THE PHYLOGENETIC RELATIONSHIPS OF THE MEMBERS OF THE *DROSOPHILA ROBUSTA* GROUP[1]

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ABSTRACT

The phylogenetic relationships among the species of the *D. robusta* group were investigated by the analysis of chromosomal differences. Six of the ten known members of the *D. robusta* group were available for the study: *D. colorata* and *D. robusta* from the United States, and *D. sordidula, D. pseudosordidula, D. lacertosa,* and *D. moriwakii* from Japan. Analysis of the metaphase chromosomes from larval ganglion cells suggests that *D. moriwakii* and *D. colorata,* with rod-shaped X-chromosomes, are the more ancestral species, while *D. sordidula, D. pseudosordidula, D. robusta,* and *D. lacertosa,* with V-shaped X-chromosomes, are derived. The ancestral position of *D. colorata* and *D. moriwakii* is further strengthened by the fact that these are the two species in the *D. robusta* group that are cytologically closest to *D. nigromelanica* of the related *D. melanica* group. Of the four derived species, *D. sordidula* was found to be the closest to the ancestral species. The phylogeny based on the analysis of the gene sequences in the homologous chromosomes agreed with that indicated by the metaphase chromosomes. Since all attempts to obtain hybrids were unsuccessful except for the cross involving *D. moriwakii* females and *D. colorata* males, photographic maps of the salivary chromosomes were used to determine homology between the chromosomes of the different species. Evidence is presented to indicate that the *D. robusta* group originated in Asia (Japan), and that there were two migrations to the New World, the first leading to *D. robusta,* and the second to *D. colorata.* It is suggested that the route of migrations was across the Bering Land Bridge, and further, that the migrations occurred during the period from late Oligocene to middle Miocene, 20–25 million years ago.

SALIVARY gland chromosomes have been successfully utilized in determining the phylogenetic relationships of members within several species groups belonging to the genus Drosophila: the *D. obscura* group by DOBZHANSKY (1951), the *D. virilis* group by STONE, GUEST and WILSON (1960), the *D. repleta* group by Wasserman (1963), the *D. melanica* group by STALKER (1966), and the Hawaiian species groups by CARSON and STALKER (1968a,b,c; 1969). This paper deals with the phylogenetic relationships of the members of the *D. robusta* group.

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This group, which belongs to the subgenus Drosophila, is primarily Asiatic, and bears a close relationship to the well-understood *D. melanica* group, and a possible close relationship to the Hawaiian Drosophila species (STALKER 1968).

The *D. robusta* group includes ten known species, two Nearctic and eight Palearctic. Of the ten, only six were available for the present study: *D. colorata* and *D. robusta* from the United States, and *D. moriwakii, D. sordidula, D. pseudosordidula* and *D. lacertosa* from Asia. *D. okadai* and *D. neokadai*, reported from Japan, (TAKADA 1959; KANEKO and TAKADA 1966) and *D. cheda* and *D. pullata* reported from mainland China (TAN, HSU and SHENG 1949) were not available. The genetics of natural populations of *D. robusta* as shown by their salivary chromosomes has been studied in great detail (CARSON and STALKER 1946, 1947; CARSON 1956; 1958; LEVITAN 1951), while information regarding the salivary chromosomes of *D. colorata*, the only other member of the group in the eastern United States, is meagre. The metaphase chromosomes of most of the Japanese members have been described (TOKUMITSU *et al.* 1967); however, little is known about their salivary gland chromosomes.

The Nearctic species *D. robusta* and *D. colorata* are restricted to the eastern deciduous forests of the United States. Their geographic distribution has been described in detail by CARSON (1958). The Asiatic members of the group are all laboratory stocks, representing the various localities as follows: One strain of *D. moriwakii* from Hokkaido; seven strains of *D. lacertosa*, one from Kyushu, two from Honshu, three from Hokkaido, and one from Taiwan; four strains of *D. sordidula*, two from Honshu, one from Hokkaido, and one from Korea; and two strains of *D. pseudosordidula* from Hokkaido.

The cytotaxonomic study of the members of the *D. robusta* group to be reported was undertaken to obtain information bearing specifically on the following questions: (1) What are the phylogenetic relationships among the species in the group? (2). Where in the phylogeny has New World-Asiatic migration taken place? (3) How many such migrations are presumed to have occurred? (4) Which of the *D. robusta* group species are most closely related to species of outside groups, particularly members of the *D. melanica* group?

MATERIALS AND METHODS

Flies were reared at 23° ± 1°C on cornmeal-molasses-agar-tegosept food. Two or three pairs of adults were allowed to mate and lay eggs in vials and were transferred to fresh food vials on alternate days. For salivary chromosome preparations, larvae were grown at the same temperature, on food enriched daily with yeast paste.

Salivary gland chromosomes were prepared by a modification of the technique described by STALKER (1964b). Salivary glands were dissected in 60% acetic acid, quickly transferred to a drop of lactic-acetic-orcein on a silicone treated slide, and allowed to stain for 3–4 minutes. A coverslip was then dropped on the gland in the stain, and was lightly tapped with the rubber tip of a pencil, to facilitate the breaking of the nuclear membranes and spreading of the chromosomes. Finally, the glands were squashed under the coverslip and the preparations sealed with Farrant's medium and stored in a freezer.

Metaphase chromosomes were studied from the ganglion cells of mature larvae, following the technique of LEWIS and RILES (1960). The preparations were lightly tapped before squashing to facilitate the separation of the ganglion cells.

Since attempts to obtain hybrids between the different species by normal mating procedures were unsuccessful except in the case of *D. moriwakii* females and *D. colorata* males, photomaps were used to compare the banding pattern in the salivary chromosomes; such maps were made according to the technique described by STALKER (1964a). When photomaps were used for interspecific comparisons of banding patterns, a large number of additional photographs which showed the same chromosome regions in different conditions of bending, puffing, stretching, and staining, were consulted. This was necessary to determine the homology of a particular section of the chromosome in the different species, since the maps themselves show only one condition of the chromosome (STALKER 1965). In the interspecific comparisons, very small rearrangements may have been overlooked; however, it is believed that the overall picture of the phylogenetic relationships among the species will not be modified by any such omissions.

Comparisons of species maps were made and the homologous regions marked, using the method explained in detail by STALKER (1965). Numbers were assigned to mark the limits of the homologous sections of the chromosomes, and the minimum number of inversions which could lead from the sequence found in one species to that found in another was determined. In each chromosome arm the gene sequence observed in *D. colorata* was chosen as the standard. Each inversion relative to that standard was assigned a letter, starting with A. The letters A,B,C, etc. were used over again for each chromosome arm. For instance inversion A in chromosome 2 bears no relationship to the similarly designated inversion in other chromosome arms. Capital letters were used for representing the fixed homozygous inversion differences, while lower case letters were used to represent the intraspecific inversions. However, similarly designated intraspecific inversions in the different chromosome arms of the various species have no relationship to one another. For instance, the inversions *a* and *b* of chromosome 4 of *D. sordidula* are in no way related to inversions *a*, *b* and *c* of chromosome 4 of *D. robusta* or inversions *a*, *b* and *c* of chromosome 3 of *D. robusta*.

In comparisons in which it was not possible to homologize the entire chromosome, partial analysis could be carried out by following the distribution of the identifiable regions in the homologous chromosomes. Where most of the length of the chromosome could be homologized, the number of interspecific inversions was estimated, disregarding the unidentified regions. In this case the number of inversions necessary to convert the gene sequence found in one species to that found in another is underestimated.

Interspecific and intraspecific crosses were attempted using males and females of the different strains available for the study. Virgins were collected and stored until maturity (8–14 days depending on the species). All attempted matings were made in shell vials containing up to ten pairs of flies, partly at 25°C under normal day and night conditions, and partly at 23°C in darkness. The flies were transferred to fresh food two or three times a week for 30–40 days. If by this time no progeny were produced, they were discarded.

RESULTS

Hybridization experiments: The results of the hybridization tests are summarized in Table 1. No hybrids were produced in any of the tests except that involving *D. moriwakii* females and *D. colorata* males. The reciprocal test involving *D. colorata* females and *D. moriwakii* males was not productive. It is evident that there is a high degree of sexual isolation between all the species of the *D. robusta* group studied so far except the allopatric pair *D. moriwakii* and *D. colorata*. The incomplete reproductive isolation between *D. moriwakii* and *D. colorata* was not surprising in view of their karyotypic and morphological similarities, which would place them in a category close to that of sibling species.

In the crosses involving *D. moriwakii* females and *D. colorata* males, of the 111 pairs tested, 7 females produced offspring; 65 female and 45 male hybrids were obtained. The hybrid males and females were tested for fertility by backcrossing

TABLE 1

Hybridization tests between members of the D. robusta *group*

Females	Males	Number of pairs tested	Hybrids
D. moriwakii	× D. colorata	111	65♀♀ 45♂♂
D. moriwakii	× D. sordidula	84	0
D. moriwakii	× D. lacertosa	93	0
D. moriwakii	× D. pseudosordidula	85	0
D. moriwakii	× D. robusta	88	0
D. colorata	× D. moriwakii	112	0
D. colorata	× D. sordidula	179	0
D. colorata	× D. lacertosa	99	0
D. colorata	× D. pseudosordidula	112	0
D. colorata	× D. robusta	112	0
D. robusta	× D. moriwakii	84	0
D. robusta	× D. colorata	137	0
D. robusta	× D. sordidula	234	0
D. robusta	× D. pseudosordidula	203	0
D. robusta	× D. lacertosa	205	0
D. sordidula	× D. moriwakii	86	0
D. sordidula	× D. colorata	204	0
D. sordidula	× D. lacertosa	205	0
D. sordidula	× D. pseudosordidula	225	0
D. sordidula	× D. robusta	273	0
D. lacertosa	× D. moriwakii	84	0
D. lacertosa	× D. colorata	127	0
D. lacertosa	× D. sordidula	205	0
D. lacertosa	× D. pseudosordidula	182	0
D. lacertosa	× D. robusta	164	0
D. pseudosordidula	× D. moriwakii	71	0
D. pseudosordidula	× D. colorata	124	0
D. pseudosordidula	× D. sordidula	110	0
D. pseudosordidula	× D. lacertosa	164	0
D. pseudosordidula	× D. robusta	250	0

with the parental species. The hybrid males were sterile, while the hybrid females laid eggs after about 14 days and produced offspring when crossed with either *D. colorata* or *D. moriwakii* males (2 females used for each backcross). Attempts were also made to cross these hybrid females to the males of the other

TABLE 2

Hybridization tests between the F_1 hybrid females (D. moriwakii♀♀ × D. colorata♂♂) and males of the other species of the D. robusta group

Females	Males	Number of flies tested Females		Males	Hybrids
F_1 hybrid	× D. sordidula	14	×	30	0
F_1 hybrid	× D. pseudosordidula	11	×	23	0
F_1 hybrid	× D. robusta	13	×	30	0
F_1 hybrid	× D. lacertosa	23	×	50	0

TABLE 3

Frequencies of gene arrangements in Chromosome 4 of D. lacertosa

Locality	N	Chromosome 4 +	a
Hokkaido			
Eniwa	44	44	..
Higashiyama	44	44	..
Nopporo	44	44	..
Honshu			
Hirokawara	44	12	32
Zao, Aamori	50	34	16
Kyushu			
Kirishima	38	15	23
Taiwan	34	..	34

species in order to produce three way hybrids [(A × B) female × C male], with no success. The results of the above crosses are summarized in Table 2.

Chromosomal polymorphism in the members of the D. robusta group: Although the number of strains of the various species available for the present study was limited and they were from laboratory stocks, intraspecific variation exhibited by the different strains was studied. Salivary gland chromosomes of approximately 20 larvae from each stock were prepared according to the method described earlier, and their inversions scored. The results are shown in Tables 3, 4 and 5.

D. moriwakii and *D. pseudosordidula* show no intraspecific variation. *D. lacertosa* has one inversion in chromosome 4. *D. sordidula* has eight inversions, five in

TABLE 4

Frequencies of gene arrangements in chromosomes 2, 3, and 4 of D. colorata

Locality	N	Chromosome 2 +	ab	Chromosome 3 +	a	Chromosome 4 +	ab
Petoskey, Michigan	46	46	..	46	..	46	..
Itasca, Minnesota	50	24	26	33	17	42	8

TABLE 5

Frequencies of gene arrangements in chromosomes 2, 3, and 4 of D. sordidula

Locality	Chromosome 2 +	a	b	c	d	e	ac	bc	bd	cd	ce	de	acd	bcd	cde	Chr. 3 +	a	Chr. 4 +	ab
Honshu																			
Kanagawa	48	.	.	.	.	.	.	.	.	.	.	.	.	.	.	33	15	..	48
Sugadaira	26	4	.	1	.	.	1	.	.	3	.	.	13	.	.	48	..	24	24
Iwakisan	23	.	.	5	.	.	.	.	.	22	.	.	.	.	.	43	7	25	25
Hokkaido	29	.	.	1	.	.	.	3	5	.	1	.	.	4	5	40	8	24	24
Korea	28	.	6	.	3	3	.	.	3	.	.	1	.	.	.	33	11	22	22

chromosome 2, one in chromosome 3, and two in chromosome 4. *D. colorata* has two inversions each in chromosome 2 and chromosome 4, and an inversion complex in chromosome 3. Nineteen intraspecific inversions, evenly distributed in all the chromosome arms, have been reported for *D. robusta* by CARSON (1958). The designations used by CARSON for the intraspecific inversions of *D. robusta* have been changed in the present paper to be consistent with those used for the other species, as shown below:

Chromosome number	CARSON (1958)	This paper
X-right	XR–1	XR–a
	XR–2	XR–b
	XR–3	XR–c
X-left	XL–1	XL–a
	XL–2	XL–b
	XL–3	XL–c
2-right	2R–1	2R–a
	2R–2	2R–b
	2L–R	2R–c
2-left	2L–1	2L–a
	2L–2	2L–b
	2L–3	2L–c
	2L–4	2L–d
	2L–5	2L–e
	2L–6	2L–f
	2L–7	2L–g
3-right	3R–1	3–a
	3R–2	3–b
3-left	3L–R	3–c

Phylogenetic changes in chromosome X-right: The X-right phylogeny for the group is summarized in Figure 1 (see also Figures 2 and 3)* Chromosome 5 of *D. colorata* is homosequential with that of *D. moriwakii*, and is homologous to the X-right of the rest of the members of the group. Chromosome 5 of *D. moriwakii* and *D. colorata* is also found to be identical to the chromosome X-left of *D. nigromelanica* of the *D. melanica* group, except for a very short region. Of all the members of the *D. robusta* group, the above two species are therefore the closest to species of outside groups, and are assumed to be the most ancestral species of the group. Based on this, chromosome 5 of *D. moriwakii* and *D. colorata* is chosen as the arbitrary standard for the group.

Starting with the *D. colorata-D. moriwakii* standard, the addition of inversion A leads to the sequence found in *D. sordidula* (A). From *D. sordidula* three branches arise. The first branch involves three inversions—B, C, and D—and the apparent deletion of the section 77–80 (Figure 2), and leads to *D. pseudosordidula* (ABCD, del. 77–80). In the second branch, the chromosome acquires inversions E and F, leading to *D. robusta* (AEF). The third branch, with the addition of the inversion G, leads to *D. lacertosa* (AG). It is possible to homologize

* Diagrams showing the specific regions involved in the inversions which give rise to the proposed phylogenies (Figures 1, 4, 7, 10) have been deposited with the Editor of GENETICS and are available on request.

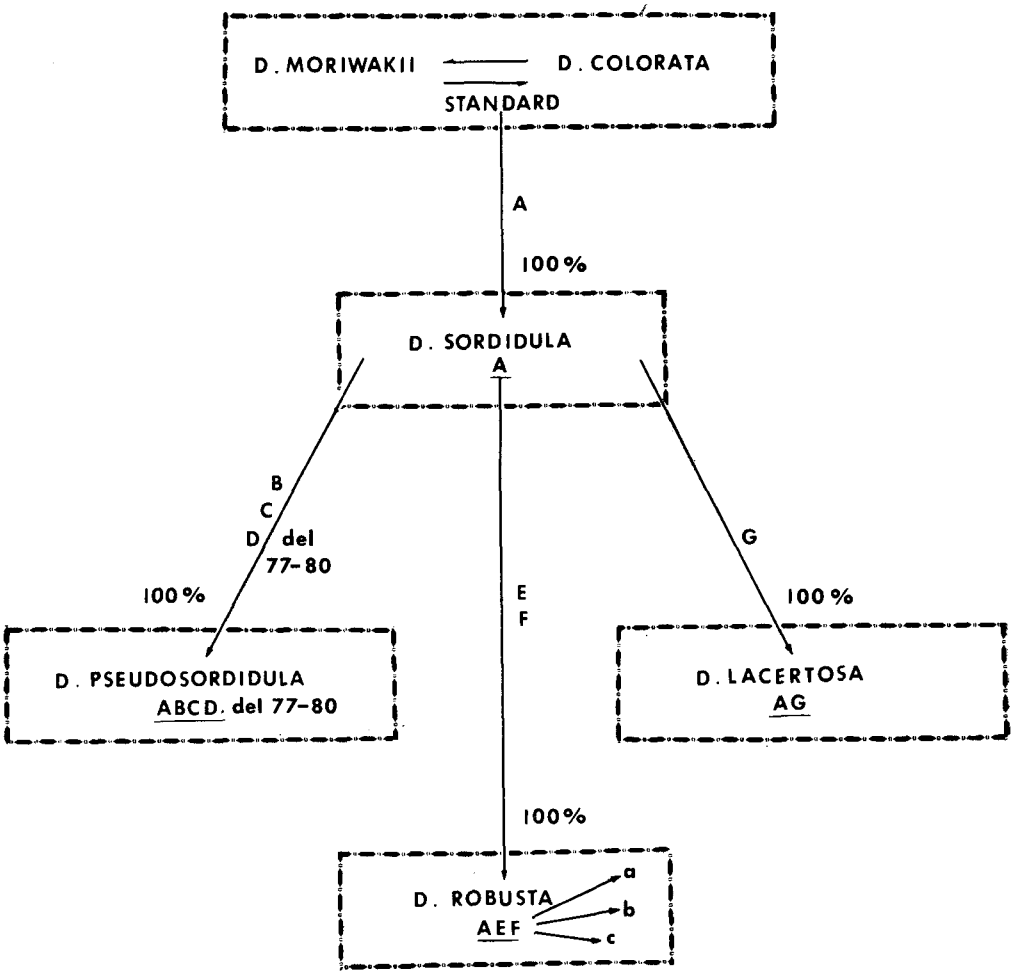
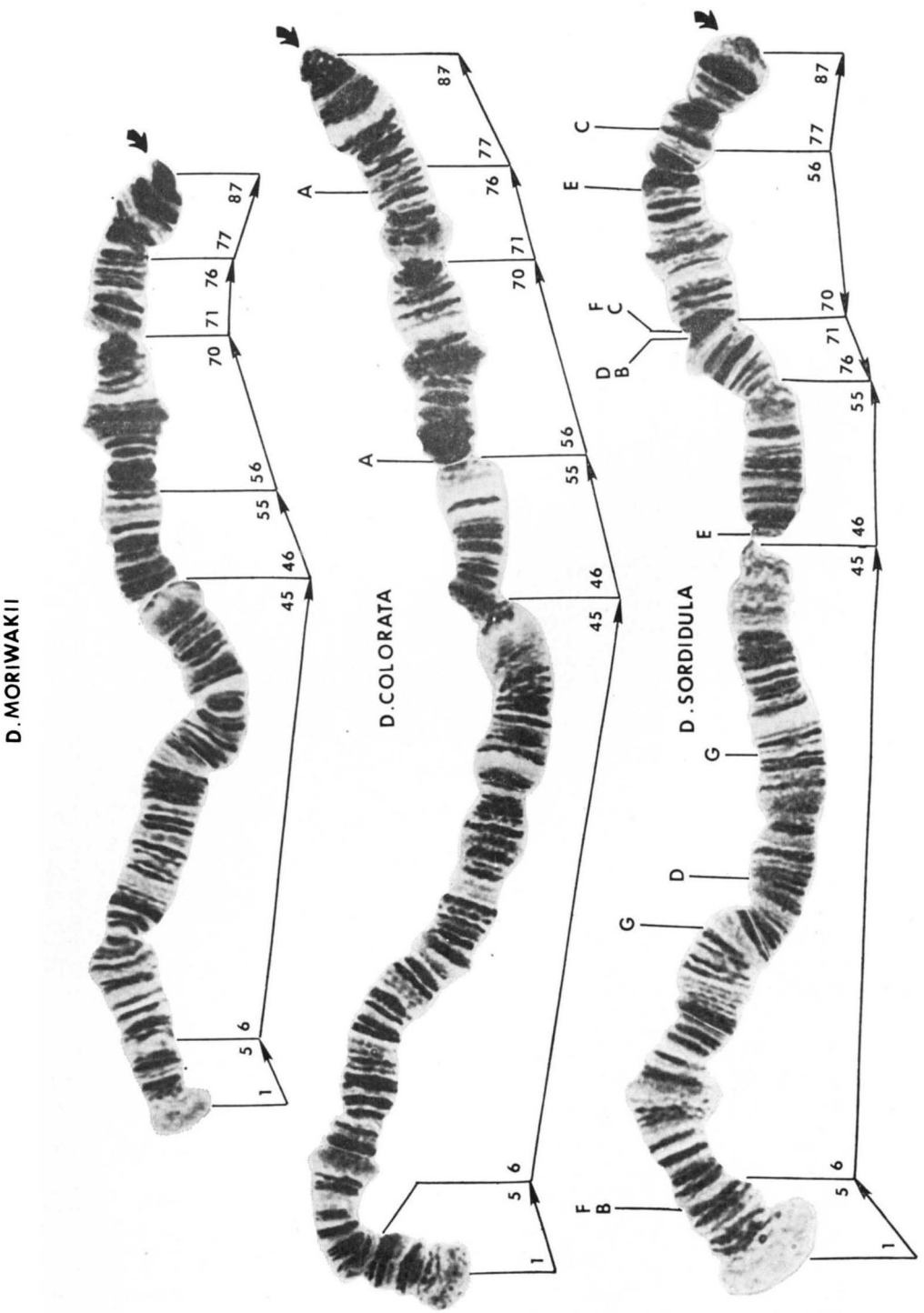


FIGURE 1.—The phylogeny based on inversions in chromosome X-right (Chr. 5 in *D. moriwakii* and *D. colorata*). The small letters inside the boxes represent the intraspecific inversions, the capital letters between the boxes represent homozygous or interspecific inversions. The percentages indicate the proportion of the chromosomes which could be homologized in any particular interspecific step.

100% of the chromosome in all the species. The X-right of all the species (chromosome 5 in *O. moriwakki* and *D. colorata*), has a total of seven interspecific inversions, and one short deletion. This relatively conservative element is known to have intraspecific inversions only in the species *D. robusta,* in which three heterozygous inversions have been reported (CARSON 1958).

Phylogenetic changes in chromosome X-left: The X-left phylogeny is summarized in Figure 4 (see also Figures 5 and 6). Chromosome X in *D. colorata* and *D. moriwakii* is homologous with the X-left of *D. sordidula* and the other three species. In the latter four species, a centric fusion has occurred between the elements homologous to the original X and the chromosome 5 of *D. colorata* and *D.*


D.MORIWAKII
D.COLORATA
D.SORDIDULA
1
5
6
45
46
55
56
70
71
76
77
87
A
B
C
D
E
F
G

moriwakii. Because of increasing difficulty in recognizing homologous regions and in order to simplify the diagram, two different numbering systems were used, one for *D. moriwakii, D. colorata,* and *D. sordidual* and the other for *D. sordidula, D. pseudosordidula, D. robusta,* and *D. lactertosa.* First, the chromosomes of *D. moriwakii* and *D. sordidula* are compared to the sequence found in *D. colorata,* which is chosen as the standard. Then, chromosome X-left of *D. sordidula* is renumbered and used as a standard to which the X-left of *D. pseudosordidula, D. robusta,* and *D. lacertosa,* are compared. The difficulty encountered in working out the homology is probably due to the numerous inversion differences between species. *D. moriwakii* was the only species in which it was possible to homologize 100% of the chromosome with that of *D. colorata.* Parts of the chromosomes in the other species have been left unidentified.

D. colorata differs from *D. moriwakii* (A) by the single inversion A. *D. colorata,* with the addition of the inversions B,C,D,E,F and G, leads to *D. sordidula* (BCDEFG). At this point in the phylogeny scheme *D. sordidula* is renumbered. Addition of four more inversions H,I,J, and K, leads to a hypothetical species (BCDEFGHIJK), which in turn gives rise to three branches. In the first branch, addition of three inversions L,M, and N, leads to *D. pseudosordidula* (BCDEFGHIJKLMN). The second branch, with the addition of a single inversion O, leads to *D. robusta* (BCDEFGHIJKO). The third branch, with the addition of a large number of inversions, leads to *D. lacertosa.* In *D. lacertosa* the chromosome has been so broken up that more than ⅓ of it could not be identified.

In *D. lacertosa,* in parts of the chromosome that could be recognized it was observed that the region 1–15 which is intact in *D. sordidula, D. pseudosordidula* and *D. robusta* has been broken up into 1–8 and 9–13. Further, the region 21–25 56 . . . . . 65 which occurs as a continuous block in *D. pseudosordidula* and *D. robusta* is rearranged as 65–62 21–25 56–61 (plate 4). In the above scheme, only inversions H and J could be identified in *D. lacertosa.* Inversions I and K could not be identified, probably because of extensive rearrangements. Alternatively, the possibility that I and K may not be present in *D. lacertosa* cannot be overlooked, in which case *D. lacertosa* would be in a branch between *D. sordidula* and the hypothetical species. However, it is assumed that these inversions are present since analyses of other chromosomes show that *D. lacertosa* comes after the hypotheticals.

Except for the three intraspecific inversions reported by CARSON (1958) in *D. robusta,* none has been found in this chromosome in any species. The first five members of the group, excluding *D. lacertosa,* have a total of at least sixteen interspecific inversions. Although in the phylogenetic analysis described above sev-

FIGURE 2.—The standard gene sequences in chromosome "X-right" of *D. moriwakii, D. colorata* and *D. sordidula* (Chr. 5 in *D. moriwakii* and *D. colorata*). Heavy arrows indicate the centromere ends of the chromosome arms. The numbers indicate regions of the chromosome sharing interspecific homology in banding pattern. The capital letters indicate limits of interspecific inversions known in each of the three species. Intraspecific inversions are indicated by lower case letters. The direction followed by the banding sequence in each of the species is indicated by arrows. Regions for which no homology could be established are unnumbered.

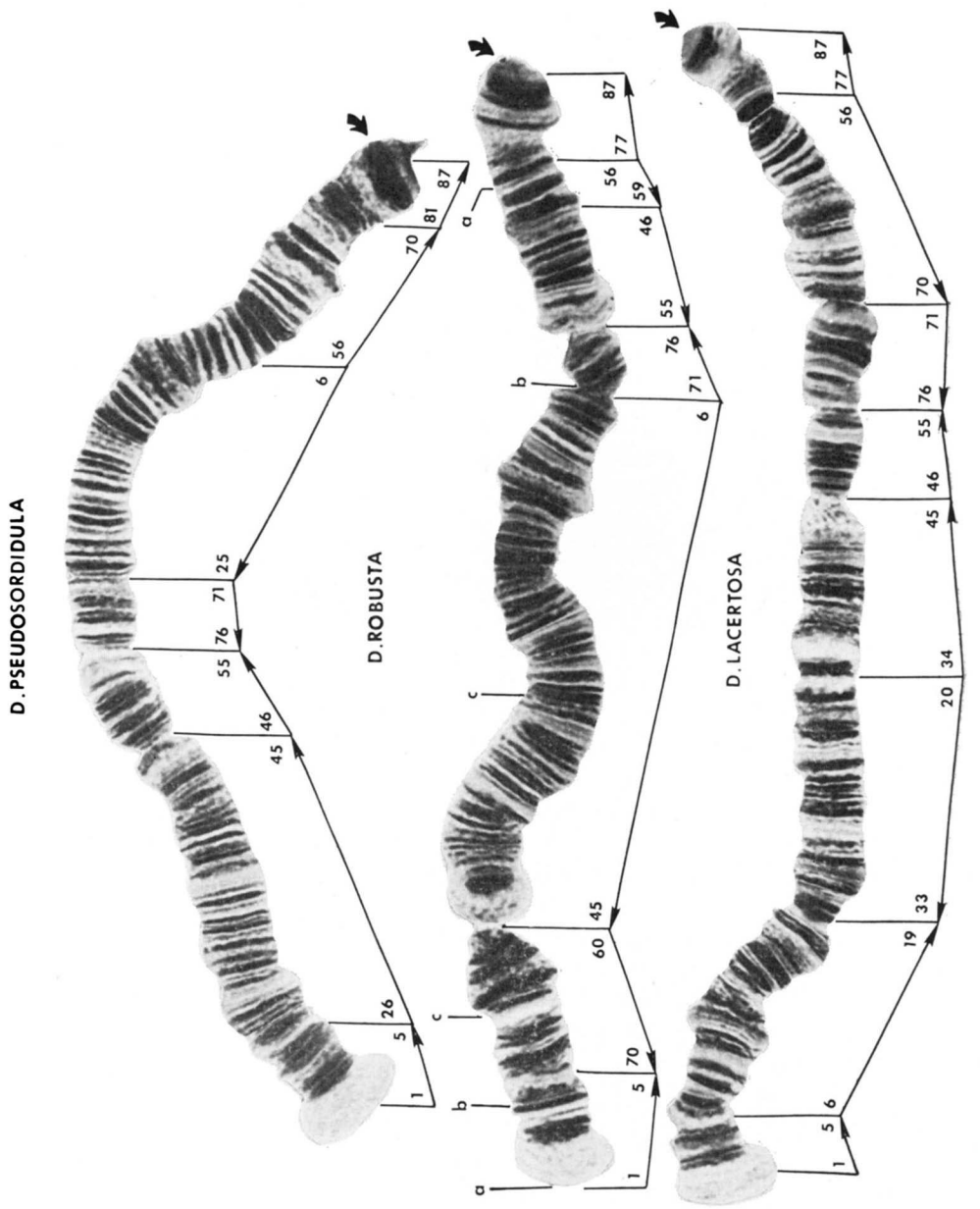


FIGURE 3.—The standard gene sequences in chromosome X-right of *D. pseudosordidula*, *D. robusta*, and *D. lacertosa*. The general meaning of the symbols is as in Figure 2.

eral breakpoints are shown as being shared by a number of interspecific inversions, this is undoubtedly due to limitations of the comparative methods used which could not distinguish breakpoints lying very closely together.

Phylogenetic changes in chromosome 3: Chromosome 3 phylogeny is sum-

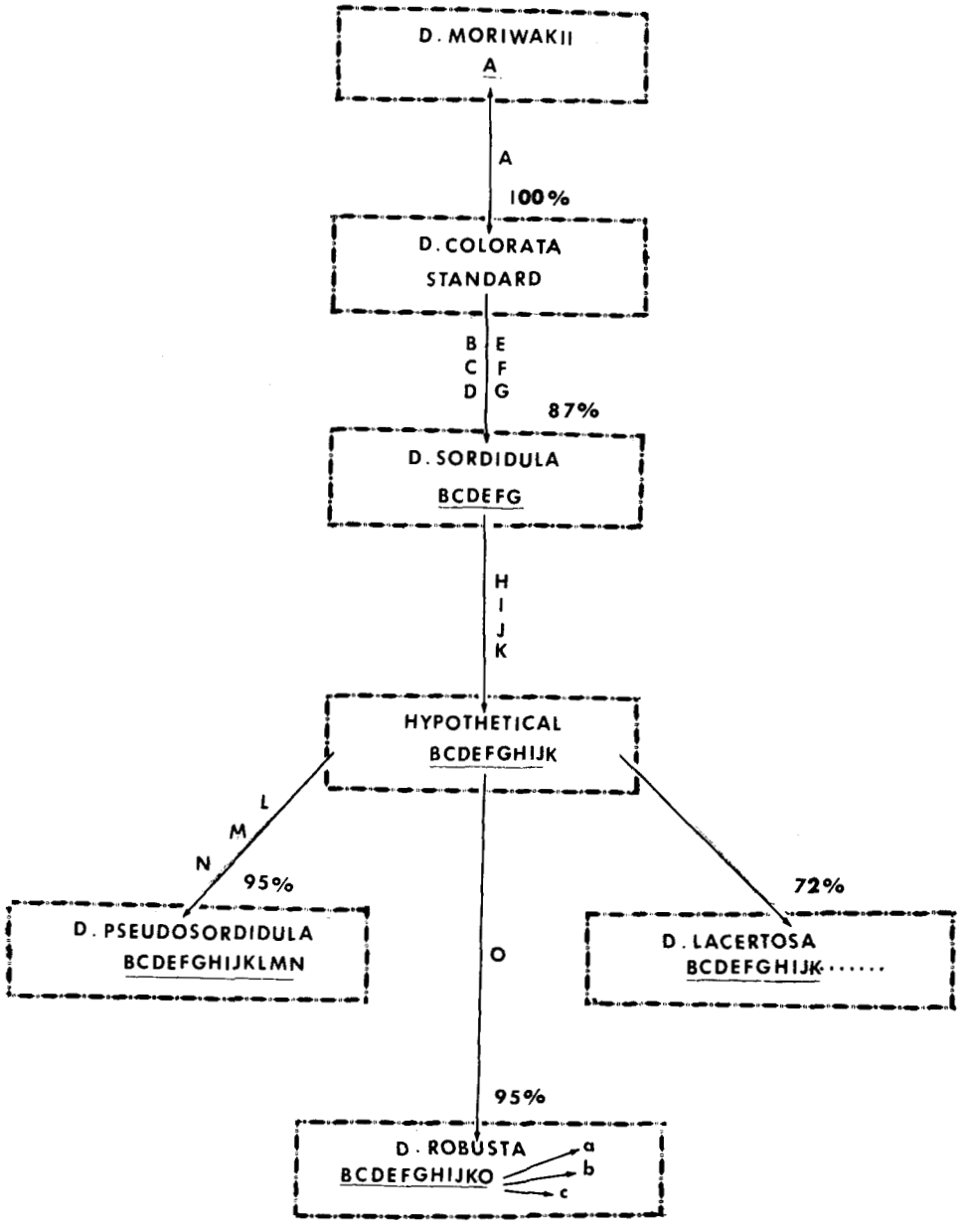


FIGURE 4.—The phylogeny based on inversions in chromosome X-left (X in *D. moriwakii* and *D. colorata*). The general meaning of the symbols is as in Figure 1; however, specific symbols A,B, or a,b, etc. in this figure have no relationship to the same symbols in other figures.

marized in Figure 7 (see also Figures 8 and 9). Chromosome 3 of *D. colorata* differs from that of *D. moriwakii* (A) by the single inversion A. The addition of five more inversions B,C,D,E, and F to *D. colorata* leads to the hypothetical spe-

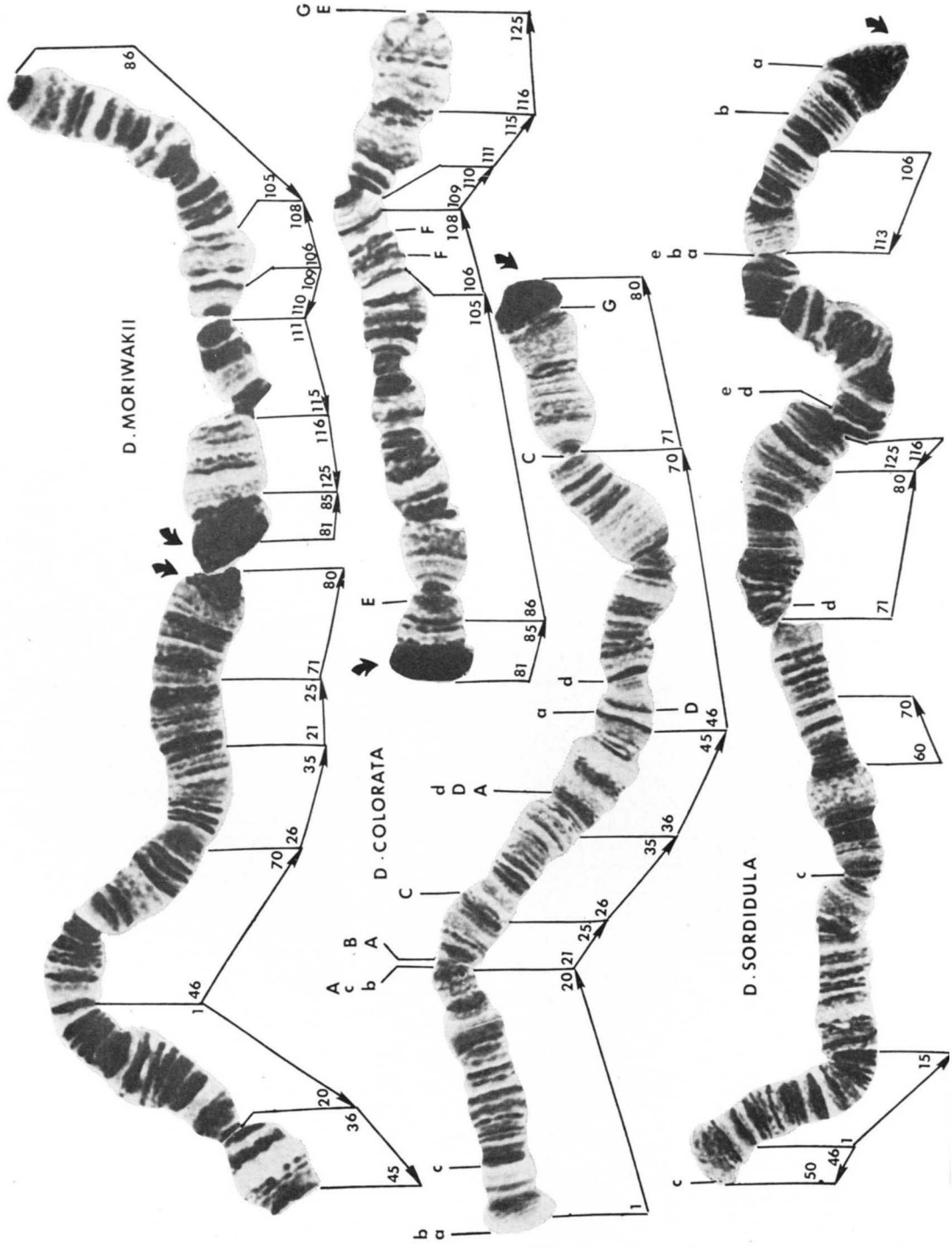


FIGURE 5.—The standard gene sequences in Chromosome "X-left" of *D. moriwakii*, *D. colorata* and *D. sordidula* (Chromosome X in *D. moriwakii* and *D. colorata*). The system of numbers

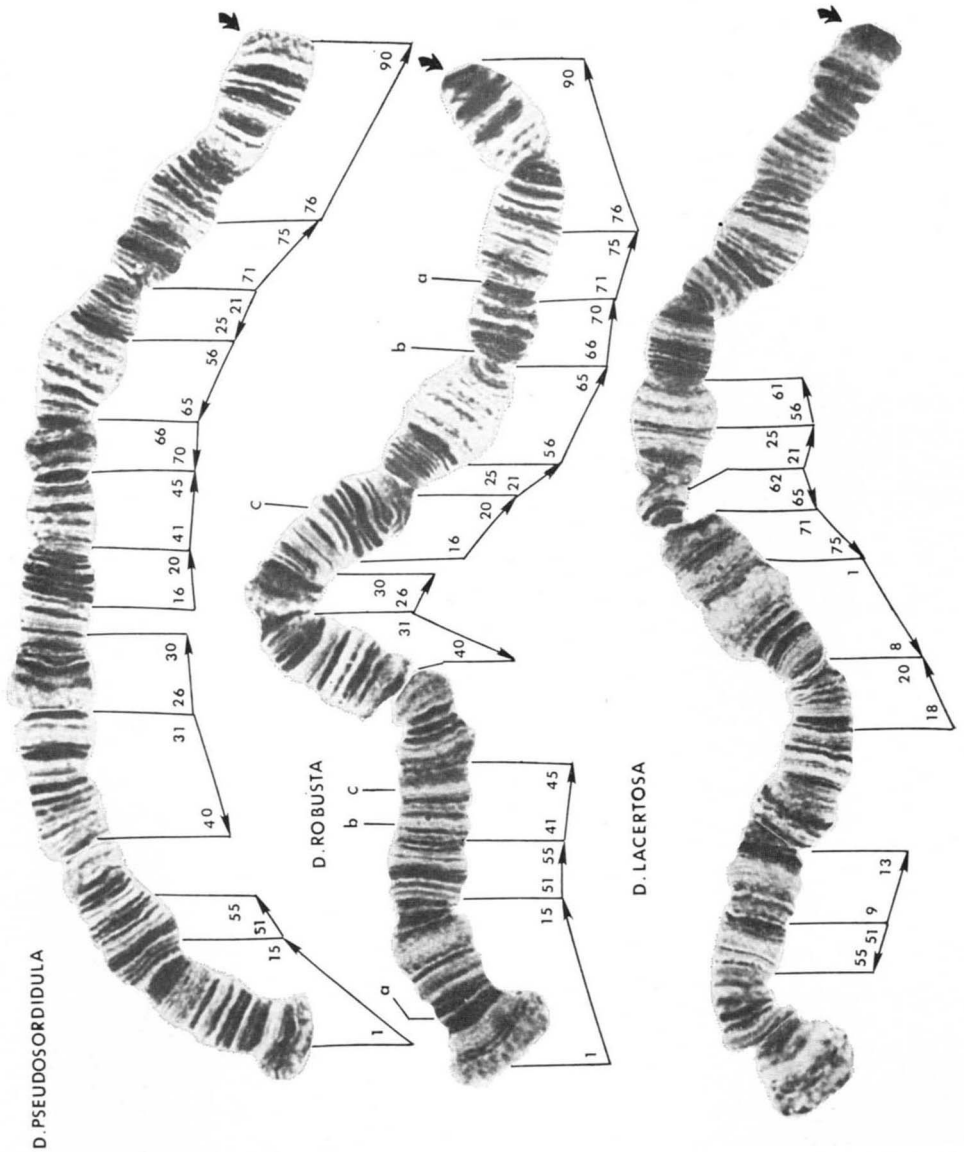


FIGURE 6.—The standard gene sequences in chromosome X-left of *D. pseudosordidula*, *D. robusta* and *D. lacertosa*. The system of numbers is comparable to that below the map of *D. sordidula* in Figure 5. The general meaning of symbols is as in Figure 2.

cies 1, (BCDEF). A branch from hypothetical 1, with the addition of inversion G and the apparent deletion of region 79–80 (Figure 8), leads to *D. sordidula* (BCDEFG), del. 79–80). The addition of two more inversions H and I to hypo-

above the map of *D. sordidula* should be compared with those of *D. moriwakii* and *D. colorata*. The numbers below the map or *D. sordidula* should be compared with those of *D. pseudosordidula*, *D. robusta* and *D. lacertosa*, in Figure 6. The two numbering systems in *D. sordidula* are independent. The general meaning of symbols is as in Figure 2.

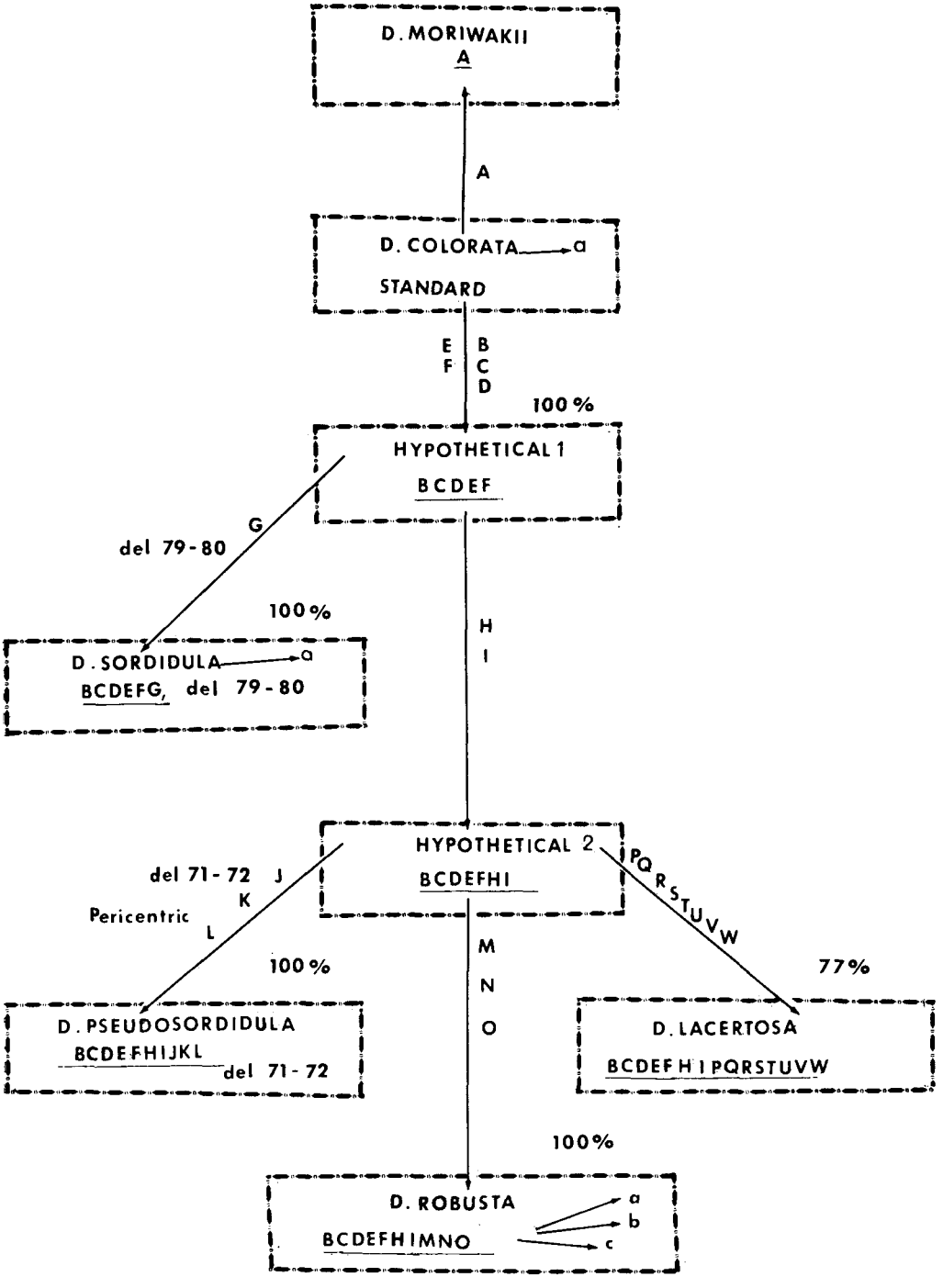


FIGURE 7.—The phylogeny based on inversions in chromosome 3. The inversion a in *D. sordidula* bears no relationship to inversion a in *D. robusta* or similarly designated inversions in the other chromosomes.

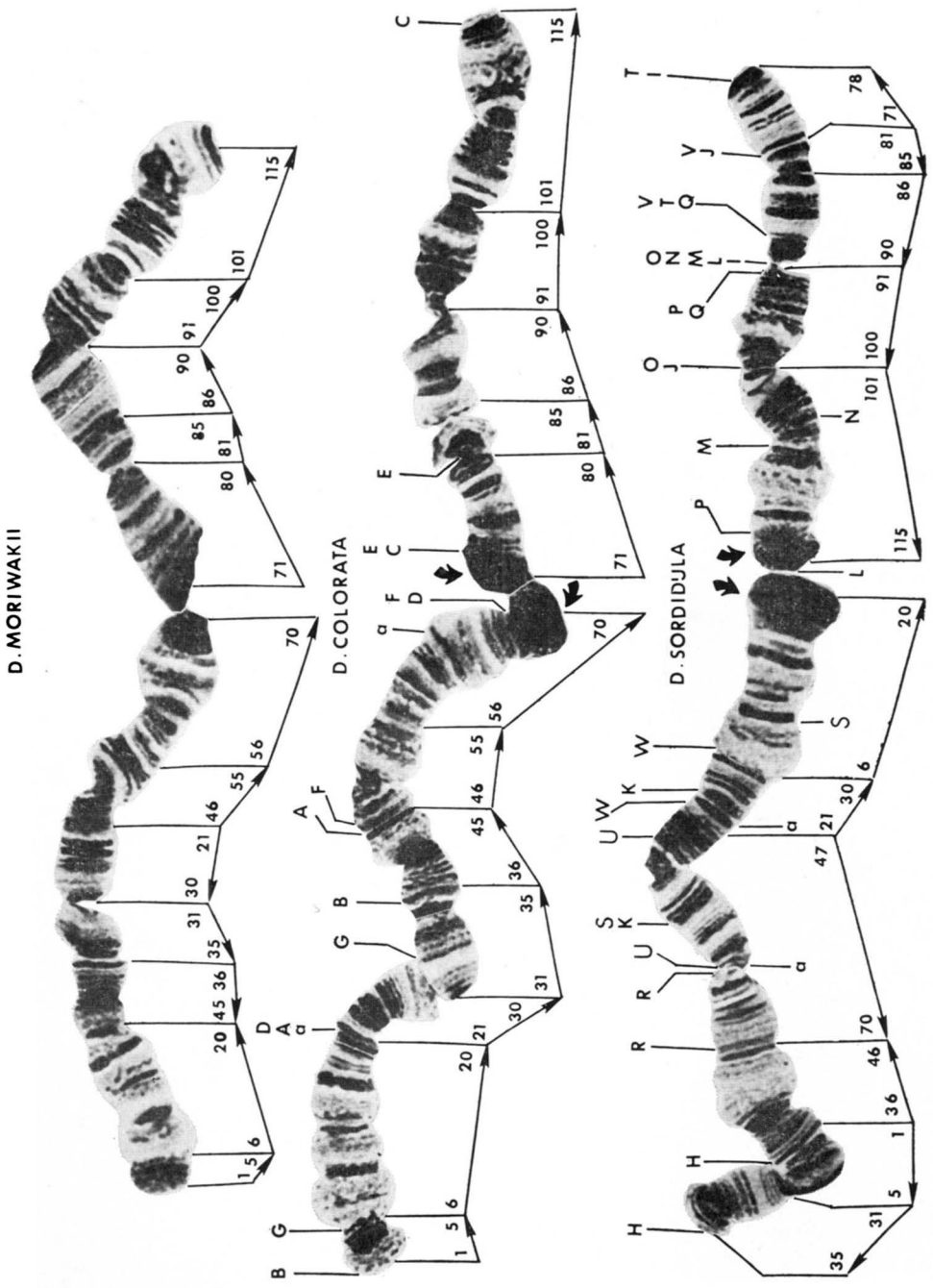


FIGURE 8.—The standard gene sequences in chromosome 3 of *D. moriwakii*, *D. colorata*, and *D. sordidula*. For explanation of symbols see Figure 2.

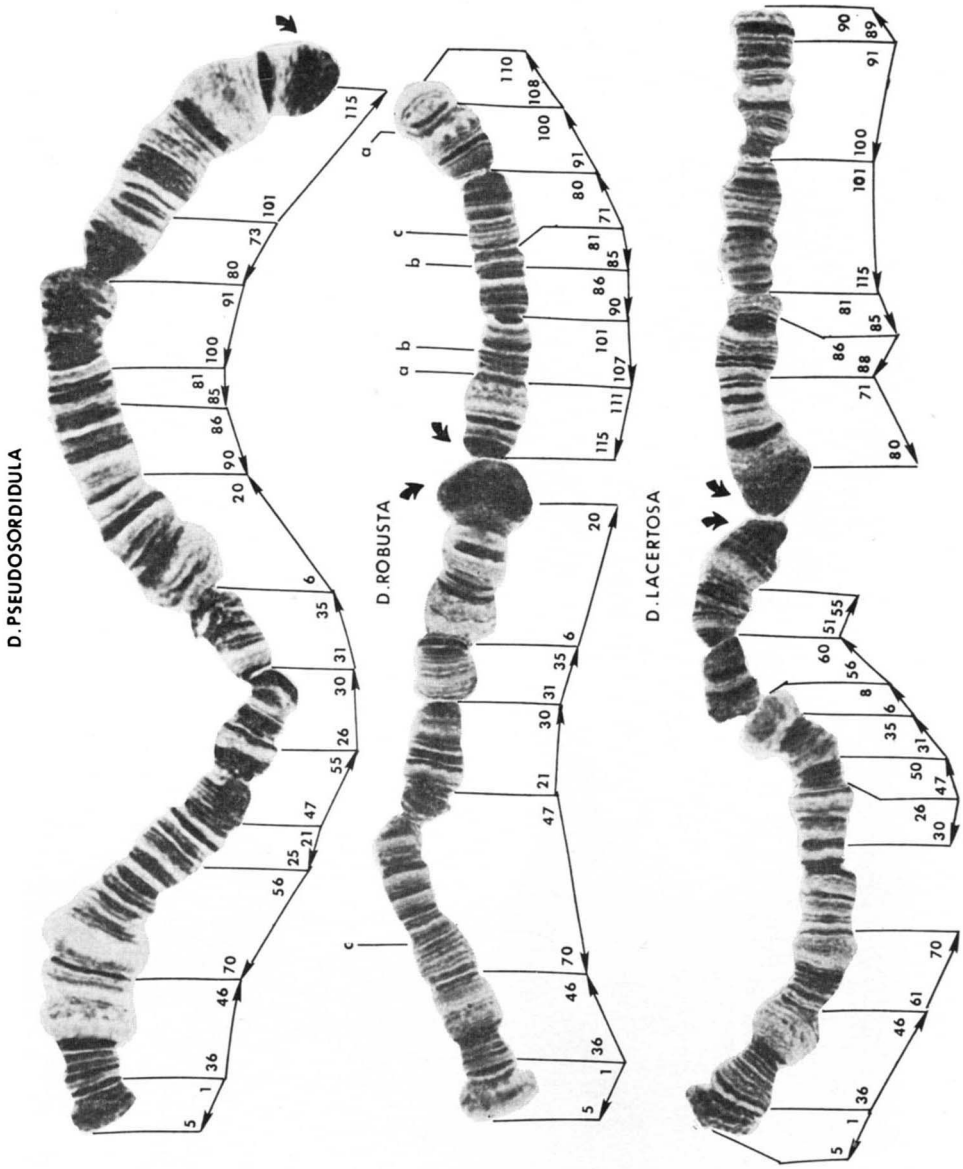


FIGURE 9.—The standard gene sequences in chromosome 3 of *D. pseudosordidula*, *D. robusta* and *D. lacertosa*. For explanation of symbols see Figure 2.

thetical 1 leads to hypothetical 2 (BCDEFHI). Three branches arise from hypothetical 2. One branch involves the three inversions J,K, and L, one of which is pericentric, and a deletion of region 71–72 (Figure 9) leading to *D. pseudosordidula* (BCDEFHIJKL, del. 71–72). The second branch, with the addition of three inversions M,N, and O, leads to *D. robusta* (BCDEFHIMNO). The third branch involves eight inversions PQRSTUVW, leading to *D. lacertosa* (BCDEFHIPQRSTUVW).

The occurrence of inversion G in *D. sordidula* and not in any other species, and the deletion of the region 79–80 in *D. sordidula* and its retention in *D. colorata* and *D. pseudosordidula* and the rest of the species, requires the hypothetical species 1, since *D. pseudosordidula, D. lacertosa,* and *D. robusta* cannot be derived from the modern *D. sordidula* karyotype. The fact that *D. pseudosordidula, D. robusta,* and *D. lacertosa* share inversions H and I (absent in all other species), and at the same time have several inversions unique for each of them, requires hypothetical species 2. Inversion F could not be identified in *D. lacertosa*; however, it is assumed to occur in *D. lacertosa* since hypothetical species 2 from which it is shown to arise on the basis of inversions H and I, has inversion F. It is possible to homologize 100% of chromosome 3 in all species except *D. lacertosa* in which only 77% of the chromosome could be homologized.

Single heterozygous inversion is found in the left arm of chromosome 3 of *D. sordidula.* An inversion complex, difficult to analyze, is observed in the left arm of *D. colorata* (SAYEED 1960; present study). In *D. robusta,* three intraspecific inversions, of which one is pericentric, have been reported by CARSON (1958). Chromosome 3 in all the species reveals a total of at least 25 intraspecific inversions. However, the actual number must be higher since chromosome 3 was only partially homologized in *D. lacertosa.*

Phylogenetic changes in chromosome 4: Chromosome 4 exists as a rod-shaped element in all the members of the group except in the case of *D. robusta* and *D. lacertosa*; in *D. robusta* it occurs as one arm (2R) of the V-shaped chromosome 2. Arm 2L corresponds to chromosome 2 in the rest of the group. In *D. lacertosa* chromosome 4 is a small V with a right and left arm produced by a pericentric inversion. The phylogeny of chromosome 4 is summarized in Figure 10 (see also Figures 11 and 12). Starting with the standard in *D. colorata,* the addition of two inversions A and B leads to the sequence found in *D. moriwakii* (AB). Again, *D. colorata,* with the addition of inversions C,D, and E, and the apparent deletion of region 70–66 (Figure 11) leads to the sequence found in hypothetical 1 (CDE, del. 70–66), from which two branches arise. One branch, without any further karyotypic change leads to *D. sordidula* (CDE, del. 70–66). The second branch, with the addition of the inversion F, leads to hypothetical 2 (CDEF, del. 70–66), from which two more branches arise. One branch, without any karyotypic change, leads to *D. pseudosordidula* (CDEF, del. 70–66). The second branch involves two more inversions G, and H, leading to hypothetical 3 (CDEFGH, del. 70–66), which in turn gives rise to two additional branches. One of these branches, with the addition of the inversion I, leads to *D. robusta* (CDEFGHI, del. 70–66). The second branch, with the addition of seven more inversions J,K,L,M,N,O, and P, one of which is pericentric, leads to *D. lacertosa* (CDEFGHIJKLMNOP, del. 70–66).

An analysis of chromosome 4 phylogeny reveals some interesting facts. It should be noted that chromosome 4 of hypothetical 1 and *D. sordidula* are identical as are those of hypothetical 2 and *D. pseudosordidula.* However, both hypotheticals are necessary to be consistent with the analysis of chromosome X-right, X-left, and 3. In the analysis of these chromosomes it has already been shown that *D. robusta* and *D. lacertosa* cannot be derived from the modern *D. pseudo-*

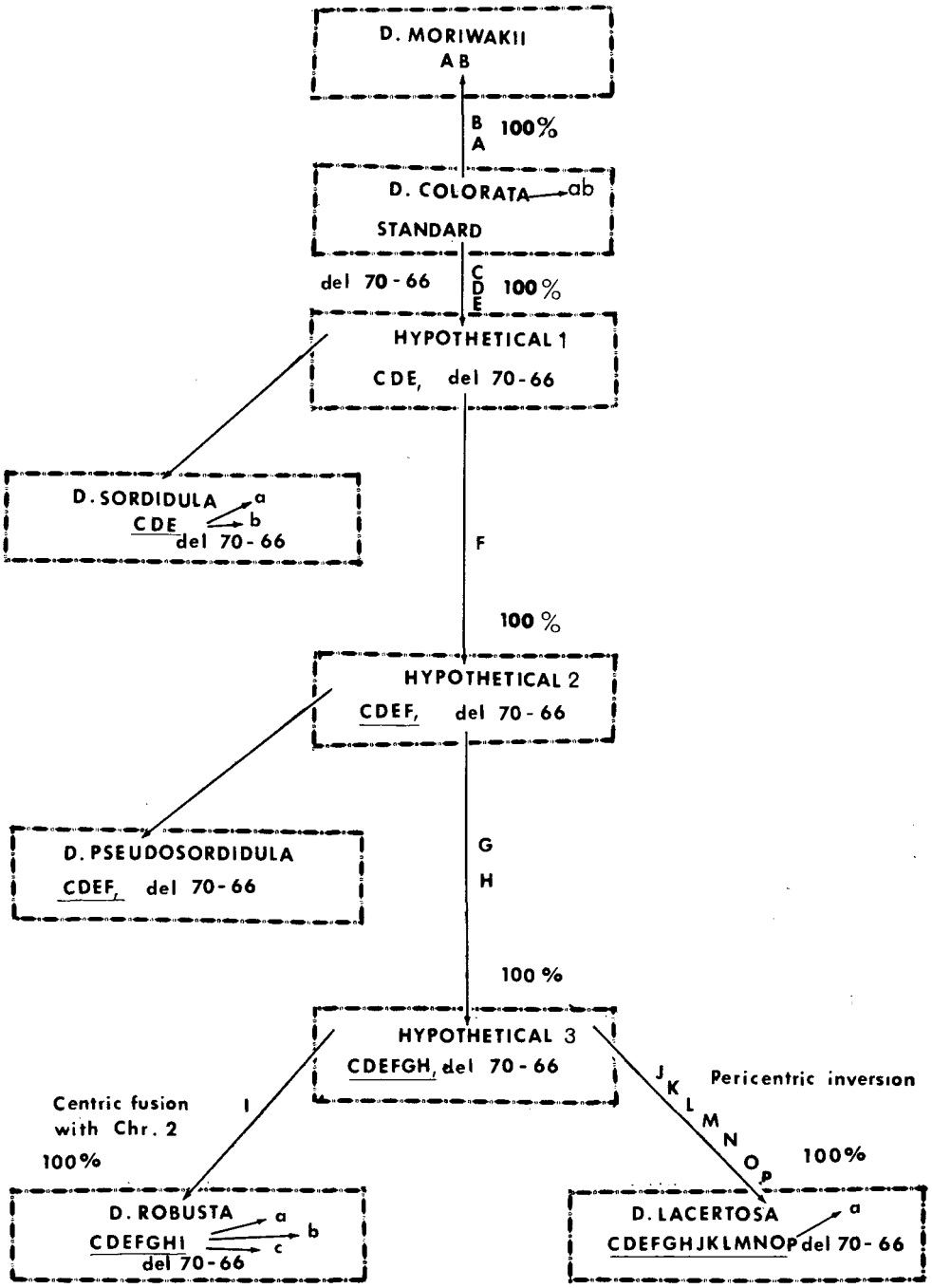


FIGURE 10.—The phylogeny based on inversions in chromosome 4. The general meaning of symbols is as in the other figures. The intraspecific inversions in *D. sordidula*, *D. robusta* and *D. lacertosa* designated by similar symbols have no relationship with one another.

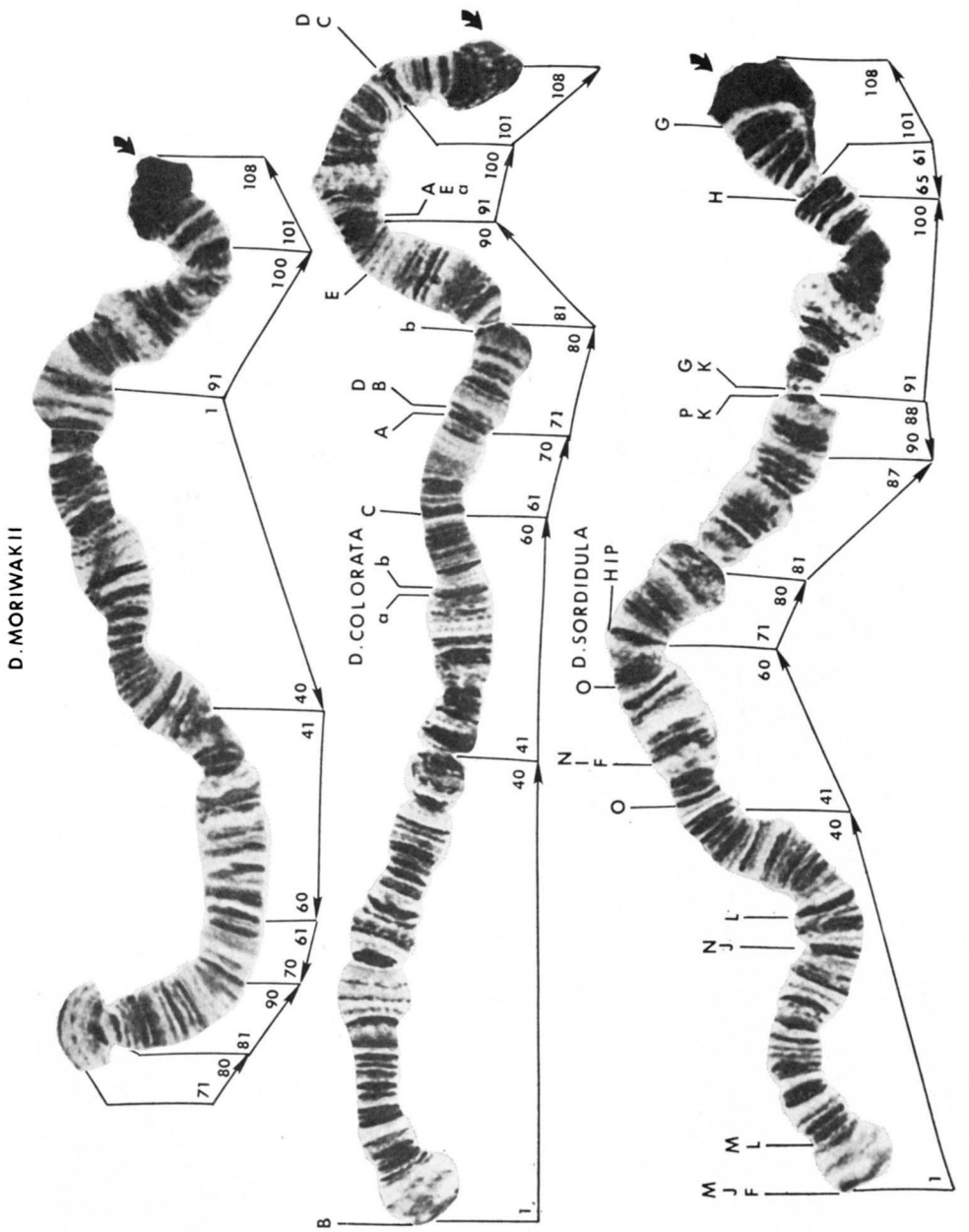


FIGURE 11.—The standard gene sequences in chromosome 4 of *D. moriwakii*, *D. colorata* and *D. sordidula*. For explanation of symbols see Figure 2.

sordidula, as those two species lack inversions which are found in the modern *D. pseudosordidula*. In the analysis of chromosome 3 it has been shown that it is not possible to derive the other species from the modern *D. sordidula*. For the

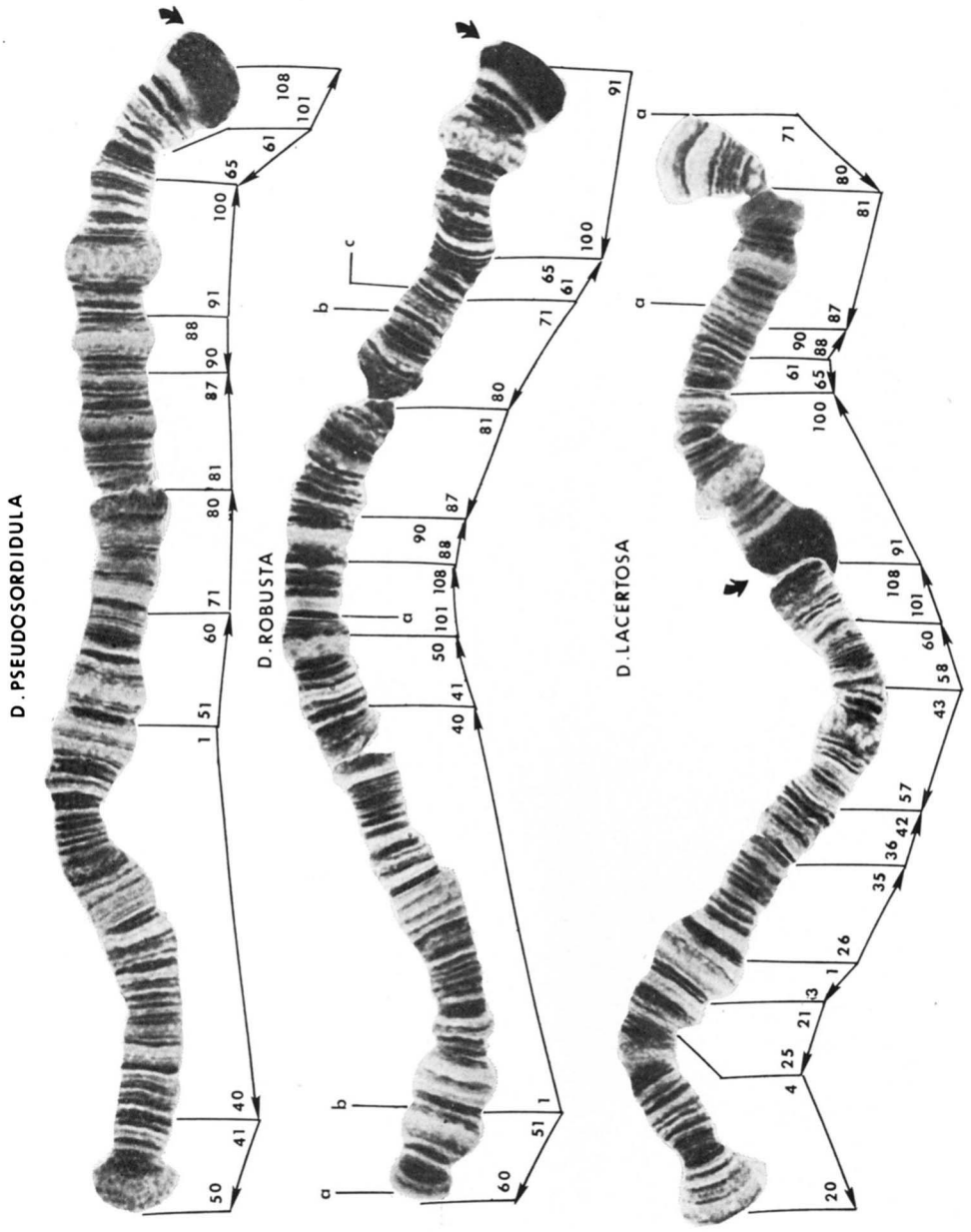


FIGURE 12.—The standard gene sequences in chromosome 4 of *D. pseudosordidula*, *D. robusta* and *D. lacertosa* (Chromosome 2-right in *D. robusta*). For explanation of symbols see Figure 2.

above reasons hypotheticals 1 and 2 are indicated in the analysis of chromosome 4 although they are not needed to explain its phylogeny. The absence of any karyotypic differences between the hypotheticals and the two species *D. sordidula* and *D. pseudosordidula* is related to the fact that chromosome 4 is a relatively

conservative one, having a total of only 16 interspecific inversions, with 100% of the chromosomes of all the species homologized. The fact that *D. robusta* and *D. lacertosa* share inversions G and H (absent in other species) and have at the same time inversions unique for each species, requires hypothetical 3. Inversion F cannot be identified in *D. lacertosa*; however, since F occurs in hypothetical species 3 from which *D. lacertosa* is shown to arise on the basis of inversions G and H, F is assumed to be present in *D. lacertosa* also. *D. sordidula* has two adjacent inversions in this chromosome invariably found as double heterozygotes. Approximately 25 larvae of each of five different strains of *D. sordidula*, including one from Korea, were examined. No homozygotes of either sequence, standard or inverted, was observed. *D. lacertosa* has a short, apparently terminal, inversion in the right arm. Three intraspecific inversions, including a pericentric one, have been reported for this chromosome by CARSON (1958) in *D. robusta*. In *D. colorata*, Itasca, two inversions, one included in the other, forming a complex configuration, were observed.

Phylogenetic changes in chromosome 2: Chromosome 2L of *D. robusta* is homologous to chromosome 2 of all the other members of the group. This chromosome has turned out to be the most difficult to homologize between species, probably because of a large number of interspecific inversions. Chromosome 2 of *D. colorata* has been completely homologized with that of *D. moriwakii*, the analysis aided by the hybrids that were obtained between them. Only partial homologies were established in the case of the other species comparisons.

The phylogeny of chromosome 2 has been summarized in Figure 13 (see also Figures 14 and 15). Chromosome 2 of *D. colorata* is again chosen as the arbitrary standard for the group. Two inversions a and b (b included in a, which is terminal), were observed in the left arm of chromosome 2 of *D. colorata*. The inverted ab sequence of these intraspecific inversions is found to be closer to *D. moriwakki* than is the standard *D. colorata* sequence, as shown in the map (Figure 14). The ab sequence in *D. colorata* differs from the sequence found in *D. moriwakii* (ab, ABCDEF) by six interspecific inversions A,B,C,D,E, and F. The addition of the pericentric inversion G to the standard sequence of *D. colorata*, which converts the metacentric chromosome 2 into an acrocentric, leads to hypothetical 1. This pericentric inversion brings the region 106 . . . . 125 adjacent to the region 71–80, thus leading to the continuous sequence 60–70 71–80 125 . . . . . 106. Hypothetical 1 gives rise to two branches. In one branch leading to *D. sordidula*, the addition of inversions has separated the region 60–70 from 71–80 and has fragmented the region 125 . . . . 106 into two separate regions 125–116 and 113–106, ending in *D. sordidula*, which has the sequence 50–46 1–15 . . . . 60–70 . . . 71–80 126–116 . . . . 113–106. The second branch from hypothetical 1, without any karyotypic change leads to hypothetical 2, which in turn gives rise to two branches. One branch, without further karyotypic change leads to *D. pseudosordidula* which has the sequence 50–46 1–15 . . . . 60–70 71–80 125 . . . . 106. In the second branch, two or more inversions separate the regions 60–70, 71–80, and 125 . . . . 106 from one another and at the same time bring together in one continuous sequence the previously separate regions 15–1 46–50 A 60–70 leading

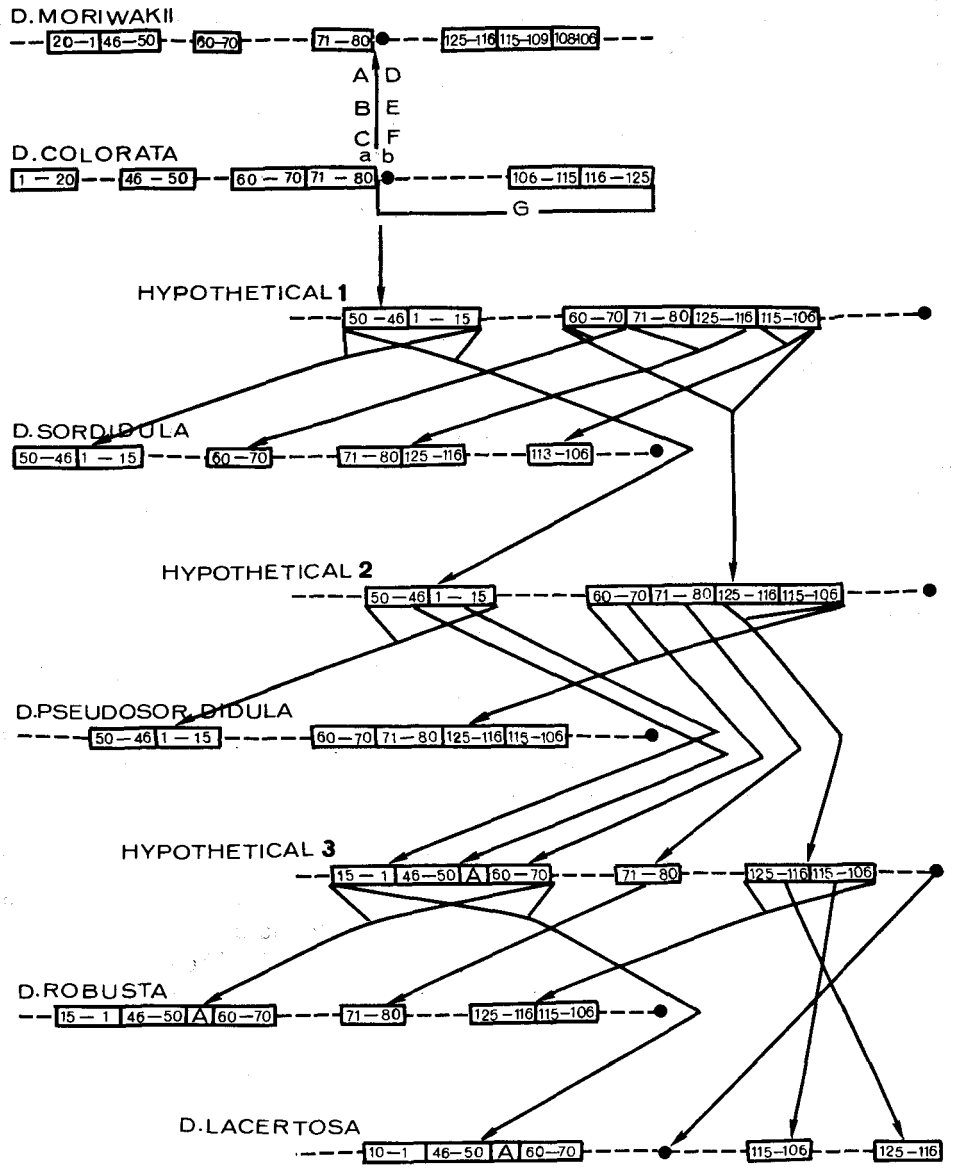


FIGURE 13.—The phylogeny based on partial analysis of chromosome 2 in the *D. robusta* group. For the sake of convenience in following the phylogeny, the identical regions in chromosome 2 of the above species are indicated by arrows. Dotted lines indicate unidentified portions of the chromosome. Letter A in the chromosomes of H_3, *D. robusta*, and *D. lacertosa* represents a short section that could be identified only in the above species and in no others. The capital letters A,B,C,D,E, and F, and the lower case letters a and b, designate the interspecific and intraspecific inversions that will convert the standard gene sequence found in *D. colorata* chromosome 2 to the standard gene sequence found in that of *D. moriwakii*. The pericentric inversion G will convert the standard gene sequence in *D. colorata* chromosome 2 into the sequence found in that of Hypothetical 1.

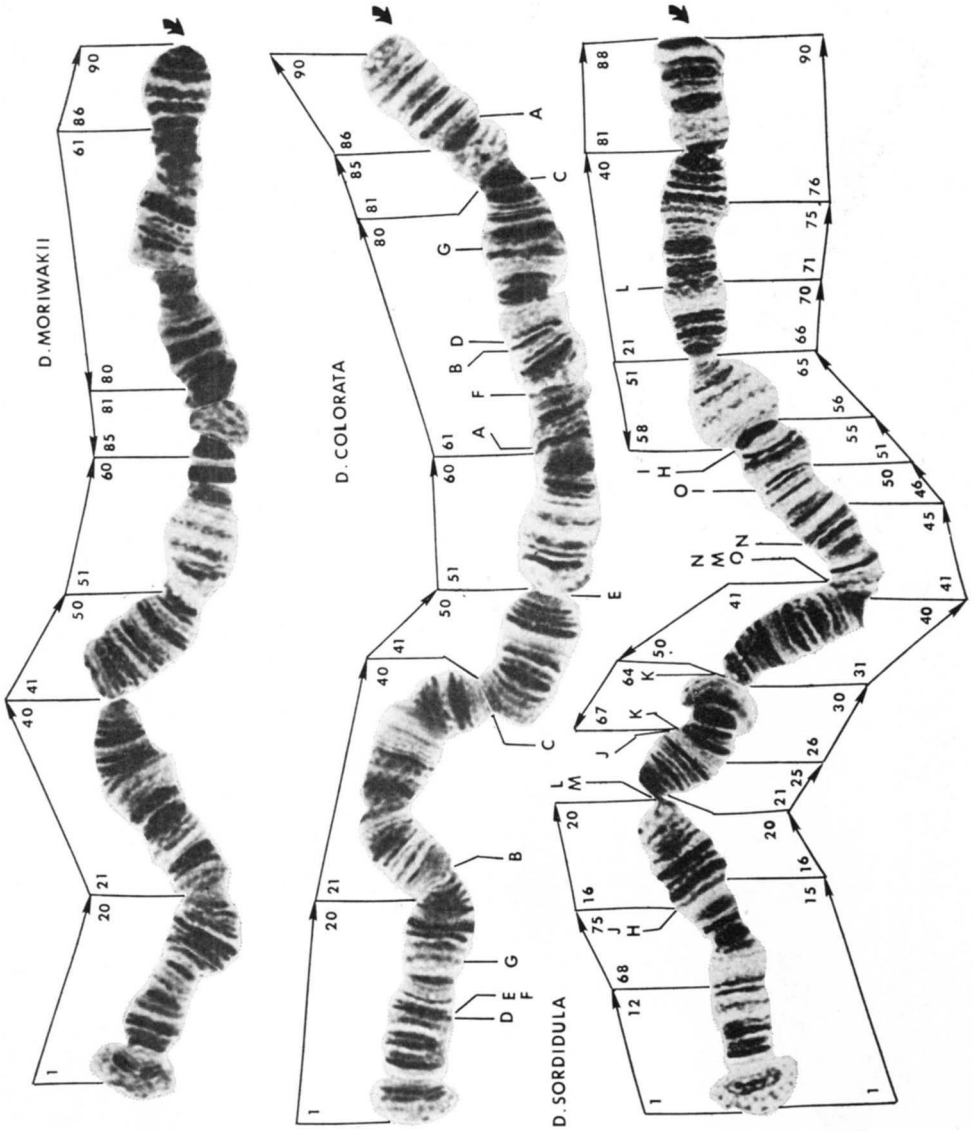


FIGURE 14.—The standard gene sequences in chromosome 2 of *D. moriwakii*, *D. colorata* and *D. sordidula*. The two chromosomes in the middle represent the left and right arms of chromosome 2 of *D. colorata*. The explanation of symbols is the same as in Figure 2.

to hypothetical 3. The region A was given a special designation instead of numbering because it could be identified only in *D. lacertosa* and *D. robusta*. Hypothetical 3, without further karyotypic change leads to *D. robusta*. In the other branch leading to *D. lacertosa* a number of inversions, including a pericentric, result in a fragmentation of the region 125 . . . . 106 into two separate regions 115–106 and 125–116 and also a fragmentation of the region 15–1 so that only

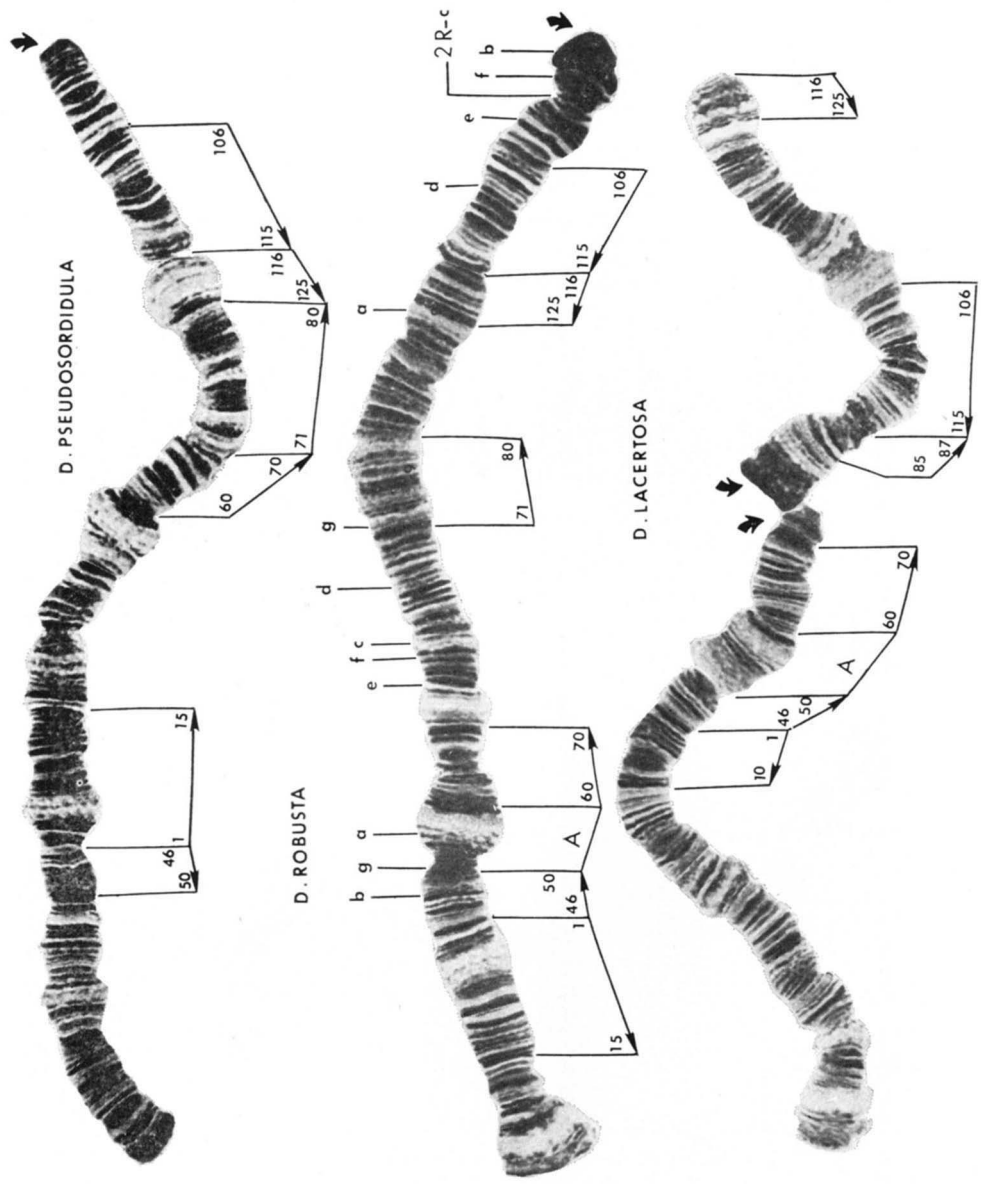


FIGURE 15.—The standard gene sequences in chromosome 2 of *D. pseudosordidula*, *D. robusta* and *D. lacertosa* (Chromosome 2-left in *D. robusta*). 2R-c is the limit of the pericentric inversion across the centromere of chromosome 2 of *D. robusta*. A represents the region in chromosome 2 that could be identified only in *D. lacertosa* and *D. robusta*, and not in any other species. For explanation of symbols see Figure 2.

10–1 portion is recognizable. This branch ends in *D. lacertosa*, which has the sequence 10–1 46–50 A 60–70 . . . . 115–106 . . . . 125–116. The region 71–80 could not be identified, presumably because it was broken up by inversions.

The phylogeny suggested by chromosome 2 depends in part on partially homologized regions of the chromosomes. It will be noted that *D. pseudosordidula* has the sequence 60–70 71–80 125 . . . . 106 which can be directly derived from *D. colorata* by a single pericentric inversion, suggesting that this sequence is closer to *D. colorata* than that found in modern *D. sordidula*. However, it has already been shown in the analyses based on the chromosomes X-right, 3 and 4, that *D. sordidula* is closer to *D. colorata* and *D. moriwakii* than are any of the other species. This indicates the existence of a hypothetical species 1 which had the same chromosome 2 sequence presently found in modern *D. pseudosordidula*, from which *D. sordidula* should have arisen. It has also been shown in the earlier analyses of salivary chromosomes XR, XL and 3 that the chromosomes of the type found in modern *D. pseudosordidula* could not have given rise to those in the species *D. robusta* and *D. lacertosa*. Hence it is necessary to postulate hypothetical 2 as ancestral to *D. pseudosordidula* and the other two species. It has already been shown in the analyses of the salivary chromosome 4 that *D. robusta* cannot give rise to *D. lacertosa* or vice versa in view of their unique inversions, and therefore must both arise from a common ancestor. Hence it is probable that *D. robusta* and *D. lacertosa* shared some of the inversions which separated the previously adjacent regions 125 . . . . 106 and 71–80, and also brought together in continuous sequence the previously separated regions 46–50, A, and 60–70. In *D. lacertosa* even though the region 71–80 was not identified, still it should be noted it is either shifted from its original position adjacent to 125 . . . . 106, or so broken up as to be unidentifiable.

In *D. robusta* this chromosome, by a centric fusion with the chromosome homologous to chromosome 4 of *D. colorata*, has been converted into chromosome 2-left. In the case of *D. lacertosa* another pericentric inversion, probably different from G (Figure 13) has occurred, converting the rod-shaped chromosome 2 to a V-shaped one. The results of the partial analysis of the banding pattern of this chromosome above show that *D. colorata* and *D. lacertosa* are separated by at least three hypothetical species in which chromosome 2 is acrocentric. Further, *D. robusta*, in which this chromosome lacks the pericentric found in *D. lacertosa*, is closely related to that species through their common ancestor, hypothetical 3. Since neither *D. robusta* nor hypothetical 3 shows the pericentric, this indicates that it arose independently in *D. lacertosa* and is not simply derived from the pericentric in *D. colorata* and *D. moriwakii*.

In *D. sordidula* five different intraspecific inversions a,b,c,d, and e, have been observed in this chromosome, independently and in random combination with one another, the details of which have been shown in Table 5. In *D. robusta* seven intraspecific inversions have been reported in this arm by CARSON (1958; Figure 15).

The metaphase chromosomes: The metaphase configurations of all the species used in the present study were from larval ganglionic cells of males and females. *D. moriwakii* and *D. colorata* have identical metaphase chromosome configuration, 2 pairs of Vs, 3 pairs of rods, and a pair of V-shaped microchromosomes. One of the rod-shaped chromosomes is the X and the other two are chromosome 4 and

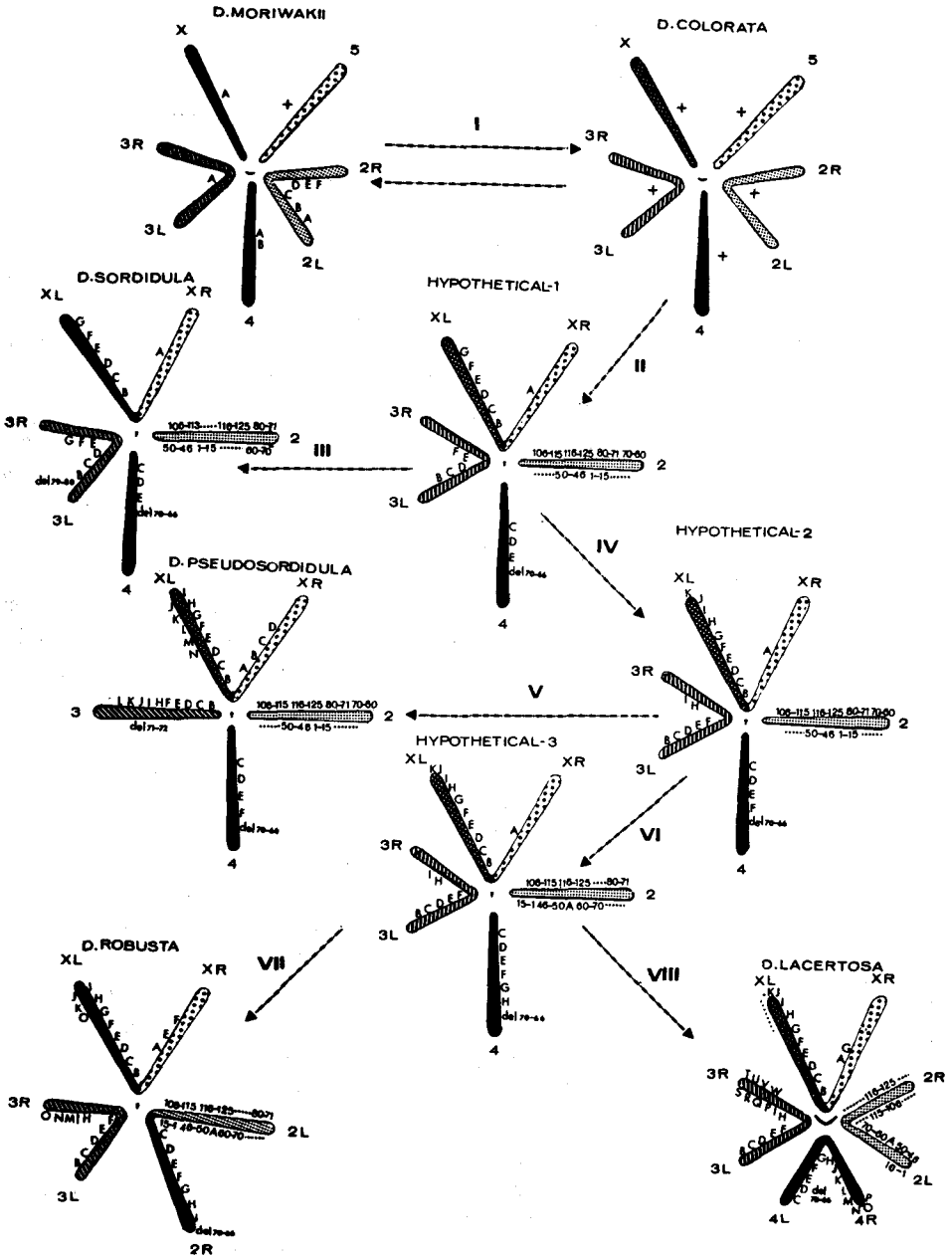


FIGURE 16.—The chromosome phylogeny of the *D. robusta* group. The letters alongside each chromosome arm represent the homozygous inversions fixed in each species. The numbers alongside chromosome 2 indicate the regions of the chromosome that were homologized and used for partial analysis. The arrows indicate the probable evolutionary phylogeny. The Roman numerals represent the eight different steps in the phylogeny of the group.

chromosome 5, while the V-shaped chromosomes are chromosome 2 and chromosome 3 (see Figure 16). The metaphase chromosomes of *D. moriwakii* have been described by TOKUMITSU *et al.* (1967) and those of *D. colorata* by SAYEED (1960). In both of the above cases one of the chromosomes here described as a V has been described as a J. The V designation is preferred by this author since there is little difference between the length of the two arms in either of the metacentric chromosomes.

D. moriwakii and *D. colorata* are the only species in the group studied so far which have a rod-shaped X. The other species have a V-shaped X resulting from the fusion of the original X and an autosome homologous with chromosome 5 of *D. moriwakii* and *D. colorata.*

D. sordidula has 1 pair of large V's (X), one pair of small V's (3), two pairs of rods (2 & 4), and a pair of dots (6); *D. pseudosordidula* has one pair V's (X), 3 pairs of rods (2, 3, & 4) and one pair of dots (6). *D. lacertosa* has one pair of large V's (X), three pairs of small V's (2, 3, & 4), and one pair of microchromosomes (6) almost the same size as the other autosomes. In *D. sordidula, D. pseudosordidula, D. robusta* and *D. lacertosa* the Y chromosome is also V-shaped like the X. This has been substantiated by CARSON and STALKER (1946; 1947) for *D. robusta,* by TOKUMITSU *et al.* (1967) for *D. sordidula* and *D. pseudosordidula,* and by the author for *D. lacertosa.*

In a study of the evolution of the species within the group two kinds of karyotypic patterns have to be considered, one with a rod-shaped, and the other with a V-shaped X. Evolutionary fragmentation of chromosomes leading to an increase in the number of centromeres presumably requires a donor chromosome to provide an extra centromere or else a centromere duplication, whereas fusion of two rods involves only the loss of a short section containing the centromere. Fusion of chromosomes resulting in a reduction in the number of centromeres has been assumed to occur in Drosophila speciation more generally than a separation of V-shaped chromosomes into rods. This is especially likely to be true when the V-shaped chromosome involves the X chromosome and an autosome (WHITE 1964). Therefore species with rod-shaped X are generally considered the more ancestral ones. Hence it is assumed that the rod-shaped X, observed in *D. moriwakii* and *D. colorata,* represents the ancestral condition for this group, and that the V-shaped X observed in the rest of the species is derived. This, in addition to the fact that *D. moriwakii* and *D. colorata* are the closest to species outside the group, tends to confirm the assumption that these two are the ancestral species in the group.

Of the four species with the V-shaped X, *D. sordidula* is found to be the closest to *D. moriwakii* and *D. colorata,* as shown by the analysis of the salivary chromosomes. *D. sordidula* metaphase chromosomes can be derived from those of *D. moriwakii* or *D. colorata* by a fusion of the X and an autosome and by a pericentric inversion of chromosome 2. *D. pseudosordidula* metaphase chromosomes can be derived from those of *D. sordidula* by a pericentric inversion in chromosome 3 which converts the V-shaped chromosome 3 into a rod-shaped one. *D. robusta* metaphase chromosomes can be derived from those of *D. sordidula* by a

fusion of chromosomes 2 and 4. *D. lacertosa* metaphase chromosomes can be derived from those of *D. sordidula* by pericentric inversions in chromosomes 2 and 4. *D. pseudosordidula* and *D. robusta* can be derived from *D. moriwakii* or *D. colorata* only through the karyotype found in *D. sordidula*, and their positions in the phylogeny are secure beyond any reasonable doubt. *D. lacertosa* shows some ambiguity in its position in the phylogeny, in view of the fact that it resembles the probable ancestral species *D. colorata* and *D. moriwakii* in having a V-shaped chromosome 2 and at the same time resembles the derived species in having a metacentric X.

However, it has already been shown by analysis of the salivary gland chromosomes that *D. lacertosa* is very distant from *D. colorata* and *D. moriwakii*, separated from them by at least three hypothetical species, and that *D. sordidula* karyotype has to be a structural intermediate between *D. colorata* and *D. lacertosa*, and that the pericentric inversion in chromosome 2 of *D. lacertosa* is not derived from the one in *D. colorata* or *D. moriwakii*. Based on this, *D. lacertosa* is considered closer to the derived species than the ancestral. The metaphase chromosomes suggest the same basic phylogenetic pattern suggested by the chromosome X-right. (see Figure 1).

The data from the critical analyses of all the chromosomes are consistent with the same phylogenetic pattern. Analysis of the metaphase chromosomes and of the relatively conservative X-right shows the basic pattern (Figure 1), *D. colorata* leading to *D. sordidula* and the latter giving rise to three branches, one leading to *D. pseudosordidula*, another leading to *D. robusta*, and a third to *D. lacertosa*. The other chromosomes, although they show the basic pattern, reveal additional phylogenetic details. In chromosome 3 it has been observed that *D. sordidula* has a unique inversion G, and a deletion of the region 79–80, not found in any of the other species. This indicates hypothetical species 1 from which *D. sordidula* arises. Analyses of chromosome X-left and chromosome 3 both indicate that the branches leading to *D. pseudosordidula*, *D. robusta* and *D. lacertosa* do not arise directly from hypothetical species 1. In chromosome X-left the above three species share the inversions H,I,J and K, which are absent in *D. sordidula* and hence in hypothetical species 1. In chromosome 3 they share the inversions H and I not found in *D. sordidula* or hypothetical species 1. This indicates a second hypothetical species 2 (Figures 4 & 7), as a common ancestor to *D. pseudosordidula*, *D. robusta*, and *D. lacertosa*, but not to *D. sordidula*. Analysis of chromosome 4 phylogeny suggests a third hypothetical species 3 (Figure 10). In chromosome 4, *D. robusta* and *D. lacertosa* share two inversions G and H, which are not found in *D. pseudosordidula*. This would indicate that there must have been a third hypothetical, a common ancestor for *D. robusta* and *D. lacertosa*, and not for *D. pseudosordidula*.

Chromosome 2 phylogeny, although based on incomplete analysis, fits well into the phylogenetic pattern indicated by the rest of the chromosomes. All the hypothetical species suggested by chromosomes X-left, 3, and 4 are indicated by chromosome 2. Based on all of the analyses above, the pattern of the phylogenetic relationships among the six species is presented in summary form in Figure. 16.

DISCUSSION

The derived phylogeny is of special interest when the geographic distribution of the species involved is taken into account. Of the ten known species of the *D. robusta* group, only two, *D. colorata* and *D. robusta* have been reported from the New World. The other eight species are known only from Asia: *D. cheda* from Korea and mainland China; *D. pullata* from mainland China; *D. lacertosa* from Japan and Taiwan; *D. moriwakii*, *D. pseudosordidula*, *D. okadai*, and *D. neokadai* from Japan; and *D. sordidula* from Korea and Japan. Such a distribution pattern, partly Palearctic and partly Nearctic, is not unique to the *D. robusta* group of Drosophila. In the *D. melanica* group, seven species have been found in the New World, while one member *D. pengi*, is known from Japan (STALKER 1966). In the *D. virilis* group, *D. virilis* is a commonly-occurring species in China and Japan, while it occurs sporadically in the United States (HSU 1952). *D. ezoana* of this group has been recently reported from Japan and the other seven species in the group are found in the New World. These patterns of distribution raise the question of the origins of such species groups. The origin of the *D. melanica* group is still unknown (STALKER 1966); the possible origin and diversification of the members of the *D. virilis* group have been discussed by HSU (1952), who suggests an oriental origin of the hypothetical ancestor of the group, with its migration to the Nearctic region while the two continents were still united.

In a consideration of the distribution of the members of the *D. robusta* group, at the outset, it would seem reasonable to divide the members into two groups, a Palearctic, and a Nearctic, separated by the ocean barrier, and assume that the ancestors must have crossed the barrier once in the life history of the group. This would lead to the assumption that in the New World *D. robusta* was derived directly from *D. colorata*. However, the phylogeny derived from the chromosome analysis shows that *D. robusta* is closer to the Japanese members of the group than to *D. colorata*. This finding suggests that at least two migrations must have occurred, one involving *D. colorata*, and the other, *D. robusta*.

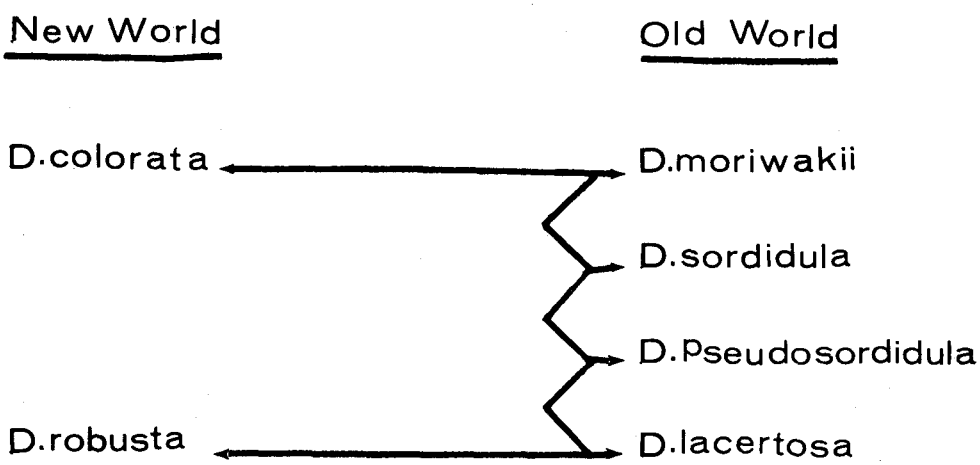


FIGURE 17.—Diagrammatic representation of the New World-Asiatic migration of the *D. robusta* group.

The number of species in the *D. robusta* group known from the orient is more than the number known from the New World, and new species are still being discovered in Japan. Active speciation seems to have occurred in Japan with the production of a well-developed species group but has not occurred in the New World, where there are no phylogenetically continuous series but only two distantly related terminal species. Further, if the group originated in the New World, *D. colorata* must be ancestrial to *D. moriwakii* and their separation should have occurred much earlier than the separation of *D. robusta* and *D. lacertosa.* However, chromosomal analysis shows that *D. colorata* and *D. moriwakii* are much closer to each other than any of the other members of the group, having incomplete reproductive isolation, and a total of only ten inversion differences between them. On the other hand *D. robusta* and *D. lacertosa* have complete reproductive isolation, and have more than 25 inversion differences between them. This suggests that the separation of *D. robusta* and *D. lacertosa* must have taken place earlier than the separation of *D. colorata* and *D. moriwakii*, and this is possible only if the group originated in the Old World.

On the basis of the above evidence, it is postulated that the group originated in Asia (possibly Japan) and speciated actively there. Two separate migrations took place from the Palearctic to the Nearctic region, leading to the establishment of *D. colorata* and *D. robusta*, which did not speciate further. The migration leading to *D. robusta* probably took place first, and the one leading to *D. colorata* much later. This might also explain why *D. robusta* has a much more extensive population in the United States than *D. colorata*, which is considered a rarer species.

The route of migration was probably across the Bering Land Bridge, by which intercontinental dispersal of many plants and animals seems to have taken place during the Cenozoic period. During the late Oligocene to the middle Miocene, a mixed conifer-broad leaved deciduous forest apparently existed around the northern Pacific region, continuously from Japan through Alaska into northwestern United States up to Oregon (Wolfe *et al.* 1967). This indicates a probable land connection between the Asiatic mainland and Japan during this period. In Japan this kind of flora had not changed much, and broad-leaved forests are found to persist even to the present day, whereas the flora of northwestern United States has been replaced by the more modern vegetation. This indicates a probable discontinuity in the land connection between Japan and the Asiatic mainland after middle Miocene.

Floral records also indicate warmer temperature conditions during Miocene, and a rapid decline in the temperature during and after late Miocene. With respect to the ecology of the members of the *D. robusta* groups, it is well known that they are associated more with forest vegetation (especially broad-leaved deciduous trees) than any other type of environment in Hokkaido and other parts of Japan. In the United States the distribution of *D. robusta* was found to be approximately co-extensive with the eastern deciduous forests (Carson 1958). *D. colorata* also has approximately the same distribution as that of *D. robusta* but occurs in much smaller numbers.

On the basis of the geological evidence, the warmer climatic conditions that

seem to have existed early in the Miocene, and the ecology of the species group, it is postulated that the migrations of the species which led to *D. colorata* and *D. robusta* probably took place during the period extending from late Oligocene to middle Miocene, about 20–25 million years ago.

This idea is in accordance with the available paleontological data. Evidence for the age of Drosophila comes from the two poorly preserved specimens of the family Drosophilidae from the amber of Chiapos, Mexico, obtained from the collections of the University of California (WHEELER 1963). These specimens were found to be of late Oligocene to early Miocene in age, about 30 million years old. An estimation of the minimum age of the Drosophilidae as a group is thus possible on the basis of amber evidence which would in turn substantiate our hypothesis that the age of *D. robusta* group is in the neighborhood of 20–25 million years.

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