ARTICLES

BASES OF MOSQUITO SYSTEMATICS

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The man we honor today is Marston Bates. It is appropriate that I should give the Memorial Address because Bates had a good deal of influence on me. When I traveled to Baltimore in the spring of 1949 to begin graduate study at the Johns Hopkins School of Hygiene and Public Health, I met Bates, who had just closed the Rockefeller laboratory at Villavicencio, Colombia, and returned to the United States. He was spending the summer at Johns Hopkins while working on a new book, *The Prevalence of People*. I had many conversations with Bates that summer and read his book *The Natural History of Mosquitoes*, which had just been published. That was my introduction to the study of mosquitoes, as I am sure is true of many of you in the audience as well.

One of Bates' continuing interests was, in his words, "the species problem." To quote from his book (1949), "The discovery of cryptic species—of sexually isolated populations with few or no tangible recognition characters—has caused a great deal of pain to some conventional taxonomists, since they feel that the whole basis of the system of Linnean nomenclature is endangered in so far as it depends on the assorting and classification of dead museum specimens. The use of genetic or biological procedures for the identification of a species is certainly a tremendous technical handicap. . . ." I also have had a continuing interest in mosquito systematics so I wish to discuss our changing data base for the classification of mosquitoes.

"What's the use of their having names," the gnat said, "if they won't answer to them?"
"No use to them," said Alice; "but its

useful to the people that name them, I suppose."
　　　　　　　　　—Lewis Carroll

As we all know, our system of nomenclature dates from the 10th edition of Linnaeus' *Systema Naturae*, published in 1758. In that work Linnaeus classified mosquitoes as *Culex*, the Latin name for mosquito; and referred 6 species to that genus, only 2 of which are accepted as mosquitoes today. Linnaeus' concept of the genus *Culex* included not only mosquitoes but also biting gnats, black flies, and dance flies. He was familiar with the immature stages of mosquitoes, as shown by his reference to the work of Reaumur (1738), but neither he nor Reaumur recognized sexual dimorphism of mosquitoes; males and females were described as different species. The two true mosquito species listed were *Cx. pipiens* and *Cx. bifurcatus;* the latter was so named, according to Harbach et al. (1985), because it was a male and the palps were thought to branch from the proboscis, giving it a bifurcate appearance.

Mosquitoes, at that time, of course, were of no great importance except for the discomfort they caused. In the years that followed many mosquitoes were described based on the morphology of the adult female; the names of Meigen, Robineau-Desvoidy, Macquart, Walker, Lynch-Arribálzaga, and Giles, among others, were prominent. The morphological concept of the species, based only on the adult female, was adequate for the time.

Mosquitoes took on a more sinister aspect when Sir Patrick Manson demonstrated the development of filarial larvae in them (1879), Sir Ronald Ross demonstrated the development of malarial parasites in them (1899), and the Reed Commission demonstrated transmission of yellow fever virus by them (Reed and Carroll 1901). Mosquitoes then became of first rate importance and studies of them increased enormously. Theobald's *Monograph of the Culicidae of the World*, published from 1901 to 1910, chronicled the intense study of mosquitoes that took place throughout the world at that time. Mosquito larvae were studied from a systematic

point of view by Meinert, Dyar, Nuttall, Smith, Theobald (1901–10) and others. Classification of larvae provided a new system which pointed out many of the faults of the old system of classification based only on adult females. A paper by Dyar and Knab (1904) entitled "Diverse mosquito larvae that produce similar adults" indicates what happened to mosquito taxonomy when stages other than the adult female were examined closely.

Although entomologists had recognized sexual dimorphism of mosquitoes for many years, male mosquitoes generally were not classified, because they did not come to bite and so, were not frequently collected, except perhaps in sweeping or at light. When larvae were collected and reared, however, males were produced abundantly and were studied for purposes of classification. The leaders in this field, at least in this country, were E. P. Felt (1904) and Harrison G. Dyar. The classification of males by genitalic characteristics again opened new vistas of mosquito classification. Many new species were described on characteristics of the male genitalia.

For those who think that taxonomists are stodgy people, consider the case of Dyar as told by Spilman (1984). "Dyar was a great digger of tunnels. In 1906–1916, from his first home near Dupont Circle in Washington he dug complex tunnels on various levels that extended approximately 200 or 500 feet and were large enough for a man to stand in. The tunnels were discovered in 1924 when a delivery truck fell through the pavement into one of them. The discoverers, not knowing the origin, thought the tunnels were used by German spies in World War I or by bootleggers during prohibition. Why did Dyar dig? He said he started digging a deep trench for his wife's hollyhocks, became interested in digging, and simply continued. He dug very wide and deep trenches, proceeded to wall and arch them with enameled brick, and finally covered and hid them with earth. In one version of the story he said they were for playrooms for his son but in another said simply that he liked the smell of fresh earth and dug for exercise. The outcome of his exploits is almost as strange. Dyar, a wealthy man, maintained two homes; in one he had a wife, in the other a mistress. His amorous duplicity was discovered when two children named Dyar met in school and began talking of their fathers. They were surprised when they discovered that their fathers worked at the Smithsonian, then more surprised that they worked in Entomology, and finally astounded that their fathers worked on mosquitoes. The secret was out—their fathers were the same man! The stories are often combined, saying

that the tunnels were dug between the two homes, but there is nothing to substantiate that embellishment.

"When Dyar died W. T. M. Forbes said in an obituary that 'there is no one to take his place.' In more ways than he could have imagined, Forbes was right."

In a remarkable book by Evelyn Groesbeeck Mitchell published in 1907, mosquitoes were identified not only in the adult and larval stages but also in the egg and pupal stages. An attempt was made to describe each life history stage of all species of mosquitoes found in the United States. This is the first work of which I am aware in which there was an attempt to classify mosquitoes by each of the major stages in their life history.

The basic tenets of morphological analysis of mosquitoes were therefore developed by the early 1900s. Since that time there have been refinements in chaetotactic analysis, scanning electron microscopy of mouthparts, eggs, and buccal armature, and description of female genitalia, and the earlier larval instars, but these have only refined the already complex morphological descriptions of species.

The phenomenon of "anophelism without malaria," the presence of presumable vectors of malaria in an area in which malaria did not occur, or occurred rarely, caused workers in the early 1920s to take a closer look at mosquitoes. It was postulated that "races" of mosquitoes that differed biologically existed within a species. Swellengrebel and de Buck (1938) in the Netherlands found that there were two kinds of *Anopheles* that differed in feeding habits, mating habits, and breeding places, but not morphologically. One fed on man and was associated with malaria. The other did not feed on man and was not associated with malaria. The investigators found a statistical difference in size between the two populations and therefore called them "long wings" and "short wings" although they could not be differentiated morphologically. When adults of the two "races" were cross-mated in the laboratory, however, the hybrid eggs either failed to hatch or the larvae died soon afterward (de Buck et al. 1934).

It is clear that the morphological basis of classification of mosquitoes was inadequate. In certain cases genetic and behavioral diversification had taken place without accompanying morphological changes. Before leaving the subject of morphological species, however, we must give credit to F. W. Edwards who, in his masterful treatment of classification of the family Culicidae, published in *Genera Insectorum* in 1932, adopted a conservative view of genera of mosquitoes and reduced the enormous

number of generic names in use to a relative few. This treatment placed all of the vectors of human malaria in the genus *Anopheles* rather than in the many genera recognized by Theobald and later workers; the same treatment was accorded *Culex* and *Aedes*, reducing the number of generic names in use to a comprehensible few, and using subgenera and lower categories for the numerous genera recognized by earlier workers.

The existence of "cryptic," or hidden, species was suggested initially by studies on biological characteristics of mosquitoes, their ability to hibernate, to lay eggs without taking blood, the time of year that various stages were found, the type of water selected for egg-laying, the propensity of adults for entering human habitations, the type of vertebrate host most often attacked, the amount of space required for mating, and even more esoteric characteristics. The finding of cryptic species led to an enormous amount of work on mosquito biology during the 1920s and later, and biological characteristics became an important element in classification during that time. The morphological species was not discarded but was importantly supplemented by biological information. Thus arose the concept of the biological species in mosquito classification. The species concept was couched in terms of biology (mating behavior and reproductive exchange between populations), although identification of species was still done morphologically except in difficult cases where distinctions could be made only after biological studies. A case in point is *Cx. molestus*, a name that was used for many years for a *Cx. pipiens*-like form that was able to mature a clutch of eggs without engorging on blood; the name *molestus* has traditionally been applied to autogenous *Cx. pipiens* irrespective of its other biological traits.

Two books that had an enormous impact on the field of Systematics were Dobzhansky's *Genetics and the Origin of Species* published in 1937 and Mayr's *Systematics and the Origin of Species* published in 1942. The focus in these books was on speciation, the formation of new species, and the processes by which evolution occurred. The species concept emerging from these works was a genetic one, whether or not there was gene flow between putative species, or whether gene flow between two putative species was even possible. In the case of Swellengrebel and de Buck's "short wings" and "long wings" (1938), laboratory studies indicated that gene flow between the two populations was not possible, at least in the direction "short wing" males mated with "long wing" females, since hybrids invariably died during the egg or early larval stages; presum-

ably, the reciprocal cross would have given similar results but "long wing" males did not mate readily in the laboratory so the reciprocal cross was difficult to carry out. Crosses of "short wing" males with females from populations of "*An. maculipennis*" from other parts of Europe in some cases produced fertile hybrid females but the male hybrids were always sterile (Table 1). Thus the widespread European putative species "*An. maculipennis*" proved to consist of populations that seemed to be more or less completely isolated from each other genetically. Since the definition of species at that time was based on lack of gene exchange between populations, *An. maculipennis* obviously was a complex of species, some of which were important vectors of malaria and others of which were not. The biological species concept had taken on a firm baseline; populations that were not capable of gene exchange were, by definition, different species; the "short wings" and "long wings" of the Dutch workers, if the laboratory results were correct, were not capable of gene exchange and were therefore different species, even though they could not be differentiated morphologically.

Table 1. Crosses of *An. atroparvus* males (Bates 1949).

Female	Hybrid progeny
An. labranchiae	females normal, males sterile
An. subalpinus	females normal, males sterile
An. melanoon	females normal?, males sterile
An. maculipennis	females and males sterile
An. sacharovi	most larvae died, few adults produced, males sterile
An. messeae	eggs or young larvae died

Studies of behavior have shown, however, that populations that are capable of gene exchange may, in fact, not interbreed. Populations of *Aedes taeniorhynchus* on the East and West Coasts of North America, for example, appear to be completely isolated spatially at the present time, which prevents gene flow between them. We have no reason to believe, however, that they have differentiated genetically to a significant degree so they are treated as being the same species, even though they are reproductively isolated. How such allopatric populations should be treated taxonomically is not clear and, in fact, they may be handled differently in different groups of organisms.

In sympatric populations, however, we should be able to determine whether in fact there is or is not evidence of gene flow. Frizzi, an Italian cytologist, was the first to prepare usable preparations of mosquito chromosomes (Frizzi,

1947). He was able to show that cryptic species could be identified by differences in banding patterns of their chromosomes, and that hybrids between populations that had different banding patterns could be recognized cytologically. It then became possible to look at populations in areas where cryptic species were sympatric to determine whether or not there was evidence of hybridization between them. Such was done in West Africa in an area where *An. gambiae* and *An. arabiensis* occurred together and it was found that hybrids were rare (4 of 1,830 females examined; White 1971). Even though the two species could not be differentiated morphologically, and hybridization was possible, as shown in the laboratory, there was evidence of a lack of mating between the species in the field. The barrier to mating is almost certainly a behavioral one although it has not been completely elucidated. From a systematic point of view, the two forms behave as different species when they come together.

The presence of hybrids, however, does not necessarily indicate effective gene flow between populations. Bates (1939) found that in the cross of *An. atroparvus* males and *An. maculipennis* females, adults were produced but both sexes were sterile. The presence of hybrids in an area where cryptic species are sympatric therefore indicates cross-mating of the two species but not necessarily effective gene flow; the lack of hybrids in such an area, however, indicates some kind of barrier to cross-mating.

Hybridization frequently can be detected morphologically, as in the *Cx. pipiens* complex. In a broad area of the United States where *pipiens* and *quinquefasciatus* come together, populations occur that are morphologically intermediate between the two forms. Laboratory studies have shown that there is no genetic barrier to mating between these forms and there is evidence of flow of *quinquefasciatus* genes into *pipiens* populations in the field (Barr 1957). In this case it is clear that *pipiens* and *quinquefasciatus* are not reproductively isolated and therefore should not be treated as separate species.

Chromatography has been used to investigate possible biochemical differences between strains of organisms. Lewallen (1957), for example, found that chromatograms of populations of *An. occidentalis* from central California differed from those of the same species from southern California. Further studies, as yet unpublished, have confirmed that these populations indeed are different; we now regard them as separate species.

A more recent technique for detection of gene flow in mosquitoes is a type of biochemical analysis using electrophoresis to separate components. Enzymes of mosquitoes frequently differ from species to species and the genes governing their formation do not show dominance. If cryptic species that are sympatric have alleles of an enzyme with different electrophoretic mobilities, hybrids can be recognized by having an allele for each of the mobilities. This technique has now been widely used for detection of gene flow between populations. Hybrids are recognized by having one allele unique to one species and a second allele unique to a second species. A population composed of two sympatric but morphologically identical species can be resolved into the numbers of each species present and the number of hybrids present. These figures will reveal the presence of reproductive barriers between the species and the strength of such barriers.

A complicating factor in the speciation of some mosquitoes is the presence of symbiotic bacteria which influence the ability of the mosquitoes to reproduce. In *Culex pipiens*, for example, a strain is able to reproduce when mated with some other strains but not when mated with still other strains; in some cases a cross succeeds in one direction but not in the reciprocal direction. We now know that this type of infertility, which we call cytoplasmic incompatibility, is due not to genetic factors of the mosquitoes but to symbiotic bacteria that they harbor (Barr 1982). In some way that we do not presently understand, an infected male mosquito is able to sire young only if mated with a female infected with the same strain of bacteria. If she is uninfected, or infected with a different strain of bacteria, she will produce eggs that are not effectively fertilized or that die before hatching. From the standpoint of the bacteria, infected females are not able to reproduce unless mated with males infected with the same strain of bacteria, so once infection has been introduced into a strain, all uninfected individuals are quickly eliminated from the strain. When infected strains from different areas are crossed, it frequently is found that crossing cannot occur in one or both directions, apparently because of incompatibility of the strains of bacteria of the two species. In Japan, for example, males of the autogenous strain of *Cx. pipiens* usually are incompatible with females of the indigenous strains of *Cx. pipiens* (Sasa et al. 1966), which suggests that the autogenous strains are not native but are introduced; the barrier to gene exchange between the strains is caused not by genetic factors but by symbiotic bacteria.

Cytoplasmic incompatibility is also known in the *Ae. scutellaris* complex and was again demonstrated to be associated with bacterial

symbionts (Wright and Barr 1981). In this case, however, the strains of bacteria found in different mosquito stocks did not seem to differ but incompatibility was seen when infected males were mated with uninfected females (Wright 1979)[3]. The original experiments (Woodhill 1949) had been done with *Ae. scutellaris* males crossed with *Ae. katherinensis* females, the *katherinensis* stock apparently being uninfected. The stocks with which those experiments were done have been lost but examination of subsequently isolated stocks of *katherinensis* showed that indeed they were not infected (unpublished). Other members of this group of species have all been found infected when examined shortly after isolation from the field. Symbiotic bacteria therefore are a complicating factor in the speciation of mosquitoes, at least in the *Cx. pipiens* and *Ae. scutellaris* groups.

A recently developed technique, which has not yet been widely utilized for the study of mosquitoes, is the use of numerical methods for comparison of species, or putative species. We now have an enormous amount of information available for well-known species; morphological, distributional, biological, behavioral, and chemical, among others. Proper use of numerical methods should allow us to evaluate the relationship between species using all of these data, rather than the restricted set of data that we consider most interesting. By using all available information we should be able to sort out species and the relationships of species better than was done in the past.

The newest technique for comparison of species is DNA hybridization. This rather complicated procedure involves hybridization of the DNA of an unknown organism with DNA probes developed from that organism (Post 1985). The technique is said to be quite specific in that it tests for identity of DNA sequences. It is not clear, however, how different genomes must be to be recognized nomenclatorially.

It would seem that the most useful concept of a species still is that it is a gene pool that evolves more or less independently of other gene pools. Most species that we recognize still can be identified morphologically although occasionally we must resort to other biological or chemical methods of identification.

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