68: 117-123; Noor, M.A.F., and D. Ortiz-Barrientos 2006, Behav. Genet. 36: 322-327; Ortiz-Barrientos, D., B.A. Counterman, and M.A.F. Noor 2004, PLoS Biol. 2: e416; Servedio, M.R., and M.A.F. Noor 2003, Annu. Rev. Ecol. Evol. Syst. 34: 339-364; Williams, M.A., A.G. Blouin, and M.A.F. Noor 2001, Heredity 86: 68-77.

Tolerance adaptation of *Drosophila melanogaster* to increased salt concentration due to new beneficial mutations.

Azad, Priti,[1, 2] and R.C. Woodruff.[1] [1]Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403; [2]Current address-School of Medicine, University of California San Diego, La Jolla, CA 92093.

Abstract

Mutation and selection are major evolutionary forces that help organisms to adapt to novel environments. Although numerous laboratory experiments with model organisms and observations from nature have demonstrated adaptations to new environmental conditions, it is difficult to distinguish whether adaptation has occurred due to new beneficial mutation or by selection on preexisting genetic variation. By using a highly inbred homozygous stock we have demonstrated adaptation of *Drosophila melanogaster* to increased tolerance to previous toxic levels of dietary salt as a result of new beneficial mutations. The adaptation occurred quickly (seven generations) suggesting that a small number of genes might have been involved and that new mutations can play an important role in adaptive evolution.

Introduction

Mutation and selection are important interactive forces responsible for major evolutionary changes in all organisms. Yet, whether evolution is driven by natural selection acting on new mutations or on preexisting genetic variation is an ongoing debate (Nei, 1987; Lande, 1988; Gillespie, 1991; Li, 1997; Lynch, 1996; Barton, 1998; Orr and Betancourt, 2000; Kim and Stephan, 2002; Orr, 2005). There are numerous examples of rapid adaptations in natural populations, including insecticide resistance, adaptive melanism in populations of rock pocket mice, pelvic armor loss in fresh water sticklebacks, evolution of speech in humans, metal tolerance in plants, and HIV resistance in humans, that may be caused by new advantageous mutations (Wood and Bishop, 1981; Macnair, 1993; Stephens *et al.,* 1998; Toma *et al.*, 2002; Daborn *et al*., 2002; Nachman and Hoekstra, 2003; Shapiro *et al*., 2004). It is difficult, however, to disentangle the effects of new mutations from preexisting genetic variation. A number of studies have shown that adaptations can occur quickly due to preexisting genetic variation (Moya *et al.,* 2005; Peichel, 2005; Hartley *et al*., 2006; Zhan *et al*., 2006). New beneficial mutations have been studied experimentally in lines of microorganisms, including adaptation of clones of *Escherichia coli* to high and low temperatures (Bennett *et al*., 1992) and yeast to a low phosphate chemostat environment (Francis and Hansche, 1972). Few experiments, however, have been performed with multicellular organisms to measure directly the influence of new beneficial mutations on fitness and adaptation (Francis and Hansche, 1972; Batallion, 2000).

Drosophila is also a widely used model system to study adaptations to new environmental conditions such as new food source, temperature fluctuations, osmotic stress, hypoxia, and starvation

(Powell *et al.,* 1982; Dodd, 1989; Lenski *et al.,* 1991; Huey *et al.,* 1991; Frankham *et al*., 1999; Haddad, 2000; Misener *et al.,* 2001; Anderson *et al.,* 2005). Experiments involving new food sources vary from adding salts, such as sodium chloride and copper sulfate, insecticides, and toxins to regular food, plus replacing regular food with a new food source such as starch and dextrose (Powell *et al.,* 1982; Frankham *et al.,* 1999; Wilson, 2001). *Drosophila* quickly adapt to such drastic changes (Wilson, 2001). To eliminate the effect of preexisting genetic variation in the founding populations in such experiments, it is essential to start with homozygous chromosomes, isogenic stocks or clonal lines. The adaptation in such experiments depends on the rate of emergence of new beneficial mutations and their subsequent selection, which in some cases can be rapid (Mackay *et al.,* 1994; Fuller *et al.,* 2005). Determining the number of genes involved in adaptive changes, their dominance and selection effect, and the molecular changes involved, could help us to understand the mechanisms underlying adaptive evolution and speciation (Nachman, 2005; Dodd, 1989).

In this study we observed that homozygous populations of *D. melanogaster* can quickly adapt by increased tolerance to previous toxic levels of dietary salt as a result of new beneficial mutations.

Materials and Methods

Drosophila Stocks

A highly inbred *sepia* (*se*, 3-26.0) stock of *D. melanogaster* with dark eyes (Lindsley and Zimm, 1992) was used to start the experiment. This inbred stock was generated by single-pair matings for 118 generations and was expected to be essentially completely homozygous (Hedrick, 2005). The stock was maintained on instant medium (Carolina Biological Supply) supplemented with yeast in a large population consisting of 10 bottles, transferring 100 males and 100 females per bottle each generation.

Determination of Initial Extinction Concentration of NaCl

To determine the initial concentration of salt that caused extinction of the inbred *sepia* stock, 15 sets of vials per treatment (with five pairs of flies in each vial) were tested at increasing NaCl concentrations (1%, 1.5%, 2%. 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, and 6%). The vials contained instant *Drosophila* medium, except that salt solutions were used instead of water. Extinction concentration was defined as the concentration of NaCl at which no larvae or adult flies survived. The initial extinction concentration was tested two times with 15 sets of vials per treatment, and consistent results were obtained.

Experimental Setup

After determining the initial extinction concentration for the inbred *sepia* stock, four cages were started by placing 200 pairs of flies in each cage as shown in Figure 1. Two experimental cages (EI, EII) were provided with instant food containing increasing concentrations of NaCl (1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, and 5.5%) each generation (two weeks). Seven food cups were replaced with new salt food every week. Two cages were maintained as controls (CI, CII) by replacing seven food cups, among 15 total cups per population cage, with new regular food every week. All the cages were kept at 25°C in an illuminated incubator (set at 12 hours dark and 12 hours light cycles).

Separate lines were also founded from all cage populations at the end of each generation as shown in Figure 1. For example, at the end of generation one, lines were formed from the two experimental cage populations maintained on 1% NaCl food and the two control cage populations maintained on regular food. Flies from these lines and from the original inbred *sepia* stock were raised simultaneously in bottles containing regular food. These lines were used to verify if the

populations in the cages had evolved by performing the terminal salt-tolerance test as described below.

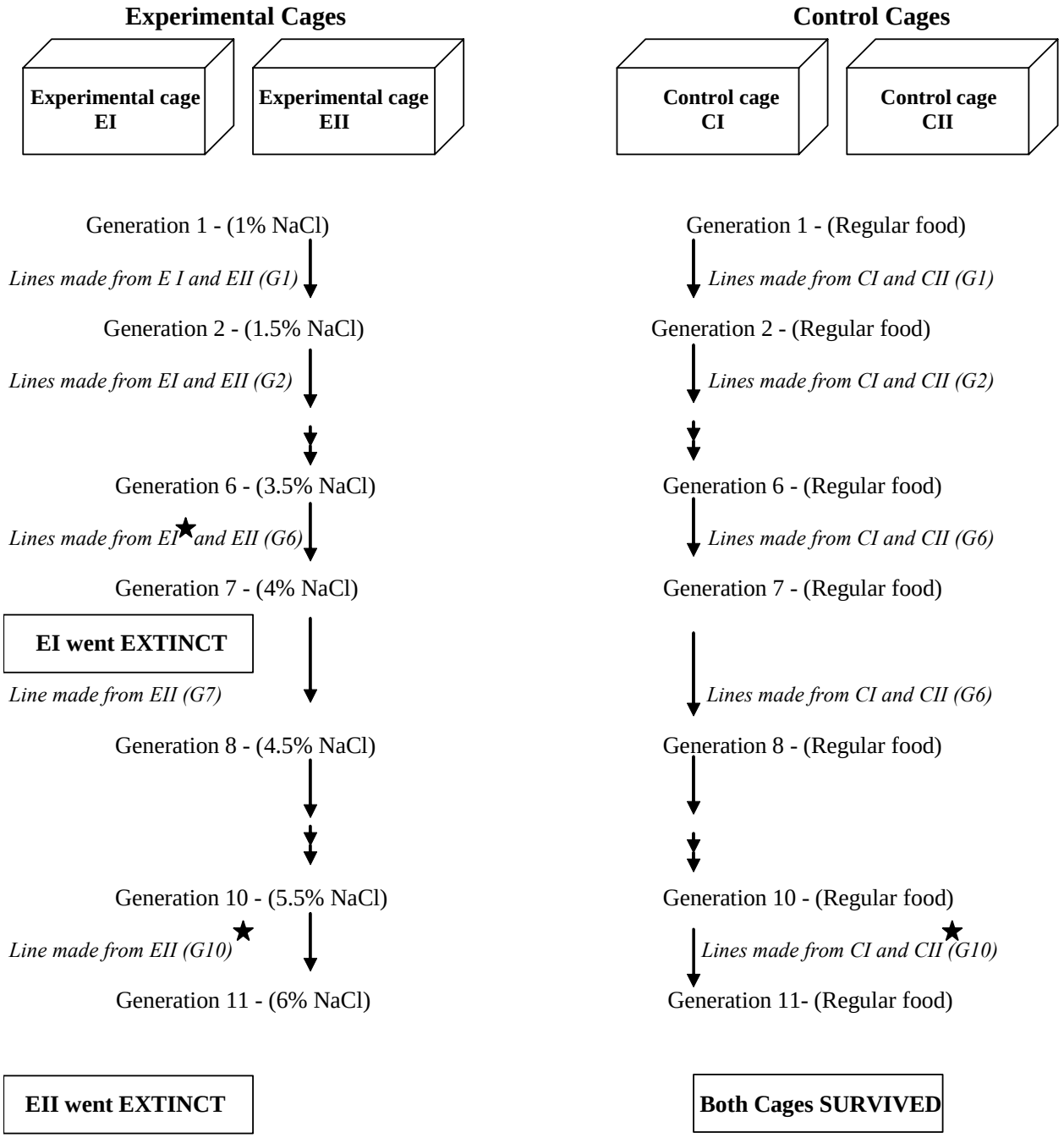

Figure 1. Experimental design. Two Experimental Cages, EI and EII (with NaCl), and two Control Cages, C I and CII (no NaCl), were started with 200 pairs of flies. Experimental and control lines were set up each generation from the previous generation population cage flies. ★ indicates that the G6 line of EI and the G10 lines of EII, CI and CII were used for performing the terminal salt-tolerance test.

Terminal Salt-tolerance Test

At the end of the experiment, flies from the lines generated from cages (G6 line of EI, G10 line of EII, and G10 lines of CI and CII) and from the inbred *sepia* stock were tested again to verify that the populations in experimental cages had evolved higher salt tolerance. Fifteen sets of five pairs of flies for each population type (original stock, and lines from control and experimental cages) were reared on regular instant food and instant food with 5.5% NaCl and the number of offspring per vial was recorded.

Confirmation of Homozygosity by Microsatellite Analysis

To check the homozygosity of the initial inbred *sepia* stock and to rule out contamination by extraneous flies, microsatellite analysis was performed at the beginning and end of the experiment using primers for the amplification of microsatellite markers on the second (DROYANETSB, DMAC9, DROPAD), third (DROABDB, DRONANOS, DMU1951) and X chromosomes (DMWHITE, DROACS2) of *D. melanogaster*. Ten individual flies from each stock (original inbred *se* stock, G10 lines of CI and CII control population cage lines, G6 line of EI, and G10 line of EII experimental population cage lines) were selected randomly at the beginning and end of the experiment and were genotyped for the eight microsatellites. PCR primer sequences and amplification conditions are described in Schug *et al.* (1998). The PCR products were run on Spreadex 300 gels using the SEA 2000 electrophoretic system (Elchrom Scientific, Switzerland). The gels were stained with SBYR Green and viewed using a Storm Phosphoimager (Amersham Biosciences, U.S.A.). The inbred *sepia* stocks, control lines, and experimental lines were homozygous initially and remained homozygous for the microsatellite markers during this study.

Statistical Analyses

Contingency χ^2 tests were performed to determine whether the experimental cage populations had evolved increased tolerance to NaCl. Comparisons were made between the number of flies emerging without NaCl and with 5.5% NaCl from the original stock with the G10 lines of CI and CII, the G6 line of EI, and the G10 line of EII. The number of flies emerging without NaCl and with 5.5% NaCl from the G10 lines of CI and CII were also compared with the G6 line of EI and the G10 line of EII.

Results

The initial salt concentration that caused extinction of the inbred *se* stock was consistently 4% NaCl. The flies in the control cages (CI and CII) (regular food without NaCl) survived throughout the entire experiment (11 generations). For the two experimental cages, cage EI flies went extinct at 4% salt in 7 generations, whereas the cage EII flies survived up to 5.5% NaCl at generation 10.

Terminal Salt-tolerance Test

As an additional test to confirm that the experimental cage populations evolved increased tolerance to NaCl, terminal salt-tolerance tests were performed on the original inbred *se* stock, the control lines (G10 lines of CI and CII) and the experimental lines (G6 line of EI and G10 line of EII). The results of the terminal tests are shown in Table 1. At 5.5% NaCl no flies emerged from the original inbred stock, the G10 line of control cage CII or the G6 line of experimental cage EI, and only one fly emerged from the G10 line of control cage CI. The G10 line from the experimental cage EII yielded adults that were 3.3% of the number of flies in vials without NaCl. There were highly

significant differences between the original inbred *sepia* stock and experimental cage EII ($P < 0.0001$), and between the experimental cage EII and control cages (CI and CII) ($P < 0.0001$). There were no significant differences between the control cages (CI and CII) and the original inbred *sepia* stock ($P = 0.36$), experimental cage EI and the original inbred *sepia* stock ($P = 0.1146$), or between experimental cage EI and control cages (CI and CII) ($P = 0.3786$).

Table 1. Results of terminal salt-tolerance test of experimental populations compared to the original *sepia* inbred stock and the control population cages. Fifteen vials of five pairs of flies for each population type were tested on regular instant food and instant food with 5.5% NaCl.

Population Type	Number of flies emerging (# of vials that produced progeny) No NaCl	Number of flies emerging (# of vials that produced progeny) 5.5% NaCl
Original Stock (O.S.)[a,f,j,l,m]	929 (15)	0 (0)
Experimental Cage I (G6 line of EI)[a,b,c,d,e]	745 (15)	2 (2)
Experimental Cage II (G10 line of EII) [b,f,g,h,i]	909 (15)	31 (9)
Control Cage I (G10 line of CI) [c,e,g,i,j,k,m]	788 (15)	1 (1)
Control Cage II (G10 line of CII)[d,e,h,i,k,l,m]	702 (15)	0 (0)

[a] E1 vs O.S: $\chi^2= 2.49$, df=1, P=0.1146; [b] EI vs EII: $\chi^2= 19.927$, df=1, P<0.0001;
[c] EI vs CI: $\chi^2= 0.391$, df=1, P=0.5316; [d] EI vs CII: $\chi^2= 1.882$, df=1, P=0.1701;
[e] EI vs (CI&CII); $\chi^2= 1.943$, df=2, P=0.3786; [f] EII vs O.S.: $\chi^2= 31.154$, df=1, P<0.0001;
[g] EII vs CI: $\chi^2= 23.746$ df=1, P<0.0001; [h] EII vs CII: $\chi^2= 23.597$, df=1, P<0.0001;
[i] EII vs (CI &CII): $\chi^2= 46.373$, df=2, p<0.0001; [j] CI vs O.S.: $\chi^2= 1.178$, df=1, p=0.2777;
[k] CI vs CII: $\chi^2= 0.890$, df=1, P=0.3454; [l] CII vs O.S.: Fisher exact P value>0.9999;
[m] O.S. vs (CI & CII): $\chi^2= 2.068$, df=2, P=0.3556. For all the contingency χ^2 comparisons, similar P values were obtained with the Fisher exact tests

Discussion

This study demonstrates that new mutations and selection can cause rapid adaptation of *D. melanogaster* to increased tolerance to a previous toxic level of NaCl in seven generations, supporting the important role of new beneficial mutations in adaptive evolution. Recent studies, have suggested that a larger fraction of mutations are beneficial than was previously believed (Joseph and Hall, 2004; Shaw *et al*., 2002; Wloch *et al*., 2001; Zeyl and Devisser, 2001). This study supports this view and suggests that new mutations along with strong selection are capable of causing adaptations in a short period of time. Epistatic interactions among various genes could also be responsible for some of these rapid adaptations. It is known that new mutations can interact with other genes and influence many traits (Anholt *et al.*, 2003).

A number of genes have been shown to be involved in adaptation to osmotic stress in *Drosophila*. Some of these include transcription factors, members of mitogen-activated protein family, heat shock proteins, and various osmolyte transporters (Sano *et al.*, 2005; Inoue *et al*., 2001; Huang *et al.,* 2002). The morphological and physiological basis of adaptive responses for salt tolerance is less clear. Various mechanisms such as reducing the area of contact by small anal papillae, the induction of osmolyte transporters, and loss of taste reception have been proposed to adapt *Drosophila* to the higher concentration of salt, but the genetic basis for the adaptations remains to be identified (Velde and Scharloo, 1988; Huang *et al.,* 2002; Ishimoto and Tanimura, 2004). Identifying the number of genes involved in salt adaptation could be helpful in addressing some of

the aspects of the debate of whether adaptation is a result of many mutations of small effects or few mutations of large effects (Fisher, 1930; Orr and Coyne, 1992; Daborn *et al.,* 2002; Nachman, 2005). In this study, adaptation due to new mutations occurred quickly, which might suggest that the adaptation involved mutations in a few genes. There are numerous examples of mutations in one or few genes that cause major morphological and behavioral changes (Carroll, 2000; Daborn *et al*., 2002).

What is clear from this study is that new mutations are capable of causing rapid adaptations to a novel environment. These results give support to the hypothesis that new beneficial mutations can have a major impact on adaptive evolution.

References: Anderson, A.R., A.A. Hoffmann, and W.M. Stephen 2005, Genet. Res. Camb. 85: 15-22; Anholt, R.R.H., C.L. Dilda, S. Chang, J.J. Fanara, N.H. Kulkarni, I. Ganguly, S.M. Rollmann, K.P. Kamdar, and T.F.C. Mackay 2003, Nat. Genet. 35: 180-184; Barton, N.H., 1998, Genet. Res. 72: 123-133; Bataillon, T., 2000, Heredity 84: 497-501**;** Bennett, F., R.E. Lenski, and J.E. Miltler 1992, Evolution 46: 16-30; Carroll, R.L., 2000, Trends Ecol. Evol. 15: 27-32; Daborn, P.J., J.L. Yen, M.R. Bogwitz, G. Le Goff, E. Feil, S. Jeffers, N. Tijet, T. Perry, D.G. Heckel, P. Batterham, R. Feyereisen, T.G. Wilson, and R.H. Constant 2002, Science 297: 2253-2256; Dodd, D.M.B., 1989, Evolution 43: 1308-1311; Fisher, R.A., 1930, *The Genetical Theory of Natural Selection*. Oxford: Oxford University Press; Francis, J.E., and P.E. Hansche 1972, Genetics 70: 59-73; Frankham, R., K. Lees, M.E. Montgomery, P.R. England, E.H. Lowe, and D.A. Briscoe 1999, Animal Conserv. 2: 255-260; Fuller, R.C., C.F. Baer, and J. Travis 2005, Integr. Comp. Biol. 45: 391-404; Gillespie, J.H., 1991, *The Causes of Molecular Evolution*. Oxford: Oxford University Press; Haddad, G.G., 2000. J. Appl. Physiol. 88: 1481-1487; Hartley, C.J., R.D. Newcomb, R.J. Russel, C.G. Yong, J.R. Stevens, D.K. Yeates, and J. La Salle 2006, Proc. Natl. Acad. Sci. USA 103: 8757-8762; Hedrick, P.W., 2005, *Genetics of Populations.* MA: Jones and Bartlett Publishers; Huang, X., Y. Huang, R. Chinnappan, C. Bocchini, M.C. Mustin, and M. Stern 2002, Genetics 160: 561-569; Huey, R.B.L., L. Partridge, and K. Fowler 1991, Evolution 45: 751-756; Inoue, H., M. Tateno, K.K. Fujimaua, G. Takaesu, A.T. Yamada, T.J. Ninomiya, K. Irie, Y. Nishida, and K. Katsumoto 2001, EMBO J. 20: 5421-5430; Ishimoto, H., and T. Tanimura 2004, Cell. Mol. Life Sci. 61: 10-18; Joseph, S.B., and D.W. Hall 2004, Genetics 168: 1817-1825; Kim, Y., and K. Stephan 2002, Genetics 160**:** 765-777; Lande, R., 1988, Science 241: 1455-1460; Lenski, R.E., M.R. Rose, S.C. Simpson, and S.C. Tadler 1991, Am. Nat. 138: 1315-1341; Li, W.H., 1997, *Molecular Evolution*. Sunderland, MA: Sinauer; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. New York: Academic Press, Inc; Lynch, M., 1996, A quantitative-genetic perspective on conservation issues. In: *Conservation Genetics.* (Avise, J.C., and J.L. Hamrick eds). Princeton, NY: Princeton University Press, 471-501; Macnair, M.R., 1993, New Phytol. 124: 541-559; Mackay, T.F.C., J.D. Fry, R.F. Lyman, and S.V. Nuzhdin 1994, Genetics 136: 937-951; Misener, S.R., C. Chen, and V.K. Walker 2001, J. Insect Physiol. 47: 393-400; Moya, A.F., E. Costas, E. Banares-Espana, L. Garcia Villada, M. Altamirano, and V. Lopez-Rodas 2004, New Phytol.166: 655-661; Nachman, M.W., and H.E. Hoekstra 2003, Mol. Ecol. 12: 1185-1194; Nachman, M.W., 2005, Genetica 123: 125-136; Nei, M., 1987, *Molecular Evolutionary Genetics*. New York: Columbia University Press; Orr, H.A., and J.A. Coyne 1992, Am. Nat. 140: 725-742; Orr, H.A., and A.J. Betancourt 2000, Genetics 157: 875-884; Orr, H.A., 2005, Nat. Rev. Genet. 6: 119-127; Peichel, C.L., 2005, Devel. Dynam. 234: 815-823; Powell, J.R., and M. Andjelkovic 1983, Genetics 103: 675-689; Sano, Y., H. Akimaru, T. Okamaura, T. Nagao, M. Okada, and S. Ishii 2005, Mol. Bio. Cell 16: 2934-2946; Schug, M.D., K.A. Wetterstrand, M.S. Gaudette, R.H. Lim, C.M. Hutter, and C.F. Aquadro 1998, Mol. Ecol. 7: 57-70; Shapiro, M.D., M.E. Marks, C.L. Peichel, B.K. Blackman, K.S. Nereng, B. Jónsson, D. Schluter, and D.M. Kingsley 2004, Nature 428: 717-723; Shaw, F.H., C.J. Geyer, and R.G. Shaw 2002, Evolution 56: 453-463;

Stephens, J.C., D.E. Reich, D.B. Goldstein, H.D. Shin, M.W. Smith, M. Carrington, C. Winkler, G.A. Huttley, R. Allikmets, L. Schriml, B. Gerrard, M. Malasky, M.D. Ramos, S. Morlot, M. Tzetis, C. Oddoux, F.S. di Giovine, G. Nasioulas, D. Chandler, M. Aseev, M. Hanson, L. Kalaydjieva, D. Glavac, P. Gasparini, E. Kanavakis, M. Claustres, M. Kambouris, H. Ostrer, G. Duff, V. Baranov, H. Sibul, A. Metspalu, D. Goldman, N. Martin, D. Duffy, J. Schmidtke, X. Estivill, S.J. O'Brien, and M. Dean 1998, Am. J. Hum. Genet. 62: 1507-1515; Toma, D.P., K.P. White, J. Hirsch, and R.J. Greenspar 2002, Nat. Genet. 31: 349-353; Velde, J.H., and W. Scharloo 1988, J. Evol. Biol. 2: 155-164; Wilson, T.G., 2001, Annu. Rev. Entomol. 46: 545-571; Wloch, D.M., K. Szafraniec, R.H. Borts, and R. Korona 2001, Genetics 159: 441-452; Wood, R., and J. Bishop 1981, *Insecticide Resistance: Population and Evolution. The Genetic Basis of Man-made Change.* London: Academic Press; Zeyl, C., and J. Devisser 2001, Genetics 157: 53–61; Zhan, J., F.L. Stefanato, and B.A. Mcdonald 2006, Mol. Plant Pathol. 7: 259-268.

A revision of the *tumiditarsus* group of the subgenus *Drosophila* and its relation to the genus *Zaprionus*.

Yassin, Amir. Laboratoire Evolution, Génomes et Spéciation, CNRS, Bât 13, BP1, av. de la Terrasse, 91198 Gif-sur-Yvette Cedex, France. E-mail: yassin@legs.cnrs-gif.fr.

Both molecular and morphological analyses have confirmed the paraphyly of the genus *Drosophila*, being defined mainly upon non-derived traits (Ashburner *et al.*, 2005). However, many attempts were made to establish groups of species and radiations of groups and to assign other drosophilid genera to them (*e.g.*, Throckmorton, 1975). Nonetheless, species groups were usually arbitrarily defined with no general taxonomical criterion, resulting in some groups including only one species as well as others encompassing more than 150 species. An example is the *tumiditarsus* species group to which a single Oriental species, *Drosophila repletoides*, belongs. The phylogenetic positioning of this species, and thus of this group, had a very long debatable history. At times, it was considered a member of the *virilis-repleta* radiation, at others of the *immigrans-Hirtodrosophila* radiation (see list of synonyms below). A recent molecular phylogenetic study of the *virilis-repleta* radiation (Tatarenkov and Ayala, 2001) using alpha-methyldopa-hypersensitive protein (*amd*) and dopa-decarboxylase (Ddc) genes discarded *D. repletoides* from the analysis due to its high degree of divergence from other species groups. Unfortunately, the same workers did not include the species in a later work using both genes aiming to analyze the phylogenetic relationships among genera of the subgenus *Drosophila* (Tatarenkov *et al.*, 2001). More recently, the species was used in a molecular phylogeny study of the Drosophilidae based on the *Amyrel* gene (Da Lage *et al.*, 2006). According to their results, *D. repletoides* was placed within the *immigrans-Hirtodrosophila* radiation, close to the genus *Zaprionus*, with a Bayesian posterior probability of 96%. The genus *Zaprionus* has long been considered a member of the *D. immigrans* group (Throckmorton, 1975). In order to test the relation of *Zaprionus* to both *D. immigrans* and *D. repletoides*, I used the three sequences available on the GenBank for the latter species (*i.e.*, *amd*, *Ddc,* and *Amyrel*) in a four-taxon analysis, taking the genus *Hirtodrosophila* (an ancient subgenus of *Drosophila*) as an outgroup. As shown in Figure 1, the result obtained from the *Amyrel* gene was further confirmed from the combined analysis: *D. repletoides* is more related to *Zaprionus*, than is *D. immigrans*, although very distant to be included within it.