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Phylogenetic relationships in the *Drosophila melanogaster* species subgroup: An ultrastructural histochemical study of the secretory granules in the larval salivary gland cells of *D. orena* and *D. sechellia*

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The *melanogaster* subgroup is divided into two complexes: the *melanogaster* complex, which includes *D. melanogaster, D. sechellia, D. simulans,* and *D. mauritiana*, with secretory granules consisted mainly of a filamentous material, and the *yakuba* complex, which includes *D. erecta, D. orena, D. yakuba* and *D. teissieri*, with secretory granules consisted mainly of a granular material. The secretory granules of *D. orena* consist almost exclusively of a granular material with high electron density which after periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining displays intense reactivity; sometimes a small "knob" - like PA-TCH-SP negative structure is revealed. The secretory granules of *D. sechellia* consist mainly of a filamentous material with medium to high electron density, while occasionally, in the periphery of the secretory granules, an electron lucent material is seen, which after post-fixation of tissues with OsO_4 disclosed high electron density and a granular appearance. Following the PA-TCH-SP staining, the filamentous material is positive, while the peripheral granular material is negative. The revised phylogenetic diagram of the *melanogaster* subgroup, based on the morphological and histochemical characteristics of the secretory granules of the salivary gland cells, and the proposed direction of evolution, is as follows: *D. erecta* \ *D. orena* \ *D. yakuba, D. teissieri* and *hypothetical species II* which yields *D. melanogaster* and *D. sechellia* which in turn yields *D. simulans* and *D. mauritiana*.

Key words: *Drosophila*, *melanogaster* subgroup, phylogenetic relationships.

INTRODUCTION

The phylogenetic relationships and the mode of evolution among several *Drosophila* species have attracted the interest of evolutionary biologists for many years (e.g. Throckmorton, 1975; Ashburner *et al*., 1984; Lemeunier *et al*., 1986; Singh, 1989; Grimaldi, 1990; Thomas & Hunt, 1993; Caccone *et al*., 1996; Kliman *et al*., 2000; Remsen & O'Grady, 2002; Kastanis *et al*., 2003). A variety of scientific approaches has been used to elucidate the phylogenetic relationships in different *Drosophila* species groups and subgroups. Such approaches include chromosomal banding patterns (e.g. Lemeunier & Ashburner, 1976, 1984; O'Grady *et al*., 2001), reproductive isolation (e.g. Lachaise *et al*., 1986; Lee & Watanabe, 1987; Coyne, 1989; Ting *et al*., 2000), analysis of cuticular hydrocarbons (Jallon & David, 1987; Coyne & Charlesworth, 1997), analysis of chorion proteins (Kalantzi-Makri *et al*., 1985), morphological and histochemical characteristics of the secretory granules in larval salivary gland cells (Thomopoulos, 1987), two-dimensional electrophoresis and allozyme variation (e.g. Cariou, 1987; Lee & Watanabe, 1987; Spicer, 1988; Coulthart & Singh, 1988; Matsuo *et al*., 1999), interspecific ovarian transplantation (Lamnissou & Zouros, 1989), analysis of mitochondrial and nuclear DNA sequences (e.g. Solignac & Monnerot, 1986; Solignac *et al*., 1986; Coyne & Kreitman, 1986; Satta & Takahata, 1990; Andersson & Lambertsson, 1991; Hale & Singh, 1991; Nigro *et al*., 1991; DeSalle, 1992; Peixo-

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to *et al*., 1992; Hey & Kliman, 1993; Kliman & Hey, 1993; Pissios & Scouras, 1993; Jeffs *et al*., 1994; Ross *et al*., 1994; Hilton *et al*., 1995; Shibata & Yamazaki, 1995; Caccone *et al*., 1996; Ballard *et al*., 2000; Arhontaki *et al*., 2002; Tsujino *et al*., 2002; Kastanis *et al*., 2003), rRNA sequencing (Pelandakis & Solignac, 1993; Lohe & Roberts, 2000), Southern analysis of heat shock proteins (Molto *et al*., 1994), and DNA hybridization and cloning (Caccone *et al*., 1988, 1992; Hartl *et al*., 1994). Despite all these efforts, the conclusions concerning the interrelationships among the different *Drosophila* species and especially the direction of evolution are not definite.

The secretory granules in the larval salivary gland cells of every *Drosophila* species studied so far, disclose a different morphological appearance as well as a distinctive distribution of the vic-glycol groups of the complex carbohydrates (Thomopoulos & Kastritsis, 1979; Thomopoulos, 1987; Thomopoulos *et al*., 1989, 1992). The unique morphology of the secretory granules provides us with a very special "tool" to characterize each species and to attempt the construction of a phylogenetic tree in different *Drosophila* species subgroups. The above-mentioned features have already been used in the investigation of the phylogenetic relationships among six species (available at that time) of the *Drosophila melanogaster* species subgroup (Thomopoulos, 1987). In the present study I report on:

i) the morphology of the secretory granules of the larval salivary gland cells of *Drosophila orena* and *D. sechellia*,

ii) the distribution of the vic-glycol groups of complex carbohydrates in their secretory granules, and

iii) the revised phylogenetic tree of the eight species of the *Drosophila melanogaster* species subgroup.

MATERIALS AND METHODS

The *Drosophila* species used in this study were obtained from the Department of Genetics, University of Patras, Greece. The insects were raised as described earlier (Thomopoulos & Kastritsis, 1979). The salivary glands were dissected out in cold *Drosophila* saline solution, pH 7.0, and immediately fixed for 1 h in cold Karnovsky's fixative (Karnovsky, 1965) buffered with 0.2 *M* phosphate buffer, pH 7.4. After washing with 0.2 *M* phosphate buffer, the glands were postfixed in aqueous 2% OsO_4 solution for 1h, rinsed with distilled water, and placed in an aqueous 0.5% uranyl acetate solution for 14-18 h. For the histochemical detection of complex carbohydrates the previous two steps were omitted. The glands were dehydrated through an ethanol series, infiltrated, and embedded in Spurr's resin (Spurr, 1969). The polymerization period was 9 h at 70-71 °C. Silver to pale gold sections (from 5-8 blocks for each species, containing 2-3 glands each) were cut using glass knives and mounted on copper, stainless steel or gold grids.

For the morphological examination of the secretory granules the sections were stained with uranyl acetate-lead citrate (Reynolds, 1963). For the localization of the complex carbohydrates with vicinal glycol groups, the periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) method of Thiery (1967) was used. Control sections were stained only with the thiocarbohydrazide-silver proteinate sequence. The sections were examined and photographed in a JEOL 100B electron microscope. Fifty photographs for each species were used for the estimation of the maximum mean diameter of the secretory granules. Approximately 10 secretory granules (excluding the smaller granules) from each photograph were used for the measurement of their diameter.

RESULTS

The secretory granules are surrounded by a single membrane and their usual shape is oval or spherical. The secretory granules of *D. orena* are mostly irregularly elongated, but both oval or spherical granules are also seen. The maximum mean diameter of the secretory granules of *D. orena* (excluding the irregularly elongated granules) reaches 1.86 mm, and that of *D. sechellia* 2.42 mm.

Drosophila orena

The secretory granules consist almost exclusively of a granular material with high electron density (Fig. 1). There are no differences in the appearance of the secretory granules in the salivary gland cells processed with or without osmium tetroxide, after the initial fixation in Karnovsky's fixative. The granular material after PA-TCH-SP staining displays an intense reactivity (Fig. 2). In a few cases, particularly after PA-TCH-SP staining, a small "knob" - like or "minicap" - like PA-TCH-SP negative structure is revealed (Fig. 3).

All bars represent 1 mm — FIGS 1-3. *Drosophila orena.*

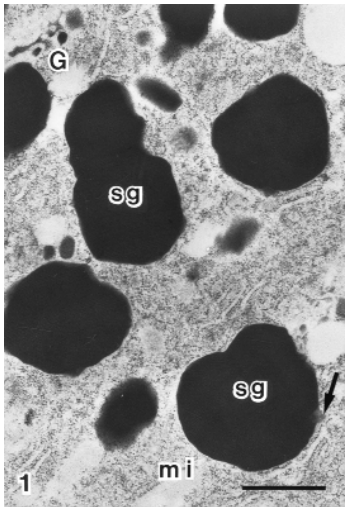


FIG. 1. Secretory granules (sg) displaying a high electron density and consisted of a granular material. In some cases "minicap" - like structures (arrows) are evident in the periphery of the secretory granules. mi=mitochondria, G=Golgi complex. Fixation: Karnovsky's fixative, no post-osmication. Staining: uranyl acetate - lead citrate (UA-LC). x 15,000.

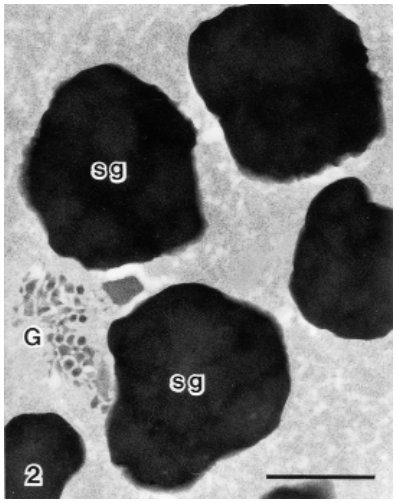
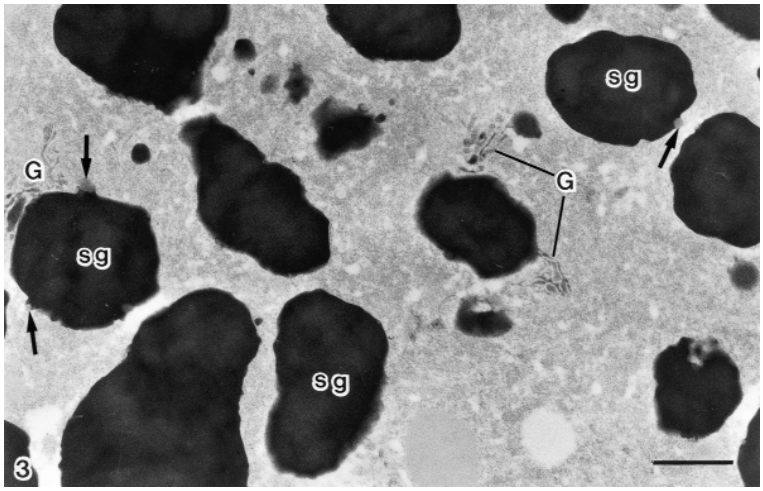


FIG. 2. Granular material of the secretory granules (sg) displaying a strong positive reactivity after PA-TCH-SP staining. G=Golgi complex. Fixation: Karnovsky's fixative, no post-osmication. x 19,700.

FIG. 3. When present, the "minicap" - like structures (arrows) are not stained after PA-TCH-SP treatment. sg=secretory granules, G=Golgi complex. Fixation: Karnovsky's fixative, no post-osmication. x 13,600.



Drosophila sechellia

The secretory granules consist mainly of a filamentous material with a medium to high electron density, while an electron lucent material is seen distributed among the filaments (Fig. 4). Occasionally, in the periphery of the secretory granules another electron lucent material (different from the electron lucent material seen among the filaments) is apparent, when the salivary gland cells were processed without post-osmication (Fig. 4). When the salivary gland cells were post-fixed with osmium tetroxide, the same material discloses a high electron density and reveals a granular structure (Fig. 5). After PA-TCH-SP staining, the filamentous material is PA-TCH-SP positive, while the peripheral granular material is PA-TCH-SP negative (Fig. 6).

FIGS 4-6. *Drosophila sechellia.*

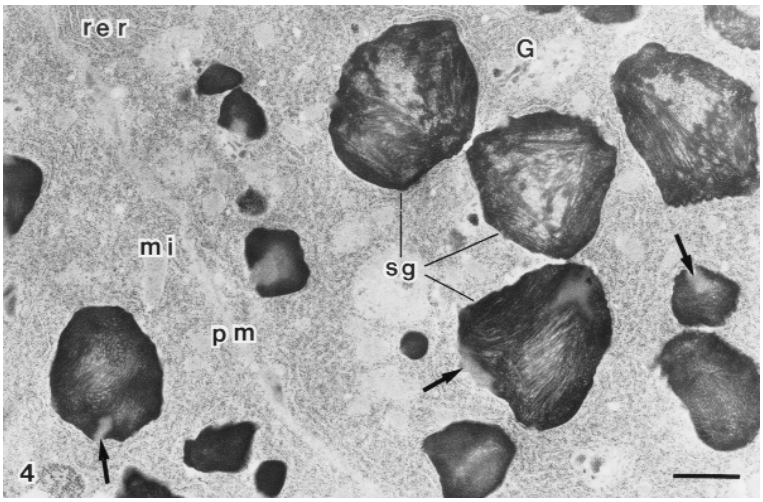
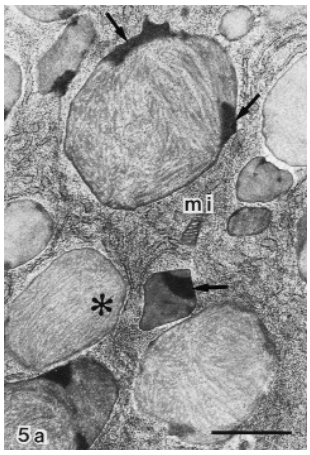


FIG. 4. A filamentous material of medium to high electron density occupying most of the secretory granules (sg), and an electron lucent material among the filaments. The material seen in the periphery of the secretory granules is electron lucent (arrows). rer=rough endoplasmic reticulum, pm=plasma membrane, mi=mitochondria, G=Golgi complex. Fixation: Karnovsky's fixative, no post-osmication. Staining: UA-LC. x 11,000.



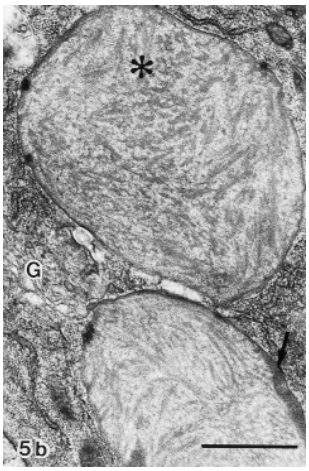
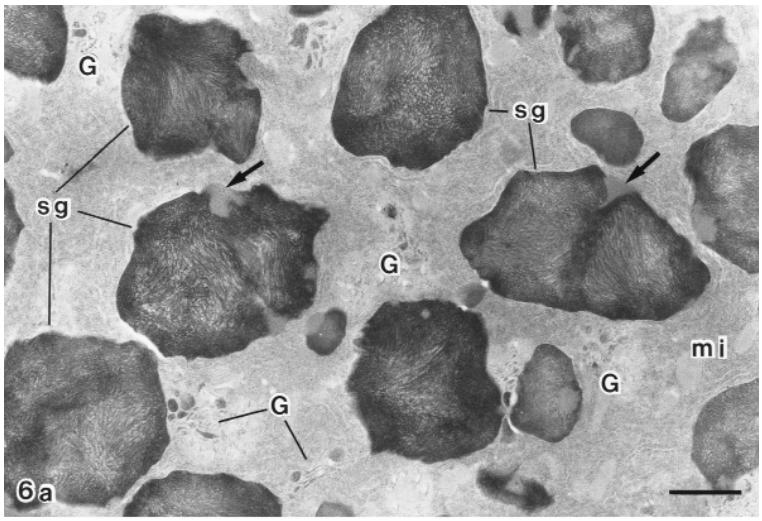


FIG. 5. A peripheral, non-filamentous material (arrows) of the secretory granules (asterisks) displaying a high electron density and revealing a granular structure. G=Golgi complex, mi=mitochondria. Fixation: Karnovsky's fixative plus post-osmication. Staining: UA-LC. a) x 17,500; b) x 20,000.



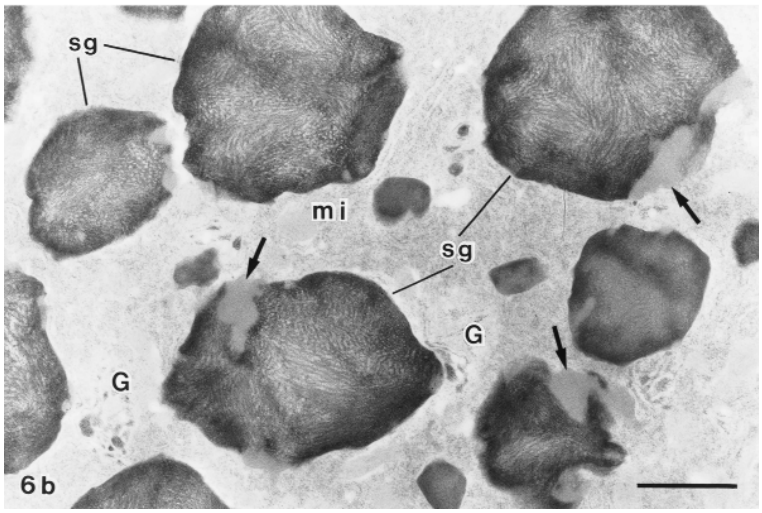


FIG. 6. After PA-TCH-SP staining, the filamentous material of the secretory granules (sg) is positive, while the granular material (arrows) displays no PA-TCH-SP reactivity. G=Golgi complex, mi=mitochondria. Karnovsky's fixative, no post-osmication. a) x 13,200; b) x 17,600.

DISCUSSION

The *melanogaster* subgroup is divided into two complexes: the *melanogaster* complex, which includes *D. melanogaster, D. sechellia, D. simulans,* and *D. mauritiana,* and the *yakuba* complex, which includes *D. erecta, D. orena, D. yakuba* and *D. teissieri*. The division of the subgroup into two complexes is in agreement with studies of experimental hybridization: the four species of the *melanogaster* complex readily give hybrids between themselves, but not with the species of the *yakuba* complex (Lemeunier *et al*., 1986). This division is also supported by the results of other studies, as well (e.g. Eisses *et al*., 1979; Lemunier & Ashburner, 1976, 1984; Cariou, 1987; Lee & Watanabe, 1987; Thomopoulos, 1987; Russo *et al*., 1995, Kastanis *et al*., 2003). Within the *yakuba* complex, the two groups of species (*erecta-orena* and *yakuba-teissieri*) share inversions absent from other species (Ashburner *et al*., 1984), and a further subdivision is evident: the *erecta-orena* and the *yakuba-teissieri* subclusters (Tsacas & Tsacas, 1984; Cobb *et al*., 1986; Singh, 1989; Russo *et al*., 1995; Caccone *et al*., 1996; Lohe & Roberts, 2000; Kastanis *et al*., 2003). Caccone *et al*. (1996) have considered the *erecta-orena* subcluster as the deepest one, followed by the *yakuba-teissieri* subcluster, with the *melanogaster* complex (*D. melanogaster, D. sechellia, D. simulans* and *D. mauritiana*) being the youngest one.

The secretory granules of *Drosophila* salivary gland cells vary considerably in morphology and in the distribution of complex carbohydrates (Thomopoulos & Kastritsis, 1979; Thomopoulos, 1987; Thomopoulos *et al*., 1989, 1992). These features have been used to establish phylogenetic relationships among six species of the *Drosophila melanogaster* species subgroup available at that time (Thomopoulos, 1987). Predictions that the other two known species of the *melanogaster* subgroup, *D. sechellia* and *D. orena*, should have secretory granules containing a PA-TCH-SP positive filamentous and granular material, respectively, are confirmed in this study (Thomopoulos, 1987).

Based on the findings of this report and the features of the other six species of the *Drosophila melanogaster* species subgroup (Thomopoulos, 1987), it is proposed that:

1. **The hypothetical I species** in the phylogenetic tree of the *melanogaster* species subgroup (Thomopoulos, 1987) **corresponds to *D. orena***, because *D. erecta* and *D. orena* are morphologically and histochemically more similar than is *D. orena* with either *D. yakuba* or *D. teissieri*. The existence of a "minicap" - like PA-TCH-SP negative material in the secretory granules of *D. orena*, similar to the material found in the periphery of the secretory granules in *D. yakuba* and *D. teissieri*, supports the view that the latter two species could be evolved from *D. orena*, and confirms earlier predictions that the secretory granules of the hypothetical I species should contain a small amount of a PA-TCH-SP negative material (Thomopoulos, 1987). It has also been found, using the salivary gland polytene chromosomes, that *D. orena* is very close *to D. erecta* (Ashburner *et al*., 1984), while, from mtDNA data, it has been calculated that *D. yakuba* and *D. teissieri* differ by about 3% (Caccone *et al*., 1988). The proposal that *D. erecta* and *D. orena* are closer than is *D. orena* with either *D. yakuba* or *D. teissieri,* is consistent with the concept that the *yakuba* complex splits into two subclusters: the *erecta* subcluster *(D. erecta* and *D. orena*) and the *yakuba* subcluster (*D. yakuba* and *D. teissieri*) (Singh, 1989). The high genetic distance between *D. erecta* and *D. orena* (compared to the other six species of the *melanogaster* subgroup) suggests a relative ancient divergence (Cariou, 1987) which is also supported by satellite DNA (Strachan *et al*., 1981) and mtDNA (Solignac *et al*., 1986) data. The genetic distance (ds=0.4) between *D. yakuba* and *D. teissieri*, half between *D. erecta* and *D. orena* (ds=1), indicates a substantial genetic differentiation and a more recent split than that between the *erecta-orena* pair (Cariou, 1987). The same conclusion has also been reached by satellite DNA (Strachan *et al*., 1981) and ribosomal and histone gene families (Coen *et al*., 1982) studies. The amylase data have suggested that the first cladogenesis in the *melanogaster* species subgroup would separate *D. erecta* and *D. orena* from the six other species (Dainou *et al*., 1987). Both molecular (Ashburner *et al*., 1984) and biogeographical (Lacchaise *et al*., 1988) data support an initial split between the *erecta* subcluster and the other six species, 15 million years ago (Mya), followed by the divergence between the *yakuba* subcluster and the *melanogaster* complex as recently as 2.5-3.0 Mya.

2. ***Drosophila sechellia* and *D. melanogaster* evolved from the hypothetical II species** (Thomopoulos, 1987) where the filamentous PA-TCH-SP positive material first appeared. The existence of PA-TCH-SP negative areas in the secretory granules of *D. sechellia*, similar to element I found in *D.*

melanogaster (Thomopoulos, 1987), and the complete absence of this material in the secretory granules of *D. simulans* and *D. mauritiana* support the view that the latter two species are closer to each other than to *D. sechellia*. This PA-TCH-SP negative material corresponds to the electron dense, granular material seen in the secretory granules of the salivary gland cells of *D. sechellia* post-fixed with osmium tetroxide, while the same structures disclose no electron density when the salivary gland cells were processed without post-osmication. On the contrary, the element I, found in the secretory granules of the salivary gland cells of *D. melanogaster*, displays an intense electron density with or without osmium tetroxide (Thomopoulos, 1987), indicating that these two morphologically similar materials could have different chemical composition.

The notion that *D. simulans* and *D. mauritiana* are closer to each other than to *D. sechellia,* as inferred from the morphological and histochemical characteristics of the secretory granules, is supported by other data as well (Lachaise *et al*., 1986; Solignac & Monnerot, 1986; Lee & Watanabe, 1987; Joly, 1987; Coulthart & Singh, 1988; Singh, 1989; Coyne & Charlesworth, 1997; Harr *et al*., 1998; Kliman *et al*., 2000; Ting *et al*., 2000). On the other hand, there are reports supporting the view that *D. simulans* is closer to *D. sechellia* (Coyne & Kreitman, 1986; Cariou, 1987; Palopoli *et al*., 1996) and *D. mauritiana* is closer to *D. sechellia* (Lemeunier & Ashburner, 1984; Caccone *et al*., 1988, 1996; Thackeray & Kyriacou, 1990; O'Grady *et al*., 2001).

Unfortunately, the existing data on the phylogenetic relationships between the species of the *melanogaster* complex are not conclusive. It has been suggested that *D. simulans* (or a common ancestor of *D. simulans, D. mauritiana* and *D. sechellia*) gave rise to *D. mauritiana* and *D. sechellia* (Coyne & Kreitman, 1986; Solignac & Monneron, 1986; Coyne, 1989; Sata & Takahata, 1990; Kliman & Hey, 1993), but there are different results contradicting this proposal. Lee & Watanabe (1987) using allozyme genetic distances have concluded that *D. sechellia* stands at a slightly distant place from *D. simulans* and *D. mauritiana.* Lachaise *et al*. (1986) have found that *D. sechellia* is slightly more isolated among *D. sechellia, D. mauritiana* and *D. simulans* and that of the four species of the *melanogaster* complex, *D. melanogaster* diverged from a common stem first, followed by *D. sechellia* and, most recently, by *D. simulans* and *D. mauritiana*. More recent results based on microsatellite (Harr *et al*., 1998) and mtDNA (Kastanis *et al*., 2003) analyses have supported the view that *D. sechellia* arose first, followed by a split between *D. simulans* and *D. mauritiana*, and therefore, *D. sechellia* can be considered as the ancestral species of *D. simulans* and *D. mauritiana*, in accordance with the results of the present study.

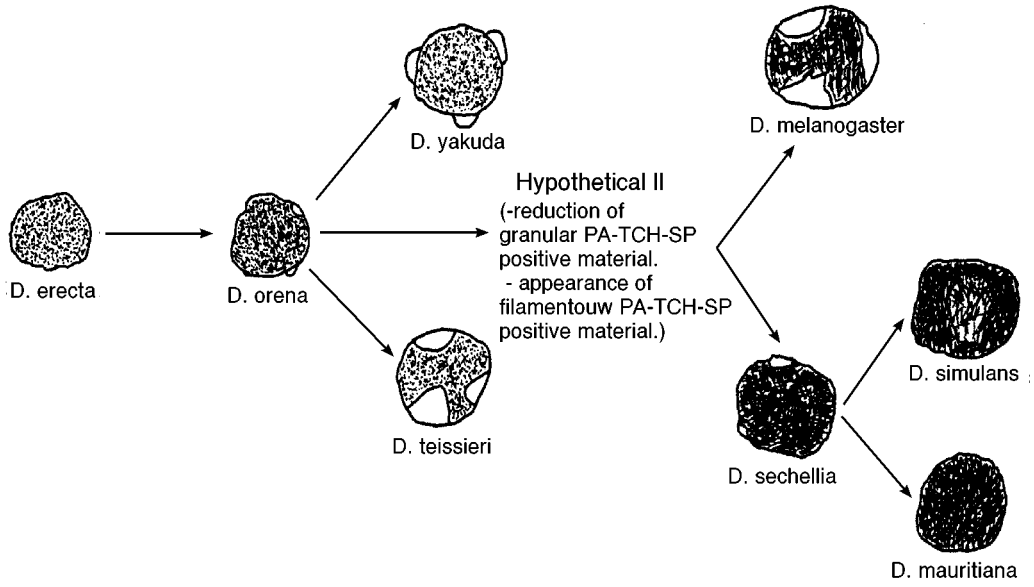


FIG. 7. Schematic presentation of the secretory granules of the species of the *melanogaster* subgroup complex and the proposed interrelationships and direction of evolution among the eight species of this subgroup, based on the ultrastructural morphology of the salivary gland secretory granules and the distribution of the complex carbohydrates. The unshaded areas correspond to the PA-TCH-SP - negative parts of the secretory granules.

Thus, the revised phylogenetic diagram and the proposed direction of evolution is as follows: *D. erecta* \ *D. orena* \ *D. yakuba, D. teissieri* and *hypothetical species II* which yields *D. melanogaster* and *D. sechellia* which in turn yields *D. simulans* and *D. mauritiana* (Fig. 7). Based on the morphological and histochemical characteristics of the secretory granules, it appears that *D. melanogaster* and *D. erecta* are at the opposite ends of the phylogenetic tree of the *melanogaster* species subgroup. The secretory granules of *D. melanogaster* are the most complex ones, consisted of three elements, whereas the secretory granules of *D. erecta* are the simplest ones, consisted only of a PA-TCH-SP positive granular material (Thomopoulos, 1987). This proposal is also supported by other studies, as well. The cytological studies of Lemunier & Ashburner (1976, 1984), for example, have shown that the polytene chromosomes of *D. melanogaster* and *D. erecta* differ by at least seven fixed autosomal inversions. Dowset (1983), using middle repetitive DNA sequences, has also shown that *D. erecta* lacks most of the *D. melanogaster* families, having another set of repetitive families which are absent from the *D. melanogaster* genome.

It has been suggested (Thomopoulos, 1987) that the most simple structure, in this case the secretory granules of *D. erecta*, corresponds to the most primitive form. Tartof (1979) also considered *D. erecta* as the oldest species of the *melanogaster* subgroup, based on the assumption that the species with the simplest rRNA gene structure was the most primitive one. *D. erecta* is an endemic African species and its selection as the most primitive species of the *melanogaster* subgroup is in accordance with the view that this subgroup emerged in Africa (Throckmorton, 1975). Despite the general belief that *D. melanogaster* is the ancestor of the species of the *melanogaster* subgroup, there are reports (Rizki & Rizki, 1980) supporting the view that *D. melanogaster* is the most recently derived species of the subgroup. In the proposed phylogenetic tree of this study, it is believed that *D. melanogaster* and *D. sechellia* are derived from the hypothetical II species, but there are no data concerning the most recently evolved species. The analysis of the region 5' of the Adh gene between nucleotides 447 and 545, has revealed that *D. sechellia* is more closely related to *D. melanogaster* than to either *D. simulans* or *D. mauritiana* (Coyne & Kreitman, 1986). It is predicted that the hypothetical II species should possess salivary gland secretory granules with two main features:

i) reduction in the amount of PA-TCH-SP positive granular material, compared to the species of the *yakuba* complex, and

ii) appearance of the filamentous PA-TCH-SP positive material, similar to that found in the species of the *melanogaster* complex.

The proposed direction of evolution is similar to that of Lee & Watanabe (1987), and particularly to that of Lachaise *et al*. (1988), with the exception of the hypothetical II species. It remains to be seen whether the new species belonging to the *melanogaster* subgroup of species should possess the above mentioned characteristics. It is noted that the newly discovered species of the *melanogaster* subgroup, *D. santomea* (Lachaise *et al*., 2000), was not available when this study was performed.

Finally, because the studies published so far have provided information not always compatible with each other, it should be emphasized that the only reasonable approach in order to clarify phylogenetic relationships is to use all available information (molecular, morphological, biogeographical, etc.) and to weigh it according to its value in a particular case (Kwiatovsky *et al*., 1994). There are several levels at which systematic studies may disagree (DeSalle & Grimaldi, 1991; Remsen & O'Grady, 2002), and therefore the utility of multiple markers in species-level phylogenetic studies is evident (Schawaroch, 2002; Machado & Hey, 2003).

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