simple in anophelines. Females in semi-gravid stage have the best polytene chromosomes in ovarian nurse cell (Coluzzi, 1968). Larvae at the IV instar stage have polytene chromosomes in salivary glands. For those anopheline species which do not have good ovarian polytenes, larval salivary chromosomes can be used, as, in general, salivary gland polytene chromosomes are not very good. The advantage with adult females is that ovaries can be removed and fixed in modified Carnoy's fluid (1 part of glacial acetic acid and 3 parts of methanol) and can be used any time. Another advantage is that the same female can be studied for other parameters like host preference and presence of sporozoites.

Recommended references for the preparation of polytene chromosomes are: for ovarian polytene chromosomes from adult females, Green and Hunt (1980), and for salivary gland polytene chromosomes from IV instar larvae, Kanda (1979). Hunt and Coetzee (1986) described storing of field-collected mosquitoes in liquid nitrogen for correlated cytogenetic, electrophoretic and morphological studies.

In *An. minimus*, similar banding patterns and complete synapsis polytene chromosomes from hybrids between *An. minimus* from Ishigaki Island and two strains (KCH-1 and KCH-2) from Kanchanaburi Province, Thailand were reported by Kanda *et al.* (1984). Similarly, Somboon *et al.* (2001) reported complete synapsis polytene chromosomes from hybrids between *An. minimus* from the Ryukyu Archipelago, Japan (species E) and species A from Chiang Mai Province, Thailand. Sucharit *et al.* (1988a) reported similar banding of polytene chromosomes from *An. minimus* M form (species A) and P form (species C) from Thailand. However, Choochote *et al.* (2002)

reported asynaptic salivary gland polytene chromosomes on 3L chromosome hybrid progeny of *An. minimus* species A (M and V form) and species C (P form) in Thailand.

1.5 Mitotic and meiotic karyotypes

All anophelines studied so far have three pairs of chromosomes, two pairs of autosomes which are either metacentric or submetacentric, and a pair of sex chromosomes: homomorphic (XX) in females and heteromorphic (XY) in males. X and Y chromosomes have been found as telocentric, acrocentric or subtelocentric, or submetacentric (depending on the position of centromere in the chromosome) in different species of anopheline. Levan (1964) is recommended for the nomenclature of chromosomes. The best mitotic chromosome plates are found in neurogonial cells of the brain in early IV instar larvae, and meiotic plates in reproductive organs of newly emerged adults. Recommended references for the preparation of mitotic and meiotic chromosomes are Breeland (1961), French *et al.* (1962) and Baimai (1977).

Baimai *et al.* (1996) reported the differentiation of metaphase karyotype of *An. minimus* species A and C from Thailand. Species A has X1, X2, Y1 chromosomes and species C has X2, Y3 chromosomes (Fig. 1). Somboon *et al.* (2001) found the variation in the sex chromosomes and in the pericentric heterochromatin of autosomes of *An. minimus* species A from Chiang Mai Province, Thailand (CM strain) and reported the metaphase karyotype of another sibling species (species E) from the Ryukyu Archipelago, Japan (Fig. 2).

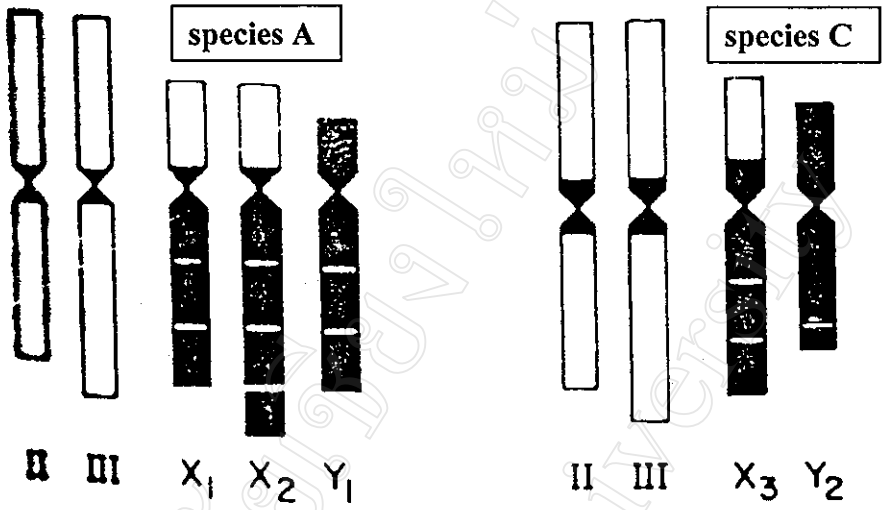

Fig. 1. Diagrammatic representation and comparison of metaphase karyotypes of *Anopheles minimus* species A and C. Only one set of autosomes II and III is presented. Variable heterochromatic portion is depicted in black or shaded. The centromeres are indicated by constrictions of each chromosome. Chromosome lengths, arm ratios, and heterochromatic portions are shown in proportion. (Data from Baimai *et al.*, 1996)

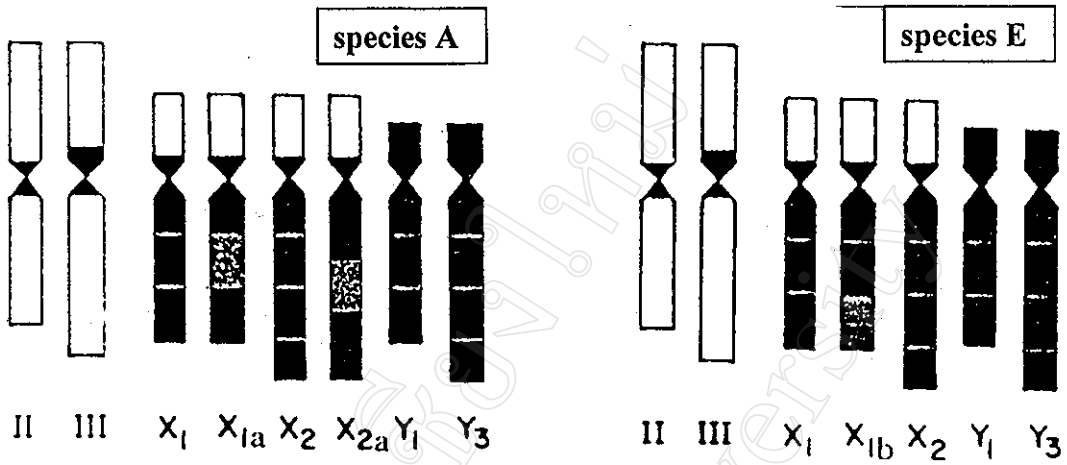

Fig. 2. Diagrammatic representation and comparison of Giemsa-stained metaphase karyotypes of CM (species A) and ISG (species E) strains of *Anopheles minimus*. Only 1 set of autosomes II and III is presented. Variable heterochromatic portion is depicted in black and shaded. The centromeres are indicated by constrictions of each chromosome. Chromosome lengths, arm ratios, and heterochromatic portions are shown in proportion. (Data from Somboon *et al.*, 2001)

1.6 Molecular approaches

Advancements in DNA recombinant technology have facilitated the development of simple and rapid molecular tools for the identification of sibling species. A detailed account of this aspect is presented by Besansky *et al.* (1992), and Hill and Crampton (1994) and Black and Munstemann (1996). The most widely-used molecular method is to identify species-specific DNA segments of the undefined highly repeated component of the genome. Clones containing these segments are identified by differential screening of genomic libraries with homologous and heterologous genomic DNAs. DNA segments from these clones are labelled and used as probes. Post and Crampton's paper (1988) on DNA probes for *Simulium damnosum* complex gives procedures (with illustrations) used in the isolation of species-specific DNA probes for the identification of species. Non-radioactive probes which can identify species from DNA dot-blots and squash-blots are now available for many complexes.

Simple non-radioactive probe assays for squash-blot hybridizations have been developed for the identification of members of the *An. gambiae* (Hill *et al.*, 1991a) and *An. punctulatus* (Cooper *et al.*, 1991) and *An. dirus* (Audtho *et al.*, 1995) complexes. Johnson *et al.* (1992) have improved the procedure to avoid the background in squash-blot hybridizations. Non-radioactive probe methods remove the hazards of radioisotope and make the assays simple and usable under field conditions. The advantage with DNA probes, as with isozymes, is that species can be identified at all stages of the mosquito life-cycle. And if kits are developed, as has been for *An. gambiae* members (Hill *et al.*, 1991b), probes can be used easily in the field.

The development of molecular-based tools for species identification is progressing fast. Differences in rDNA units are being used extensively to develop species-specific primers. Restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD-PCR) (Williams *et al.*, 1990), single strand conformational polymorphism visualization (Hiss *et al.*, 1994) are also being generated as diagnostic tools. Molecular tools are generally developed once the members of a complex are identified. The taxon *An.* (*Nysorhynchus*) *albitarsis* was, however, identified as a complex of four sibling species by examination of natural populations from Paraguay, Argentina and Brazil, by applying RAPD-PCR analysis (Wilkerson *et al.*, 1995).

Several molecular techniques have been applied to study the *An. minimus* complex. Sucharit and Komalamisra (1997) reported amplification of random regions of genomic DNA using 10-base primer in the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) could be used to differentiate *An. minimus* species A and C from Thailand. Sharpe *et al.* (1999) confirmed the presence of species A and C in western Thailand by using the sequencing of the D3 region of the 28s ribosomal DNA and PCR-base methods, allele-specific amplification and single-strand conformation polymorphism, and suggested the possible presence of a sympatric third species (represented by their specimen no. 157). Somboon *et al.*, 2001 has designated *An. minimus* species E by using the amino acid sequences of the D3 rDNA (Table 1 and Fig. 3).

Van Bortel *et al.* (2000, 2002) have developed a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for identification of *An. minimus* species A and C and the related species in Vietnam. In addition, *An.*

minimus species A and C have been reported from southern China by using single-strand conformation polymorphisms (SSCPs) and sequence data for the D3 rDNA and the mitochondrial COII locus (Chen *et al.*, 2002).

2. Other techniques used for identification of species complexes

2.1 Biological distinction

Biological distinction and/or behavioral variation of intraspecies member of mosquitoes in both laboratory colony and field population are still one of the reliable, traditional parameters to incriminate the sibling species or variety status of some cryptic mosquito species. Nonetheless, the biological and behavioral distinctions alone do not imply the exact status.

Van Bortel *et al.* (1999) reported behavioral differences in *An. minimus* species A and C in northern Vietnam. Species C is likely to be more exophagic and zoophilic than species A, and the highly endophilic behavior of species A, which was 5 times more abundant in indoor resting collections than species C.

2.2 Cuticular hydrocarbon profile

Cuticular hydrocarbon analysis for sibling species identification involves determining species-specific differences in the hydrocarbons contained in the wax layer of insect cuticle. The wax layer lies beneath the outermost cuticular layer. Carlson and Service (1979) were the first to use this technique to identify *An. gambiae s.s.* and *An. arabiensis* (two members of the *An. ganbiae* complex). In the

following years several complexes were examined and diagnostic hydrocarbon profiles were identified. However, this technique has not been found in the literature of *An. minimus* mosquitoes.

Species/strain (country)	Site number 11111111111222 29334445556677788024555557793 49 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··

Table 1. Alignment of all available D3 rDNA sequences of *Anopheles minimus*, *An. flavirostris*, and *An. aconitus* (data from Somboon *et al.*, 2001), 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).

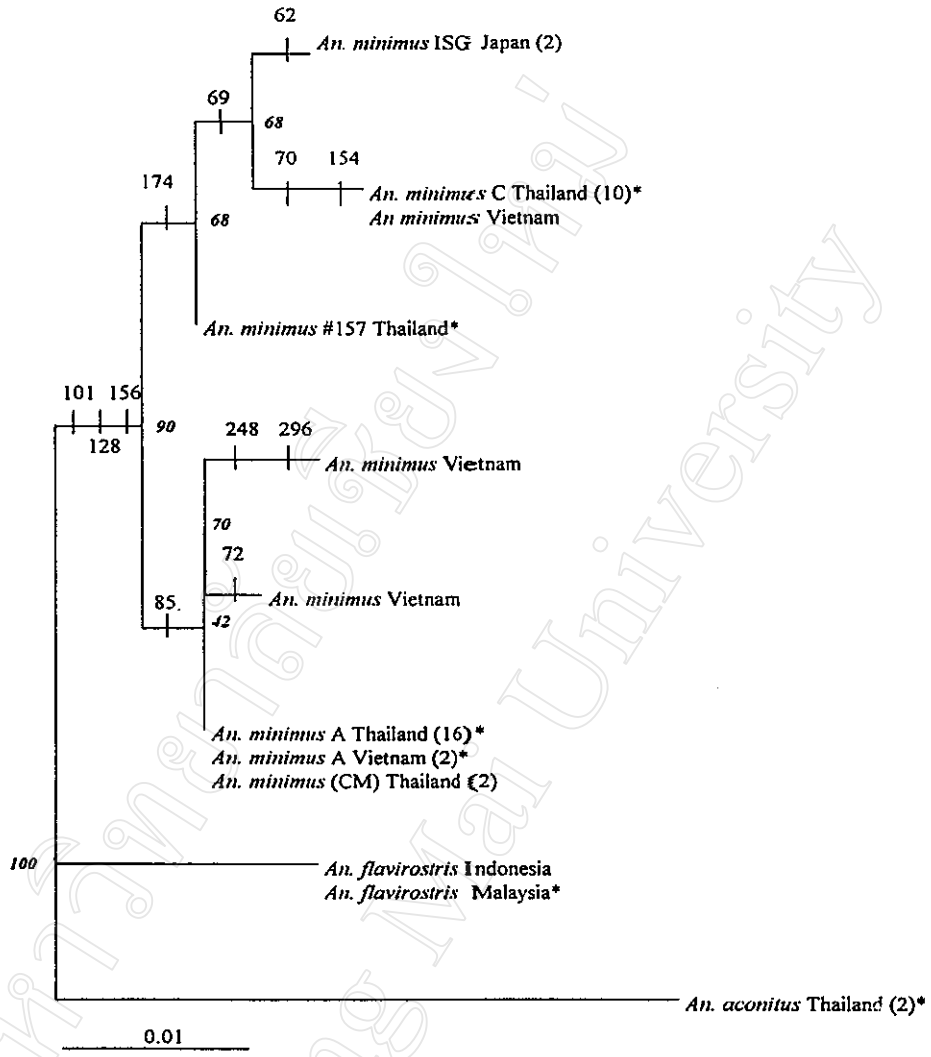

Fig. 3. A neighbor-joining tree of the D3 region of rDNA for all available *Anopheles minimus* sequences using *An. aconitus* and *An. flavirostris* as the outgroup (data from Somboon *et al.*, 2001). When greater than 1, the number of individuals with the same sequence is given in brackets. An asterisk 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 1) along the branches. The scale bar indicates the estimated genetic distance (percent).