New African species in the *Drosophila obscura* species group: genetic variation, differentiation and evolution

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The *Drosophila obscura* species group was known in the Afrotropical region from a single species, *D. microlabis*. This species was recently rediscovered in Kenya in addition to the discovery of three new *obscura* group species: *D. kitumensis*, *D. cariouae* and *D. kimbasi*. The patterns of allozyme variation in two African species, *D. microlabis* and *D. kitumensis* is compared to that of nine species of the *obscura* group. Genetic distances have been estimated from allelic frequencies at 35 loci and phylogenies inferred by methods assuming both constant as well as variable evolutionary rates. *D. microlabis* and *D. kitumensis* are evolutionary related and close to the Palearctic *D. tristis* and *D. ambigua* even if relatively distant. The data support Lakovaara and Saura's hypothesis recognising three species subgroups within the *obscura* group. In addition, we propose a fourth one for the new African species, the *microlabis* species subgroup.

INTRODUCTION

The *Drosophila obscura* species group is widely distributed in the Palearctic and Nearctic regions. In the Afrotropical region it was known from a single species, *D. microlabis* Seguy, described in 1938 (Seguy, 1938) from a single male collected from Kenya (Marakwet, on the Elgeyo Escarpment) by the Omo expedition (Arambourg *et al.*, 1935). More recently, Tsacas (1974) confirmed that this species was a member of the *obscura* group and assigned it to the *obscura* species subgroup. The Museum of Paris specimen remained, for half a century, the sole indication of the presence of the *obscura* species group in the Afrotropical region.

During a recent field investigation in Kenya (D. Lachaise, M. L. Cariou and M. Ashburner, Sept. 1984) *D. microlabis* was rediscovered from Mt Elgon, on the west side of the Kenya (or Gregory) Rift Valley. In addition, three new *obscura* group species were discovered: one also from Mt Elgon, *D. kitumensis* Tsacas and two sympatric species from Mt Kenya on the East side of the Rift Valley, *D. kimbasi* Tsacas and *D. cariouae* Tsacas. All of these species have been

recently described (Tsacas *et al.*, 1985). Thus, there are at least four species of the *obscura* species group in Kenya, and we suspect that many more may occur in the East African highlands. All these species were collected at high altitudes, above 2000 meters. Neotropical species of the *obscura* species group (*affinis* subgroup), *D. azteca* and *D. tolteca* as well as the Neotropical populations of *D. pseudoobscura* (Throckmorton, 1975) are also found only at high altitudes. Thus, there is strong presumption that the *obscura* species group, which is basically Holarctic in its distribution (Wheeler, 1981), has extended its geographic range over both the Afrotropical and Neotropical regions but only in the temperate climatic conditions of mountains.

With the new African species the *obscura* species group now includes 35 species. Although this is one of the most extensively species groups of *Drosophila* that have been studied (see, Buzzati-Traverso and Scossiroli, 1955; Lakovaara and Saura, 1982 for a review) many questions remain concerning the relationships between the species. The division of the group into two species subgroups is itself still questionable. On the basis of morphological and allozymic characters, the *obscura* group has long been subdivided into two

species subgroups: the *affinis* subgroup, with nine exclusively Nearctic species, and the widespread Palearctic species *D. helvetica*—the *obscura* subgroup, which includes 14 Palearctic species and seven Nearctic species. Several of the Palearctic species are widespread, *D. subobscura*, *D. obscura*, *D. ambigua* and *D. bifasciata*, which probably is the most widely distributed species within the group. Lakovaara and Saura (1982) have argued that the division of the *obscura* group into two subgroups is not a "natural one" and suggested dividing the *obscura* species subgroup into two lineages: the *pseudoobscura* species subgroup and the *obscura* species subgroup to include the Nearctic and Palearctic species, respectively.

In the present work, we have compared patterns of allozyme variation in two African species, *D. microlabis* and *D. kitumensis*, with that of nine species of the *obscura* group (*sensu lato*). Genetic distances have been estimated on the basis of allelic frequencies at 35 loci, and phylogenies have been inferred by methods assuming both constant as well as variable evolutionary rates. The data support Lakovaara and Saura's hypothesis. In addition we propose a fourth species subgroup for the new African species, the *microlabis* species subgroup.

MATERIAL AND METHODS

The African species and their habitats

Drosophila cariouae and *D. kimbasi* were both collected from Mt Kenya (2470 m) at Main Gate of Mt Kenya National Park, by banana traps placed above a domestic rubbish dump just behind gate hut. The former species was also collected by banana trap in *Podocarpus*/bamboo forest about 0.5 km from the Main Gate. One strain of *D. cariouae* failed to establish successfully after the first generation, while *D. kimbasi* was described from a single male. Therefore only two of the four African species could be studied, *D. microlabis* and *D. kitumensis*.

D. microlabis was collected in banana traps and by sweeping at Mt Elgon Lodge (2200 m), 1.5 km from Main Gate of Mt Elgon National Park (fig. 1). Most traps and sweep sites were in a domestic habitat, i.e., garden with grass and shrubs but with small fruit dump (tomatoes). In spite of an intensive search, the species was not collected in wild habitats in the Mt Elgon slope forest at higher elevations. On Mt Elgon the collection sites followed the main track from the Main Gate of the National Park (2260 m) to the moorland

(4200 m) below Koitoboss peak. This track runs between the Komothon River (to the north) and the Kassowai River (to the south). It is generally the same region as was covered by the Omo Expedition. *D. microlabis* was abundant at Mt Elgon lodge and is apparently a widely distributed species, it probably ranges from Mt Elgon to the Elgeyo Escarpment where it was collected in 1935. The species can easily be grown on standard corn-flour culture medium and a mass culture was established from 30 wild caught inseminated females.

D. kitumensis was collected from the area in front of the Kitum Cave (2500 m) in submontane forest with *Diospyros abyssinica* below the *Podocarpus* zone, 5 km from Main Gate of the Mt Elgon National Park (fig. 1). Kitum Cave is a large and deep excavation, much visited by elephants and buffaloes, located in valley of Rongai River. The area immediately in front of the cave is very disturbed by animals and is a jumble of rocks dominated by *Discopodium penninervium* (Hochst) (Solanaceae), the climbing plant *Stephania abyssinica* (Dillon and Rich) Wolp. var. *tomentella* (oliv.) Diels, *Urtica massaica* Mildbr. and with much dead/rotting wood. The entire valley in front of the cave was wet and cooled by air from the cave and was obviously different in its flora from the surrounding forest and from other cave valleys of the East slope of Mt Elgon (Makingeny Cave, Chepnyalil Cave) where *D. kitumensis* was not observed.

D. Kitumensis was only found at the east edge of Cave entrance, in a sap rot on a large tree, *Ekebergia rueppeliana* (Fresen) A. R. (Meliaceae). Adult flies were associated with this sap rot and only *D. kitumensis* was collected there by sweeping. This quite soft and wet substrate contained larvae, presumably of the same species; it is likely that the sap rot of *E. rueppeliana* is the breeding site of *D. kitumensis*. Similar natural breeding sites, sap and slime fluxes of trees (Oak, sycamore, willow, fir and elm) have been reported for *D. pseudoobscura*, *D. persimilis* (Carson, 1951), *D. ambigua* (Prevosti, 1959), *D. obscura* (Gordon, 1942) and *D. subobscura* (Carson *et al.* 1985).

D. kitumensis is difficult to breed, nevertheless, a strain was established from 13 wild-caught females and kept on standard culture medium.

Other species

For *D. subobscura* we studied a natural population from the French Pyrénées (Riberot locality), a strain from Crete (Greece), another from Switzerland and two standard strains, Küstnacht

Figure 1 Localities of the four African species of the *Drosophila obscura* species group in the highland forests on the eastern and western flanks of the Kenya (Gregory) Rift Valley (Altitudinal zonation of Mt Elgon and Mt Kenya after data in Schnell, 1977; Hamilton and Perrot, 1981; Geological map summarising the volcanic history of the Kenya Rift after King and Chapman, 1972).

(Lankinen and Pinsker, 1977) and Chcu homogeneous for most enzyme loci.

Two natural populations collected in valleys in the French Pyrénées (Riberot and Estours) and one strain from Switzerland represented *D. obscura*. One population of *D. helvetica* from Gif (France) was used. We also analysed some labora-

tory stocks for between species comparisons: three for *D. bifasciata* from Norway (strain 24), Italy (Pavia) (strain 25) and U.S.S.R. (Alma Ata) and only one for the following species: *D. tristis* (Switzerland), *D. ambigua* (Mt Parnes, Greece), *D. madeirensis* (Madeira Island), *D. guanche* (Canary Islands) and *D. pseudoobscura* (USA).

Genetic assays

Electrophoresis was predominantly carried out on starch gel following the general procedures of Shaw and Prasad (1970) and Ayala *et al.* (1972) with some modifications. The α -amylase was analysed using a 5 per cent acrylamide gel as described in Dainou *et al.* (1987).

Electrophoresis was performed on adult flies except for *Est1*, *Lap1* and *Acp2* (third instar larvae) and *Lap2* (pupae). Allozymes were designated by their electrophoretic mobility relative to that of the most common allozyme of *D. subobscura*, numbered 100. For each locus, 30 to 85 individuals were tested for *D. microlabis*, *D. kitumensis* and the natural populations of *D. subobscura* and *D. obscura*. The laboratory strains were studied only as a reference for the between species comparisons and fewer individuals were scored, from 15 to 25.

Four kinds of Nei's genetic distances (D_1) have been used (the usual standard, D_{1st} , another when only the most frequent allele was considered, D_m , and two others) as well as two kinds of another distance, D_2 (depending on whether data of the most common allele or all alleles were used in each locus), all described in Krimbas and Sourdis (1987).

Four different methods for constructing trees were used: UPGMA (Sneath and Sokal, 1973), Farris (1972), Fitch and Margoliash (1967) and a modified version of Farris' method (Tateno *et al.*, 1982). The three last methods were further modified by us in such a way that trees with negative distances were rejected (Sourdis and Krimbas, 1987).

Concordance of reconstructed trees with the original data could eventually indicate fidelity to the "real" phylogeny. The measure used was the sum of the squares of the differences in distances between the reconstructed and the original (experimental) distances. These distances have been normalised in every matrix to one in order to get comparable data.

RESULTS

The most common allozymes of the 35 putative loci screened in the 19 populations and species studied are presented in table 1. Variation is great between species and none of the 35 loci is strictly monomorphic. Polymorphic loci are those already known to be polymorphic in the *Drosophila obscura* group. The total number of alleles detected is 180,

some of these are rare and only detected in recently collected natural populations. The *Amy* locus appears to be highly variable, with 14 allozymes identified.

From the electrophoretic data, it follows that the two African species, *D. microlabis* and *D. kitumensis* are both clearly distinguished from the other species of the *obscura* group by different alleles fixed at three loci, *Pgm*, *Gdh* and *Amy*. In addition they only share rare alleles with one or two species at three more loci, *Mdh*, *Pgi* and *Est6*. Of the 35 loci analysed, eight are diagnostic between *D. microlabis* and *D. kitumensis*: 6*Pgd*, *Xdh*, *Ao*, *Odh*, *Acp1*, *Acp2*, *Alph* and *Lap1*.

Estimates of heterozygosity and percentage of variable loci are given in table 2. The percentage of polymorphic loci ranges from 3 per cent (laboratory strains) to 43 per cent (*D. microlabis*) with a mean at 20.1. *Drosophila kitumensis* is polymorphic at 26 per cent of its loci. The mean number of alleles per polymorphic locus is 2 and the mean number of alleles per locus per species is respectively 1.46 for *D. microlabis* and 1.26 for *D. kitumensis*. The mean expected heterozygosity for *D. microlabis* (0.094) and *D. kitumensis* (0.067) comes within the range of variation found for other species of the *obscura* group (table 2).

D_2 (D_2 polymorphic) and Nei's standard (D_{1st}) genetic distances based on allele frequencies are presented in table 3. The genetic distances within the same species (*D. subobscura*, *D. obscura* and *D. bifasciata*) are very small (from 0.004 to 0.11) indicating that strong differentiation does not exist between either local or geographically distant populations of these species. This is in good agreement with previous studies (Lakovaara and Saura, 1971; Lakovaara *et al.*, 1976; Loukas *et al.*, 1979; Pinsker and Buruga, 1982).

Extensive isozyme divergence among species is further indicated by the great magnitude of genetic distances. Nei's distance ranges from 0.269 to 2.416 and D_{2p} from 0.105 to 0.571. The two least divergent species are the African *D. microlabis* and *D. kitumensis*, they are genetically closer to one another than any other couple of species known to have some degree of relatedness on the basis of other criteria, e.g. *D. tristis* and *D. ambigua* ($D_{1st} = 0.404$), or the triplet *D. subobscura*, *D. guanche*, *D. madeirensis* ($0.522 < D_{1st} < 0.838$).

Very high distances are obtained when *D. helvetica* is paired with any other species; the highest value is between this species and *D. kitumensis* ($D_{1st} = 2.416$).

Different trees can be constructed using either, or both, different measurements of genetic distance

Table 1 Electrophoretic mobilities (relative to *D. subobscura*) of the most common allozymes at 35 loci in African and other species of the *D. obscura* group. micr: *D. microlabris*; kitu: *D. kitumensis*; subo: *D. subobscura*, 1: chcu, 2: Küstnacht, 3: Greece, 4: France, 5: Switzerland; guan: *D. guanche*; made: *D. madeirensis*; obs: *D. obscura*, 1: Switzerland, 2: Ribero, France, 3: Estours, France; tris: *D. tristis*; ambi: *D. ambigua*; bifa: *D. bifasciata*; helv: *D. helvetica*; pseu: *D. pseudoobscura*

Locus	micr	kitu	subo 1	subo 2	subo 3	subo 4	subo 5	guan	made	obs 1	obs 2	obs 3	tris	ambi	bifa 24	bifa 25	bifa Al.	helv	pseu
<i>Idh</i>	97	97	100	100	100	100	100	99	102	100	100	100	97	97	94	94	94	104	104
<i>6Pgd</i>	106	102	100	100	102	100	100	101	104	102	102	102	102	102	102	102	102	100	105
<i>Pgm</i>	90	90	100	100	100	100	100	100	100	101	101	101	101	101	104	104	102	100	100
<i>αGpdh</i>	100	100	100	100	100	100	100	100	101	100	100	100	100	100	104	104	104	100	100
<i>Fum1</i>	97	97	100	100	100	100	100	100	100	97	97	97	97	97	97	97	97	102	97
<i>Fum2</i>	102	102	100	100	100	100	100	100	100	100	100	100	105	108	108	108	108	108	108
<i>Xdh</i>	100	101	100	100	100	100	100	101	100	100	100	100	101	100	100	100	99	98	101
<i>Aldo</i>	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
<i>Hk1</i>	103	103	100	100	100	100	100	98	98	101	101	101	100	101	103	103	103	104	104
<i>Hk2</i>	101	101	100	100	100	100	100	100	100	101	101	101	101	100	100	100	100	100	101
<i>Hk3</i>	103	103	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99
<i>Pgi</i>	98	98	100	100	100	100	101	100	103	100	100	100	100	100	101	101	101	103	100
<i>Mdh</i>	103	103	100	100	100	100	100	100	102	102	102	102	104	106	106	106	106	114	102
<i>Me</i>	97	97	100	100	100	100	100	97	101	93	93	93	99	98	99	99	99	100	93
<i>Ao</i>	100	101	100	100	100	100	100	100	101	100	100	100	100	100	100	100	100	100	100
<i>Od</i>	103	98	100	100	100	100	100	100	100	102	102	102	103	103	102	102	102	101	101
<i>Adh</i>	100	100	100	100	100	100	95	100	100	120	120	120	120	120	110	110	110	95	100
<i>G6pdh</i>	97	97	100	100	100	100	97	100	102	97	97	97	97	97	93	93	93	164	105
<i>Ca</i>	96	95	100	100	100	100	100	96	94	96	96	98	96	96	94	94	94	102	100
<i>Est6</i>	96	96	100	100	100	100	100	97	100	97	97	97	97	97	94	94	94	100	100
<i>Est</i>	96	96	100	100	100	100	100	98	99	98	98	98	98	98	98	98	98	102	99
<i>EstC</i>	102	102	100	100	100	100	100	100	100	94	94	94	102	102	98	98	98	104	94
<i>Sod</i>	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
<i>Gapdh</i>	100	100	100	100	100	100	100	100	100	100	100	100	105	100	100	100	100	108	100
<i>Gdh</i>	92	92	100	100	100	100	100	102	103	97	97	97	97	97	102	102	102	105	105
<i>Acph1</i>	94	95	100	100	100	100	100	94	97	94	94	94	94	100	115	112	112	115	97
<i>Sdh</i>	95	95	100	100	100	100	100	101	101	100	100	100	100	101	101	101	101	101	100
<i>Su</i>	97	97	100	100	100	100	97	103	103	97	97	97	97	97	97	97	97	112	97
<i>Gor</i>	102	102	100	100	100	100	100	102	102	102	102	102	102	102	103	103	103	103	100
<i>Amy</i>	118	118	100	100	100	100	90	90	95	102	102	102	97	97	90	90	93	105	101
<i>Alph</i>	100	102	100	100	100	100	98	98	98	105	100	100	102	102	94	94	94	98	102
<i>Acph2</i>	94	97	100	100	100	100	100	100	103	103	103	103	99	99	95	95	95	92	102
<i>Lap1</i>	102	105	100	100	100	100	100	100	101	100	100	100	104	100	99	99	99	102	102
<i>Lap2</i>	100	100	100	100	100	100	98	98	98	97	97	97	100	101	101	101	101	98	98
<i>Est1</i>	98	98	100	100	100	100	96	96	98	96	96	96	98	100	102	100	100	102	98

The following enzymes were run: malate dehydrogenase (*Mdh*), malic enzyme (*Me*), fumerase (*Fum*), α-glycero-phosphate dehydrogenase (*αGpdh*), phosphoglucose isomerase (*Pgi*), glucose-6-phosphate dehydrogenase (*G6pdh*), aldolase (*Ald*), glutamate oxalate transaminase (*Gor*), isocitrate dehydrogenase (*Idh*), glutamate dehydrogenase (*Gdh*), octanol dehydrogenase (*Od*), sorbitol dehydrogenase (*Sdh*), glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*), succinate dehydrogenase (*Su*), superoxide dismutase (*Sod*), acid phosphatases (*Acph1*, *Acph2*), carbonic anhydrase (*Ca*), one esterase with α-naphthyl propionate as substrate (*Esp*), hexokinases (*Hk1*, *Hk2*, *Hk3*), xanthine dehydrogenase (*Xdh*), 6-phosphogluconate dehydrogenase (*6Pgd*), phosphoglucomutase (*Pgm*), alcohol dehydrogenase (*Adh*), aldehyde oxidase (*Ao*) and esterases (*Est1*, *Est6*, *EstC*), leucine aminopeptidases (*Lap1*, *Lap2*) alkaline phosphatase (*Alph*).

Table 2 Amount of genetic variation in *D. microlabis* and *D. kitumensis* compared with different species analysed in our work and published data. The total number of loci studied is given within brackets

	Present study		Published data		References
	H exp.	% var. loci (35)	H exp.	% var. loci	
<i>D. microlabis</i>	0.094	43	—	—	
<i>D. kitumensis</i>	0.067	26	—	—	
<i>D. subobscura</i>					
1. Chcu	0.023	3	0.084	34 (67)	Cabrera <i>et al.</i> , 1983
2. Küstnacht	0.065	20	—	—	
3. Greece	0.022	11	—	—	
4. France (Riberot)	0.089	26	0.234	85 (20)	Saura <i>et al.</i> , 1973
Canary Island	—	—	0.082	57 (44)	Cabrera <i>et al.</i> , 1980
Yugoslavia	—	—	0.114	75 (28)	Marinkovic <i>et al.</i> , 1978
Sweden-Italy	—	—	0.142-0.118	94 (17)	Pinsker and Sperlich, 1979
Madeira-Azores Islands	—	—	0.070-0.081	32-35 (63)	Larruga <i>et al.</i> , 1983
Finland	—	—	0.076	47 (32)	Lakovaara and Saura, 1971b
<i>D. guanche</i>	0.110	29	0.066	38 (68)	Cabrera <i>et al.</i> , 1983
<i>D. madeirensis</i>	0.045	11	0.103	57 (67)	Cabrera <i>et al.</i> , 1983
<i>D. obscura</i>					
1. France (Riberot)	0.098	23	—	—	
2. France (Estours)	0.077	17	—	—	
3. Switzerland	0.072	17	—	—	
Finland	—	—	0.110	(33)	Lakovaara and Saura, 1971a
<i>D. tristis</i> (Switzer.)	0.055	20	—	—	
<i>D. ambigua</i>					
Greece	0.071	26	—	—	
Spain	—	—	0.032	12 (56)	Cabrera <i>et al.</i> , 1983
<i>D. bifasciata</i> (Norway)	0.080	20	0.242	(21)	Saura (1974)
<i>D. pseudoobscura</i> (USA)	0.043	9	0.009	5 (63)	Cabrera <i>et al.</i> , 1983
			0.150	(43)	Prakash, 1977
<i>D. persimilis</i> (USA)	—	—	0.108	(43)	Prakash, 1977
<i>D. miranda</i> (USA)	—	—	0.085	(43)	Prakash, 1977
<i>D. helvetica</i> (France)	0.012	3	—	—	

or different algorithms. One way of evaluating different trees is by determining the tree that best represents the data, although the "best fit" may not necessarily be the best hypothesis of evolutionary relationships. Therefore we have used several other criteria based on extraneous information concerning group taxonomy to judge tree reliability; these were, whether or not different populations of the same species clustered, the relative positions of *D. madeirensis* and *D. guanche* close to *D. subobscura* (Krimbas and Loukas, 1984) and the relative closeness of *D. tristis* and *D. ambigua* (Lakovaara *et al.*, 1972; Lakovaara and Keränen, 1980).

Independently of branch lengths, the alternative topologies depicted in fig. 2 differ in the hatched zone, that is in the branching of *D. microlabis* and *D. kitumensis* and, *D. tristis*, *D. ambigua* and *D. pseudoobscura*. The trees are ranked according to their goodness-of-fit. They all satisfy the reliability criteria, except number 3 where *D. tristis* and *D. ambigua* are separated, and number 6, which is rejected because *D. madeirensis* and *D. guanche* do not cluster with *D. subobscura*.

It is worth emphasising that the five best trees all position the African species close to one another and quite close to the European species-pair, *D. tristis*-*D. ambigua*, and also to some extent to the American *D. pseudoobscura*.

The unrooted tree obtained from *D*₂ polymorphic distance and the modified Farris clustering has the best fit (fig. 3). A phylogenetic interpretation of this unrooted tree is reasonably consistent with the biochemical and chromosomal affinities previously inferred regarding the position of the Palearctic species of the *obscura* group (see Lakovaara and Saura, 1982, for a review). *D. subobscura* is close to its two endemic relatives *D. guanche* and *D. madeirensis* although the relative position of these species differs from that found by Cabrera *et al.* (1983) and Loukas *et al.* (1984). This could result from the use of different sets of loci, or the use here of a better method for tree construction, but may also be indicative of ambiguity regarding their relative genetic divergence.

D. subobscura is significantly distant from *D. obscura*, *D. tristis* and *D. ambigua* are obviously

Table 3 D_2 estimator of distance (above diagonal) and Nei's standard genetic distance, D_{1st} (below diagonal) based upon 35 enzymatic loci between the African species and 17 populations of other species in the *D. obscura* group

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
<i>microlabis</i>	1	×××	0.11	0.34	0.34	0.34	0.33	0.32	0.35	0.28	0.28	0.28	0.23	0.27	0.37	0.38	0.37	0.52	0.34
<i>kitumensis</i>	2	0.27	×××	0.36	0.36	0.36	0.35	0.33	0.36	0.32	0.32	0.32	0.24	0.31	0.42	0.43	0.43	0.57	0.35
<i>subobscura</i> 1	3	1.34	1.63	×××	0.02	0.01	0.02	0.03	0.15	0.16	0.27	0.24	0.25	0.22	0.34	0.34	0.34	0.36	0.24
2	4	1.31	1.64	0.01	×××	0.02	0.04	0.16	0.17	0.27	0.25	0.25	0.27	0.22	0.35	0.35	0.34	0.37	0.25
3	5	1.35	1.56	0.01	0.02	×××	0.03	0.15	0.15	0.27	0.24	0.24	0.26	0.21	0.34	0.34	0.33	0.37	0.24
4	6	1.35	1.56	0.01	0.02	×××	0.04	0.15	0.16	0.28	0.25	0.25	0.27	0.22	0.35	0.35	0.34	0.37	0.24
5	7	1.25	1.51	0.09	0.10	0.11	×××	0.17	0.18	0.27	0.25	0.25	0.27	0.22	0.34	0.34	0.34	0.37	0.25
<i>guanche</i>	8	1.16	1.40	0.57	0.55	0.52	0.73	×××	0.12	0.22	0.22	0.22	0.25	0.23	0.33	0.33	0.33	0.41	0.29
<i>maderensis</i>	9	1.41	1.52	0.70	0.71	0.70	0.83	0.53	×××	0.29	0.28	0.29	0.30	0.27	0.35	0.35	0.35	0.38	0.28
<i>obscura</i> 1	10	0.95	0.21	0.98	0.97	0.94	0.91	0.87	1.55	×××	0.05	0.05	0.19	0.22	0.36	0.37	0.36	0.52	0.22
2	11	0.89	1.14	0.85	0.84	0.82	0.85	0.85	1.45	0.05	×××	0.03	0.21	0.23	0.36	0.37	0.36	0.52	0.19
3	12	0.89	1.15	0.86	0.85	0.82	0.85	0.87	1.46	0.05	0.01	×××	0.21	0.23	0.35	0.36	0.36	0.51	0.19
<i>tristis</i>	13	0.79	0.84	1.20	1.18	1.11	1.20	1.09	1.72	0.56	0.61	0.60	×××	0.13	0.30	0.31	0.30	0.47	0.31
<i>ambigua</i>	14	0.89	1.02	0.97	0.96	0.97	0.92	0.85	1.28	0.53	0.59	0.58	0.40	×××	0.21	0.21	0.20	0.41	0.35
<i>bifasciata</i> 24	15	1.35	1.47	1.46	1.39	1.48	1.30	1.20	1.44	1.13	1.00	1.02	1.29	0.78	×××	0.03	0.03	0.42	0.45
25	16	1.49	1.51	1.49	1.42	1.46	1.33	1.25	1.53	1.25	1.11	1.12	1.31	0.80	0.04	×××	0.01	0.42	0.46
A1	17	1.53	1.53	1.53	1.46	1.49	1.36	1.35	1.56	1.29	1.14	1.16	1.33	0.82	0.08	0.04	×××	0.41	0.46
<i>helvetica</i>	18	1.80	2.42	1.30	1.32	1.35	1.16	1.23	1.22	2.00	1.87	1.88	1.90	1.41	1.33	1.40	1.46	×××	0.42
<i>pseudoobscura</i>	19	1.22	1.11	0.97	0.98	0.97	1.04	1.11	1.04	0.88	0.81	0.83	1.07	1.30	1.68	1.73	1.74	1.06	×××

Figure 2 Alternative topologies derived using different algorithms using D_2 and Nei's genetic distances depict different relationships between species within the hatched zone. The trees are numbered according to their goodness-of-fit measures. mic: *D. microlabis*, kit: *D. kitumensis*, tris: *D. tristis*, amb: *D. ambigua*, obs: *D. obscura*, pseu: *D. pseudoobscura*, hel: *D. helvetica*.

Figure 3 Evolutionary relationships of the African species to others of the *Drosophila obscura* group as deduced from allozyme divergences. The unrooted tree is constructed by the modified Farris method clustering of D_2 polymorphic distances.

here related species. *D. bifasciata* appears genetically quite distinct from *D. subobscura* and the *obscura* relatives (*D. obscura*, *D. tristis*, *D. ambigua*). *D. helvetica* is the most distant species and is not appreciably related to any of those analysed here.

The position of *D. pseudoobscura* is more problematic. Lakovaara and Saura (1982) found that the Nearctic *obscura* subgroup species were not appreciably related to the Palearctic species or to the Nearctic *affinis* subgroup. Our data show that genetically, *D. pseudoobscura* is closest to *D. obscura* ($D_{1st} = 0.8$) and is related to *D. tristis* and *D. ambigua*. The old laboratory strain of *D. pseudoobscura* used may not be representative of the species. However, Cabrera *et al.* (1983) also found some relatedness between *D. pseudoobscura* and *D. ambigua*. Moreover, hybridizations provide some evidence relating *D. pseudoobscura* to some Palearctic species: *D. pseudoobscura* and or *D. persimilis* crossed to either *D. bifasciata*, *D. tristis* or *D. ambigua* yield either sterile hybrids or pupae (Koske, 1953; Buzzati-Traverso and Scossiroli, 1955) while the European species generally produce no interspecific hybrids when crossed to one another (except *D. subobscura* $\times$ *D. madeirensis* and *D. madeirensis* $\times$ *D. guanche*, Krimbas *et al.*, 1984).

DISCUSSION

The discovery in the Kenya highlands of a complex of *Drosophila* species belonging to the *obscura* group allows new insight into the evolutionary history of this group.

Of the four African *obscura* relatives two were found on the Eastern slope of Mt Elgon to the West of the Kenya Rift Valley and the others two on the western slope of Mt Kenya to the east of the Rift. The four species were found at similar altitudes, between 2200 and 2500 metres and the question arises whether or not these two species pairs have resulted from a vicariant event, due to the aridification of the Rift as a climatic barrier.

The Kenya (Gregory) Rift Valley is a sector of the Rift system of eastern Africa which has been marked by continuous volcanic activity throughout its history from Miocene times to the present day. The largest of the central volcanoes are on the flanks of the Rift, but whereas those to the west (e.g., Mt Elgon) are Miocene in age, those to the east (e.g., Mt Kenya) are mainly Pleistocene to Recent (King and Chapman, 1972).

Drosophila microlabis and *D. kitumensis* from Mt Elgon are evolutionarily related. They always branch together regardless of the genetic distance or tree-building method used. This agrees with the morphological data (fig. 4): the two species have very similar genitalia and long sex combs while both *D. cariouae* and *D. kimbasi* have short sex combs and different male genitalia (Tsacas *et al.*, 1985). These data support the vicariance hypothesis. There are, however, two reservations, first, there is no evidence for the relatedness of *D. cariouae* and *D. kimbasi*; secondly, the single male assigned to a fifth African species, collected with *D. cariouae* in the *Podocarpus*/bamboo forest on Mt Kenya, is strikingly close in its genital morphology to *D. kitumensis* from Mt Elgon.

Regarding the relationship of these species with its non-African *obscura* relatives, the most distantly related species is *D. helvetica*. Although included in the *affinis* subgroup *D. helvetica* is clearly not closely related to any of these Nearctic species. However, a relationship of the African species and the *affinis* subgroup cannot be discarded. With respect to the *obscura* and *pseudoobscura* lineages *D. microlabis* and *D. kitumensis* are closer to the Palearctic *D. tristis* and *D. ambigua* than to the American *D. pseudoobscura*. However, the relevant genetic distances are large and any relationship is relatively distant. This conclusion is consistent with the observation that neither *D. microlabis* nor *D. kitumensis* will hybridise with any other *obscura* group species (all these studied in this paper and also *D. subsilvestris* have been tested) nor with each other (Tsacas *et al.*, 1985).

Electrophoretic evidence clearly shows strong heterogeneity within what is generally admitted as the *obscura* subgroup. Therefore, we concur with Lakovaara and Saura (1982) in recognising three species-subgroups instead of two, in the *obscura* group and further propose to introduce a fourth subgroup to include the African species, the *microlabis* species subgroup (fig. 5).

In summary, the extent of the newly discovered African lineage in the *obscura* species group provides new insight into the origin and evolutionary development of the *obscura* species group. On the basis of a "symplesiomorphic" acrocentric X chromosome, homologous to the X of *D. melanogaster*, Patterson and Stone (1952) considered *D. subobscura* to be a representative of the "ancestral" lineage of the group. Assuming this, Lakovaara and Saura (1982) stated that if such a X chromosome were to be found in *D. microlabis*, this species might belong to an "ancestral" lineage as well. Preliminary results indicate that this is not

Figure 4 Cladogram (UPGMA method) based on morphological affinities between the four African species of the *obscura* species group. Similarity was estimated for each pair of species by the proportion of common characters on the total 17 characters considered, including sex combs and clasper combs, wing indices, oral bristles, ejaculatory bulbs and various features of both phallus and parameres.

the case (Krimbas *et al.*, in prep.) and that the two African species have eight euchromatic arms more or less similar to those of *D. ambigua*, *D. tristis* and *D. bifasciata*. Also, Marinkovic *et al.* (1978) suggested that the American *obscura* subgroup species diverged from the *D. subobscura* lineage. Neither the present allozyme data nor those of Cabrera *et al.* (1983) support this hypothesis but rather suggest that the central taxon group might well be the *obscura-tristis-ambigua* lineage, because both American (*D. pseudoobscura*) and African species (*D. microlabis* and *D. kitumensis*) show some degree of relatedness to it. Species such as *D. bifasciata* (and to some extent *D. helvetica*) that have very large geographic ranges, from Western Europe to Japan, have also been considered to be potential evolutionary links between the *obscura* and the *affinis* subgroups (Lakovaara and Saura, 1982). Hence, the proto-*obscura* lineage

might have been “*tristis-ambigua* like” or a “*bifasciata* like” species.

The origin of the *obscura* species group remains controversial, especially with respect to the chronology of the splitting events of the major lineages. In view of affinities with the *melanogaster* species group, Throckmorton (1975) derived the *obscura* species group from a primeval proto-*melanogaster/obscura* trunk. On the basis of diversity criteria the origin of the *melanogaster* species group is assumed to be oriental (Bock and Wheeler, 1972; Bock, 1980; Tsacas, 1984; Lemeunier *et al.*, 1986). A plausible scenario is to consider that from that centre of origin the proto-*obscura* stock first split into two ancestral lineages, one of them diversifying in East Africa and the other extending to Europe, where it differentiated giving rise subsequently to both the New World *pseudoobscura* and *affinis* lineages.

Figure 5 Multivariate analysis (Centered data analysis, Lefebvre, 1976) based on allele frequencies at 35 loci. Plotting of populations and species on the plane defined by the 1st and 4th axes (37 per cent of total variability) clearly discriminate distinct lineages within the *obscura* species group.

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