LUCIANO BULLINI (*)

Genetic differentiation and speciation in European and African malaria vectors (**)

Anopheline mosquitoes are among the most important organisms both for theoretical and applied biology. Since the end of the 19th century, when their role in the transmission of human malaria was surely established, an enormous amount of scientific work has been produced, concerning their morphology, physiology, genetics, ecology, parasitology, systematics, etc. This was favoured by the fact that these mosquitoes are excellent experimental animals: easy to breed, with a short generation span, a high reproductive potential, hundreds of valuable characters available for morphological analysis, a low chromosome number (2n=6), good polythene chromosomes both in the larval salivary glands and in the adult ovarian nurse cells; moreover, their investigation can utilize sophisticated techniques such as induced copulation, age grading, etc. It is significant that the first discovery of sibling species (i.e. morphologically fairly identical populations, reproductively isolated) was performed on an anopheline mosquito: the European malaria vector *Anopheles maculipennis* s.l.

Anopheline mosquitoes represent a select material for research in evolutionary biology, including the experimental study of speciation processes (i.e. of the genetic changes whereby new species come into existence). In the present paper, data are presented on genetic differentiation and speciation of three *Anopheles* species complexes: *An. maculipennis* (European members), *An. claviger*, and *An. gambiae*, that played or are still playing a crucial role in the transmission of human malaria in Europe and Africa.

(*) Department of Genetics and Molecular Biology, University of Rome «La Sapienza», Rome, Italy.

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The Anopheles maculipennis *and* An. claviger *complexes*

As mentioned above, the *Anopheles maculipennis* complex was the first case of sibling species recognition. According to the older literature, malaria in Europe was transmitted by a single species: *Anopheles maculipennis*. A study of the distribution and ecology of this mosquito revealed, however, all sorts of puzzling irregularities, the main one of which was the so-called "anophelism without malaria" (i.e., the high frequency of *An. maculipennis* in areas of Europe where malaria was absent). A number of "ecological" or "biological" races were hypothesized, differing in host preferences (anthropophily, zoophily), breeding site characteristics (fresh or brackish water), height of nuptial swarm (eurigamy or stenogamy), etc. Further research demonstrated that consistent differences existed in the egg morphology of populations differing in biological characteristics (Falleroni, 1926). Laboratory crosses showed various levels of genetic incompatibility between populations, e.g. gametic or zygotic mortality, hybrid inviability, hybrid sterility (Corradetti, 1934). Later, fixed inversions were found at the polythene chromosomes level, that could be used as reliable diagnostic characters (Frizzi, 1947, 1953). On the basis of these and other studies, at least seven sibling species were assessed in Europe: *An. messeae*, *An. beklemishevi*, *An. melanoon*, *An. maculipennis*, *An. labranchiae*, *An. atroparvus*, and *An. sacharovi* (1).

Rather similar is the case of the *An. claviger* complex. At first, some differences were found in the morphology of the eggs, larvae and pupae, leading to the description of the "varieties" *missirolii* and *petragnanii* (Del Vecchio, 1939a, b; Lupascu, 1941). Later, hybrid inviability was revealed by crossing experiments, together with some ecological and behavioural differences (Coluzzi, 1962). To date, at least two sibling species have been detected: *An. claviger* (corresponding to the *missirolii* form), with a wide palearctic range, and *An. petragnanii*, restricted to the western Mediterranean subregion.

In both complexes: *An. maculipennis* and *An. claviger*, species recognition is often impossible on a morphological basis. The available characters allow the identification only in certain life stages and not always of all individuals. For instance, egg characters such as exochorion color pattern and floats shape are poorly diagnostic between *An. atroparvus* and *An. labranchiae*, and between *An. messeae* and *An. subalpinus*, or misleading as in the case of the supposed Moroccan

(1) The names presently used for a number of members of the *An. maculipennis* complex are not corresponding to the official rules; however, the scientific and practical interest of these species requires their nomenclature to be manipulated with extreme care to avoid serious confusion. A redefinition of the names in the *An. maculipennis* complex in accordance with their traditional use is strongly needed; this should be sanctioned by the International Commission of Zoological Nomenclature. In the present paper, species are indicated following the prevailing use in the modern literature; in the case of *An. subalpinus*, a taxon described from Albania and considered as a variety of *An. melanoon* by White (1978), we have tentatively used this name for a species found in northeast Italy, related to *An. messeae* and *An. melanoon* (Bullini *et al.*, 1980).

TABLE 1 - Allozyme key for the electrophoretic identification of Italian species of the *Anopheles maculipennis* and *An. claviger* complexes, based on four loci: malate-dehydrogenase-1 (*Mdh-1*), malic enzyme (*Me*), hydroxybutyrate dehydrogenase (*Hbdh*), and octanol dehydrogenase (*Odh*). Data from Bianchi Bullini *et al.*, 1980; Cianchi *et al.*, 1981, and unpublished.

Loci	Genotypes	Species
1. *Mdh-1*	*103/103*	*An. claviger*
	98/98	*An. petragnani*
	other genotypes	2
2. *Me*	*96/96*	*An. messeae*
	104/104	*An. atroparvus*
	other genotypes	3
3. *Hbdh*	*92/100, 92/108, 100/100, 100/108, 108/108*	*An. maculipennis*
	other genotypes	4
4. *Odh*	*95/95*	*An. sacharovi*
	109/109	*An. melanoon*
	100/100	*An. labranchiae*

endemic species *An. sicanlti*, actually belonging to *An. labranchiae* (Bullini *et al.*, 1980; De Zulueta *et al.*, 1983), from which it differs in the egg morphology.

At the polythene chromosome level, identification is not possible among *An. maculipennis*, *An. melanoon*, *An. messeae* and *An. subalpinus*, and between *An. labranchiae* and *An. atroparvus*, that are homosequential species (same banding patterns).

A considerable improvement in the detection of *Anopheles* sibling species has been provided by the use of multilocus electrophoresis. The European members of the *An. maculipennis* and *An. claviger* complexes can be reliably identified, in both sexes and at all life stages, on the basis of alternative allozymes at one or more loci (Saura *et al.*, 1979; Bianchi Bullini *et al.*, 1980a, b; Bullini *et al.*, 1980; Cianchi *et al.*, 1980, 1981; Bullini and Coluzzi, 1982). Natural interspecific hybrids can also be identified by this method, making it possible to estimate their frequency in the field in different ecological contexts.

Allozyme keys based on such diagnostic loci were prepared for species identification in the *maculipennis* and *claviger* complexes (Bianchi Bullini *et al.*, 1980b; Cianchi *et al.*, 1981), as shown in Table 1. A fully reliable species identification requires specimens to be electrophoretically tested for at least two diagnostic loci; different allozyme keys should then be used together. This method is particularly useful when the number of specimens to identify is high, as in parasitological and ecological field work, or when control programs against medical pests are planned over large areas (Bullini, 1984).

The analysis of allele frequencies at 27 enzyme loci made it possible to compare natural populations belonging to various species of the *maculipennis* and *claviger* complexes, and to estimate their genetic variability and differentiation Cianchi *et al.*, 1981, and unpublished). In Table 2, the average values of genetic variability found in these two complexes are reported, expressed as expected mean heterozygosity per locus (*He*), proportion of polymorphic loci with the 1% crite-

TABLE 2 - Parameters of genetic variability in the *Anopheles maculipennis* and *An. claviger* complexes, on the basis of 27 enzyme loci. *He* = expected mean heterozygosity per locus; *P* = proportion of polymorphic loci, at the 1% criterion; *A* = mean number of alleles per locus.

Species	*He*	*P*	*A*
An. maculipennis	0.19	0.68	2.62
An. melanoon	0.20	0.70	2.44
An. subalpinus	0.24	0.78	2.85
An. messeae	0.16	0.48	1.59
An. atroparvus	0.18	0.63	2.41
An. labranchiae	0.19	0.78	2.59
An. sacharovi	0.09	0.55	1.67
An. claviger	0.11	0.46	1.76
An. petragnani	0.16	0.74	2.11

TABLE 3 - Values of Nei's standard genetic identity (above the diagonal) and distance (below the diagonal) between species of the *Anopheles maculipennis* complex. Data from Cianchi *et al.*, 1981.

	An. maculipennis	*An. melanoon*	*An. subalpinus*	*An. messeae*	*An. atroparvus*	*An. labranchiae*	*An. sacharovi*	*An. claviger*	*An. petragnani*
An. maculipennis	—	0.83	0.85	0.72	0.70	0.75	0.51	0.20	0.22
An. melanoon	0.19	—	0.86	0.74	0.66	0.74	0.59	0.14	0.16
An. subalpinus	0.16	0.15	—	0.89	0.71	0.76	0.55	0.16	0.18
An. messeae	0.33	0.30	0.12	—	0.68	0.73	0.49	0.13	0.17
An. atroparvus	0.36	0.42	0.34	0.38	—	0.78	0.31	0.16	0.15
An. labranchiae	0.29	0.30	0.28	0.32	0.25	—	0.56	0.15	0.16
An. sacharovi	0.66	0.53	0.59	0.71	0.67	0.57	—	0.22	0.16
An. claviger	1.62	1.93	1.85	2.04	1.86	1.87	1.51	—	0.55
An. petragnani	1.49	1.86	1.73	1.77	1.90	1.83	1.84	0.60	—

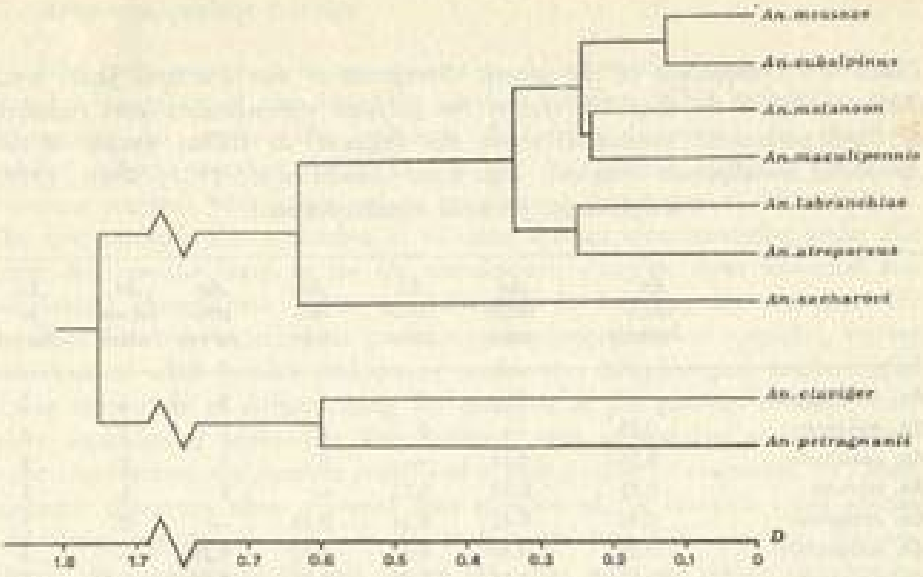

Fig. 1 — Genetic relationships among species of the *Anopheles maculipennis* and *claviger* complexes, estimated on the basis of 27 enzyme loci. D = Nei's standard genetic distance.

rion (P), and mean number of alleles per locus (A). Genetic divergence between these taxa, quantified using Nei's indices of standard genetic identity (I) and distance (D) is given in Table 3. From these values, a dendrogram was prepared, showing the genetic relationships between the considered species of the *maculipennis* and *claviger* complexes (Fig. 1).

A positive correlation was observed in the *maculipennis* complex comparing interspecific gene and chromosome divergence, the former being expressed by Nei's D, the latter by the number of fixed paracentric inversions (Table 4). A highly significant correlation ($r = 0.961$, $P < 0.001$) was also found between genetic and morphological divergence (Cianchi *et al.*, 1981). The former was quantified by Nei's D, whereas the latter was estimated using the index M, obtained by adding, for each species pair, the percentages of specimens morphologically identifiable at the different life stages and at the male genitalia (e.g. a value of $M = 1.80$ can correspond to a species morphologically identifiable from another at two life stages, respectively at 100% and 80% level). The data, reported in Table 5, would indicate that the building-up of morphological and genetic divergence have followed a parallel trend of accumulation in the evolution of both *maculipennis* and *claviger* complexes.

TABLE 4 - Comparison of the genetic divergence at the structural genes level (Nei's *D*, below the diagonal) and at the polytene chromosomes level (number of fixed paracentric inversions, above the diagonal) in Italian species of the *Anopheles maculipennis* complex. Data from Cianchi *et al.*, 1981; White, 1978; and Coluzzi, personal communication).

	An. maculipennis	*An. melanoon*	*An. subalpinus*	*An. messeae*	*An. atroparvus*	*An. labranchiae*	*An. sacharovi*
An. maculipennis	—	0	0	0	1	1	3
An. melanoon	0.19	—	0	0	1	1	3
An. subalpinus	0.16	0.15	—	0	1	1	3
An. messeae	0.33	0.30	0.12	—	1	1	3
An. atroparvus	0.36	0.42	0.34	0.38	—	0	3
An. labranchiae	0.29	0.30	0.28	0.32	0.25	—	3
An. sacharovi	0.66	0.53	0.59	0.71	0.67	0.57	—

TABLE 5 - Values of genetic divergence (Nei's *D*, below the diagonal) and of morphological divergence (*M*, above the diagonal) between the Italian species of the *Anopheles maculipennis* and *An. claviger* complexes. Data from Cianchi *et al.*, 1981.

	An. maculipennis	*An. melanoon*	*An. subalpinus*	*An. messeae*	*An. atroparvus*	*An. labranchiae*	*An. sacharovi*	*An. claviger*	*An. petragnanii*
An. maculipennis	—	1.80	1.80	1.35	1.25	1.95	3.00	5.00	5.00
An. melanoon	0.19	—	0.50	1.70	2.00	2.00	3.00	5.00	5.00
An. subalpinus	0.16	0.15	—	1.60	2.00	2.00	3.00	5.00	5.00
An. messeae	0.33	0.30	0.12	—	1.25	1.95	3.00	5.00	5.00
An. atroparvus	0.36	0.42	0.34	0.38	—	1.40	3.00	5.00	5.00
An. labranchiae	0.29	0.30	0.28	0.32	0.25	—	3.00	5.00	5.00
An. sacharovi	0.66	0.53	0.59	0.71	0.67	0.57	—	5.00	5.00
An. claviger	1.62	1.95	1.85	2.04	1.86	1.87	1.51	—	3.20
An. petragnanii	1.49	1.86	1.75	1.77	1.90	1.83	1.84	0.60	—

The Anopheles gambiae *complex*

The main vectors of human malaria in Africa south of Sahara are represented by members of the *Anopheles gambiae* complex. This includes at least six species: *An. gambiae*, *An. arabiensis*, *An. melas*, *An. merus*, *An. quadriannulatus*, and the so-called species D. In the *An. gambiae* complex, speciation processes occurred with a minimal or even absent morphological differentiation. The first evidence that a number of different species were coexisting under the name *An. gambiae* came, as for the *maculipennis* complex, from ecological and behavioural observations, indicating differences in breeding site characteristics (fresh or brackish water), host preferences (anthropophily or zoophily), resting behaviour of adult females (indoors or outdoors). Morphological studies failed almost completely in differentiating the members of the *gambiae* complex, even when sophisticated approaches were utilized, such as scanning electron microscope observations, multivariate analysis of a high number of characters, etc. Some biometric characters allow a partial discrimination of the brackish water species (*An. melas* and *An. merus*) from the fresh water ones (*An. gambiae*, *An. arabiensis*, *An. quadriannulatus* and species D). The latter are apparently identical at all life stages, and show a remarkable intraspecific variation at a number of morphological characters.

Laboratory experiments revealed various degrees of hybrid sterility in crosses involving different combinations of the six members of the *gambiae* complex (Davidson, 1964; Davidson and White, 1972). Sterility, limited to the hybrid males, is only partial in some cross-types and is virtually absent in two out of the thirty possible crosses.

Species recognition became effective only when the study of polythene chromosomes revealed that different rearrangements, based on paracentric inversions, were fixed in the various members of this complex (Coluzzi, 1966; Coluzzi and Sabatini, 1967, 1968, 1969; White, 1973; Davidson and Hunt, 1973; Petrarca, 1985).

At the electrophoretic level, alternative allozymes at one or more loci were found between the various species (Mahon *et al.*, 1976; Miles, 1978, 1979; Bullini and Coluzzi, 1982; Cianchi *et al.*, 1983a). Allozyme keys, based on these diagnostic loci (e.g. Table 6), make it possible to identify the species of the *gambiae* complex at different life stages and in both sexes, and to recognize natural interspecific hybrids (Cianchi *et al.*, 1983a).

The analysis of allozyme variation at 25 loci in populations from West Africa, belonging to *An. gambiae*, *An. arabiensis* and *An. melas*, indicated a generally lower level of variability than that found in the *maculipennis* complex (Table 7). The values of genetic distance observed within the two complexes are of the same magnitude, Nei's *D* ranging from 0.11 to 0.41 in the *gambiae* complex, from 0.12 to 0.71 in the *maculipennis* (from 0.12 to 0.42 excluding *An. sacharovi*, the species of the *maculipennis* complex most differentiated morphologically).

TABLE 6 - Allozyme keys for the electrophoretic identification of species of the *Anopheles gambiae* complex, based on four loci: superoxide dismutase-1 (*Sod-1*), glutamate-oxaloacetate transaminase-1 (*Got-1*), mannose-phosphate isomerase (*Mpi*), and acid phosphatase (*Acph*). Data froen Miles, 1978, Cianchi *et al.*, 1983, and unpublished.

Loci	Genotypes	Species
1. *Sod-1*	*95/95*	*An. merus*
	95/100, 100/100	2
	105/105	species D
2. *Got-1*	*95/95*	*An. quadriannulatus*
	other genotypes	3
3. *Mpi*	*100/100, 100/103, 100/105, 103/103, 103/105, 105/105*	*An. gambiae*
	90/90, 90/97, 97/97	4
4. *Acph*	*94/94, 94/100, 100/100*	*An. arabiensis*
	106/106	*An. melas*

TABLE 7 - Genetic variability in species of the *Anopheles gambiae* complex from West Africa. *He* = expected mean heterozygosity per locus; *P* = proportion of polymorphic loci, at the 1% level; *A* = mean number of alleles per locus.

Species	*He*	*P*	*A*
An. gambiae	0.12	0.42	1.67
An. arabiensis	0.11	0.33	1.52
An. melas	0.10	0.44	1.70

In the *gambiae* complex, unlike the *maculipennis*, no correlation exists when comparing the divergence at the structural genes level with the number of fixed inversions found between species (Table 8). This would indicate a major role of paracentric inversions in the speciation of the *gambiae* complex. Such inversions would favour the preservation of coadapted multilocus complexes, selected in populations living in ecologically marginal zones (Coluzzi, 1982). This hypothesis has been recently supported by the discovery of partially or totally reproductively isolated populations within *An. gambiae* s.s. from the Gambia (Bryan *et al.*, 1982) and from Mali (Touré *et al.*, 1983). These populations, very similar at the structural genes level (Cianchi *et al.*, 1983b), show highly differentiated frequencies at some paracentric inversions, and appear adapted to different habitats and environmental conditions (Coluzzi, 1984).

TABLE 8 - Comparison of the genetic divergence at the structural genes level (Nei's *D*, below the diagonal) and at the polythene chromosomes level (number of fixed paracentric inversions, above the diagonal), of species of the *Anopheles gambiae* complex. Data from Gianchi *et al.*, 1983b, and unpublished; Coluzzi *et al.*, 1979, and unpublished.

	An. gambiae	*An. arabiensis*	*An. melas*	*An. merus*
An. gambiae	—	5	4	2
An. arabiensis	0.15	—	6	7
An. melas	0.25	0.11	—	7
An. merus	0.29	0.36	0.41	—

Concluding remarks

The study of genetic differentiation in a number of European and African malaria vectors has led to results of both theoretical and practical importance, concerning: a) genetic methods of sibling species identification; b) the building-up of morphological and genetic divergence in the evolution of these mosquitoes; c) the development of isolating mechanisms; d) the role of paracentric inversions; e) the patterns of speciation in different species complexes.

The low or absent morphological differentiation in the speciation of these *Anopheles* appears to be a general phenomenon. The practical difficulty in recognizing species morphologically fairly identical, but strongly differentiated in their vectorial capacity, parasitological importance, etc., has been successfully overcome by the use of polythene chromosome analysis and more recently by multilocus electrophoresis.

The building-up of morphological and genetic divergence in the evolution of the *maculipennis*, *claviger* and *gambiae* complexes has been a gradual phenomenon: an evidence that strongly disagrees with the expectation of the punctuated equilibria theory, proposed by Gould and Eldredge (1977).

As to the development of reproductive isolating mechanisms, crossing experiments indicate that within the three complexes post-mating barriers are only partial and often not very effective. However, highly efficient anti-hybridization mechanisms are acting in the field, as indicated by the very rare occurrence of interspecific hybrids. The gene pools of these species seem to be maintained separated mainly by pre-mating barriers, as in other mosquitoes (e.g. *Culex*, *Aedes*). These barriers were presumably direct targets of natural selection when incipient species came into contact, whereas post-mating barriers are apparently only accidental by-products of the genetic divergence accumulated between populations.

Paracentric inversions, widespread both as intraspecific polymorphisms and

as fixed rearrangements between related species, have been often regarded as an incidental accompaniment of the evolutionary processes (e.g. Carson *et al.*, 1967). Coluzzi's studies on the *Anopheles gambiae* complex clearly show that such inversions favour the preservation of coadapted multilocus complexes, selected in populations living in ecologically marginal zones, and therefore play a major role in the adaptive and speciative processes of these mosquitoes (Coluzzi *et al.*, 1979; Coluzzi, 1982). The different amount of fixed paracentric inversions found between members of the *maculipennis* and *gambiae* complexes with similar levels of genetic divergence indicates that these rearrangements had a different importance in the two complexes. Their evolutionary role has been certainly higher in the *gambiae* complex (average number of fixed inversions between the considered species = 5.2 with a Nei's average genetic distance of 0.24), than in the *maculipennis* (average number of fixed inversions between the considered species = 1.2, with a Nei's average genetic distance of 0.38). In the latter complex, species differentiation occurred in wider areas (Palearctic and Nearctic regions), with frequent opportunities for prolonged isolation between populations. These extrinsic barriers to gene flow made it possible for populations of the *maculipennis* complex to become adapted to different ecological and climatic conditions and to diverge genetically from each other, not depending on paracentric inversions for these processes. On the contrary, the evolution of the *gambiae* complex occurred in an area (the Afrotropical region) with fewer opportunities for prolonged isolation between populations; in such conditions, paracentric inversions have substituted for extrinsic barriers, at least to some extent, in allowing the adaptation and differentiation of populations.

A similar evolutionary pattern seems to have occurred in the Afrotropical mosquitoes of the *Anopheles marshallii* complex, whose species, like those of the *An. gambiae* complex, show a high number of fixed paracentric inversions together with a very low interspecific genetic distance (Lambert, 1983).

Summary. — Data are presented on genetic variation and speciation of European and African malaria vectors belonging to the *Anopheles maculipennis*, *An. claviger* and *An. gambiae* complexes. In view of the low or absent morphological differentiation involved in the speciation of these *Anopheles*, allozyme keys are given for sibling species identification.

The genetic variability found in the various species is compared. The analysis of the building-up of morphological and genetic divergence reveals that both have gradually accumulated during the evolution of the three complexes, in contrast with the expectation of the punctuated equilibria theory.

The incomplete post-mating barriers within the *maculipennis*, *claviger* and *gambiae* complexes, demonstrated by crossing experiments, strongly contrast with the efficient isolating mechanisms acting in the field (very rare occurrence of interspecific hybrids). Pre-mating barriers, promoted by natural selection, are considered as the main cause of the reproductive isolation of these *Anopheles* species.

Gene and chromosome interspecific divergence, estimated respectively by Nei's *D* and by the number of fixed paracentric inversions, is compared in the *maculipennis* and *gambiae* complexes. A much higher number of chromosome rearrangements is found in the latter, whereas gene divergence is higher in the former. These findings are accounted for with the different importance of paracentric inversions in the speciation of these mosquito complexes.

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