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Variation in fiber number of a male-specific muscle between *Drosophila* species: a genetic and developmental analysis

Virginie Orgogozo,[a,1,*] Noelle M. Muro,[a] and David L. Stern[a]

[a]Department of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ 08544, USA

[*]Author for correspondence (email: Virginie.Orgogozo@normalesup.org)

[1]Present address: Université Pierre et Marie Curie, Bât. A, 5ème etage, case 29, 7 Quai Saint Bernard, 75005 Paris, France

SUMMARY We characterize a newly discovered morphological difference between species of the *Drosophila melanogaster* subgroup. The muscle of Lawrence (MOL) contains about four to five fibers in *D. melanogaster* and *Drosophila simulans* and six to seven fibers in *Drosophila mauritiana* and *Drosophila sechellia*. The same number of nuclei per fiber is present in these species but their total number of MOL nuclei differs. This suggests that the number of muscle precursor cells has changed during evolution. Our comparison of MOL development indicates that the species difference appears during metamorphosis. We mapped the quantitative trait loci responsible for the change in muscle fiber number between *D. sechellia* and *D. simulans* to two genomic regions on chromosome 2. Our data eliminate the possibility of evolving mutations in the *fruitless* gene and suggest that a change in the *twist* might be partly responsible for this evolutionary change.

INTRODUCTION

Contractile organs filled with myofibrils of actin and myosin that slide across each other, known as muscles, are present in all Bilateria. The major vertebrate muscle type, the skeletal muscle, is an elongated structure attached to the skeleton and innervated by motor neurons. There are many similarities between vertebrate skeletal muscles and *Drosophila* adult somatic muscles in terms of the cellular mechanisms and genes involved in their anatomy, physiology, and development, suggesting that skeletal/somatic muscles were present in their common ancestor more than 550 Ma (Oota and Saitou 1999; Taylor 2006). Muscles originate from the mesoderm. During development of both *Drosophila* and vertebrate muscles, the mesoderm is progressively subdivided into different regions to form distinct muscle types via the Wg/Wnt, Shh/Hh, and Dpp/BMP pathways (reviewed in Taylor 2006). The muscle precursor cells, named myoblasts, express bHLH transcription factors of the MyoD/Twist family that control their differentiation. During myogenesis, myoblasts migrate to specific positions near the epidermis (Fernandes et al. 1991) and fuse to form syncitial muscle fibers that elongate and attach to the epidermis (reviewed in Taylor 2006). Vertebrate skeletal muscles and *Drosophila* adult somatic muscles usually consist of several muscle fibers assembled into a bundle. Enhancement of muscle contractile force can result from an increase in the number of muscle fibers or from the enlargement of individual muscle fibers.

Elucidating the molecular mechanisms that determine the total number of fibers within a skeletal muscle is of fundamental importance for agriculture. For more than a hundred years, cattle with greatly enlarged muscles have caught the attention of beef producers. Double muscling in cattle appears to be caused primarily by an increase in the number of muscle fibers (reviewed in Bellinge et al. 2005). The relative increase in fiber number is observed early in pregnancy (Swatland and Kieffer 1974) and results in a calf possessing nearly twice the number of muscle fibers at the time of birth. The phenotype is caused by inactivating mutations in the *myostatin* gene (Grobet et al. 1997; Kambadur et al. 1997; McPherron and Lee 1997; Bellinge et al. 2005), which encodes a cytokine synthesized by muscle cells that inhibits myoblast proliferation (Bellinge et al. 2005; Joulia-Ekaza and Cabello 2006). However, double muscling is associated with many disadvantages and physiological abnormalities, such as reduction in stress tolerance, fertility, and calf viability (reviewed in Bellinge et al. 2005). Elucidation of the mechanisms that control total muscle fiber number might provide alternative methods to obtaining high-muscling phenotypes in cattle and in other commercially important species.

Changes in muscle size have also occurred frequently during animal evolution (see, e.g., differences in muscle size between humans and apes; Payne et al. 2006) and it is probable that many of these changes correspond to evolution of muscle fiber number. A dwarf morph of Artic charr in an Icelandic

lake for example has been shown recently to contain fewer muscle fibers than its relatives (Johnston et al. 2003).

Little is known of the developmental mechanisms that regulate muscle fiber number. In vertebrates, it is not yet known how the number and location of muscle fibers is determined during development. The *myostatin* gene is likely to play an important role in the determination of muscle fiber number in vertebrates because mutations or inactivation of the *myostatin* gene have been associated with an increase in body mass muscle not only in cattle but also in mice, fish, and humans (reviewed in Joulia-Ekaza and Cabello 2006). In *Drosophila*, the number of fibers per adult muscle appears to correspond to the number of "founder cells" that are specified during development (Dutta et al. 2004). During *Drosophila* metamorphosis, almost all larval muscles degenerate. Most adult muscles develop de novo from a pool of myoblast cells that are set aside during embryogenesis (Bate et al. 1991; Broadie and Bate 1991; Currie and Bate 1991) and that can be identified as undifferentiated cells with persistent *twist* expression (Bate et al. 1991). These myoblast cells proliferate during larval life and are associated with imaginal discs and nerves. At the onset of metamorphosis, at about 26 h after puparium formation (APF), a few cells named founder cells are selected among the undifferentiated population of myoblasts. According to their morphology and gene expression, founder cells appear to prefigure adult muscles (Rivlin et al. 2000; Kozopas and Nusse 2002; Dutta et al. 2004) and they are identifiable by their expression of the *dumbfounded lacZ* transgene (Kozopas and Nusse 2002; Dutta et al. 2004). The *dumbfounded*/*kirre* gene is involved in myoblast fusion during embryogenesis (Ruiz-Gomez et al. 2000). Like embryonic founder cells, adult founder cells must attract and fuse with fusion-competent myoblasts to form mature individual multinucleated muscle fibers. When fusion is compromised, embryonic and adult founder cells develop into mononucleate myosin-expressing fibers at the correct positions (Rushton et al. 1995; Dutta et al. 2004). The selection of founder cells is mediated by several components of the fibroblast growth factor (FGF) signaling pathway, including Heartless, Sprouty, and Heartbroken (Dutta et al. 2005). Therefore, the FGF signaling pathway appears to play a major role in the determination of the number of muscle fibers in *Drosophila*.

The muscle of Lawrence (MOL), a male-specific abdominal muscle, is present in many drosophilid species and has apparently been lost multiple times during *Drosophila* evolution (Gailey et al. 1997). For example, the MOL is absent in half of the species of the *Drosophila melanogaster* species subgroup (Gailey et al. 1997 and this article). In the species where it is present, we show here that the number of muscle fibers per MOL differs between species. We compared MOL development between species to determine the developmental stage at which the species diverge. We then mapped the genomic regions that are responsible for the change in muscle fiber number between *Drosophila sechellia* and *Drosophila simulans*, which possess a high and low number of MOL fibers, respectively.

MATERIALS AND METHODS

Fly strains

We used the following strains: *D. melanogaster* Oregon R, *D. melanogaster* Tai, *D. simulans* Oxnard (kindly provided by M. Ashburner), *D. simulans* sim2 (kindly provided by A. Clark), *D. simulans f;nt,pm;st,e* (kindly provided by C. Jones), *D. sechellia* 3588 (Tucson Species Stock Center strain #14.021-0248.04, kindly provided by A. Clark), *D. sechellia* .07 (Tucson Species Stock Center strain #14.021-0248.07), and *Drosophila mauritiana* (Tucson Species Stock Center strain #14.021-0241.07). Flies were raised under the same uncrowded conditions on standard media at 25°C.

Tissue preparation, immunostaining, and microscopy

Adults were dissected between 2 and 8 days after eclosion, to ensure that the larval dorsal abdominal muscles that persist transiently after eclosion (Kimura and Truman 1990) had been eliminated. White prepupae were cut open along the anterior–posterior axis and pinned on Sylgard (Dow Corning Corp., USA). Forty-seven hours APF pupal and adult dorsal abdomens were dissected in PBT (0.5% Triton X-100 in PBS). Preparations were then fixed in 4% or 5% formaldehyde, washed thoroughly, blocked with PBT +5% normal goat serum (NGS) for 1 h, incubated with primary antibodies for at least 4 h, washed thoroughly, blocked with PBT +5% NGS for 1 h, and incubated with secondary antibodies for at least 4 h. The tissues were then washed and mounted in Vectashield mounting medium (Vector Laboratories).

We used the following dilutions: phalloidin-Oregon green, 1 μM (Molecular Probes), TO-PRO-3, 1/1000 (Molecular Probes), Hoechst, 10 μg/ml (Molecular Probes), mouse anti-Futsch, 1/50 (22C10; DSHB, Iowa City, IO), rabbit anti-β3-tubulin, 1/1500 (a kind gift from R. Renkawitz-Pohl), rabbit anti-Twist, 1/500 (kind gift of S. Roth), Cy3-anti-rabbit or anti-mouse 1/1000 (Jackson Laboratories), and Alexa488-anti-rabbit or anti-mouse 1/1000 (Molecular Probes). Anti-Futsch antibodies stain axonal projections (Fujita et al. 1982; Hummel et al. 2000).

Images were collected on a PerkinElmer RS3 spinning disk microscope or on a Nikon Eclipse E1000 microscope, and processed using Adobe Photoshop software. Figures 1 and 2 show the maximal projection of several confocal *z* sections. In Fig. 1A, the 22C10 signal detected between the MOL and the epithelium, which corresponds to the peripheral nervous system, was reduced to better show the MOL innervation. In Fig. 1, the DNA staining is shown only for the phalloidin-positive regions, so that the rows of MOL nuclei are clearly visible.

Muscle fiber counts

To count individual fibers with accuracy, a transparency was placed on a computer screen showing each stack of microscope images in animation from top to bottom sections and MOL nuclei were drawn on the transparency.

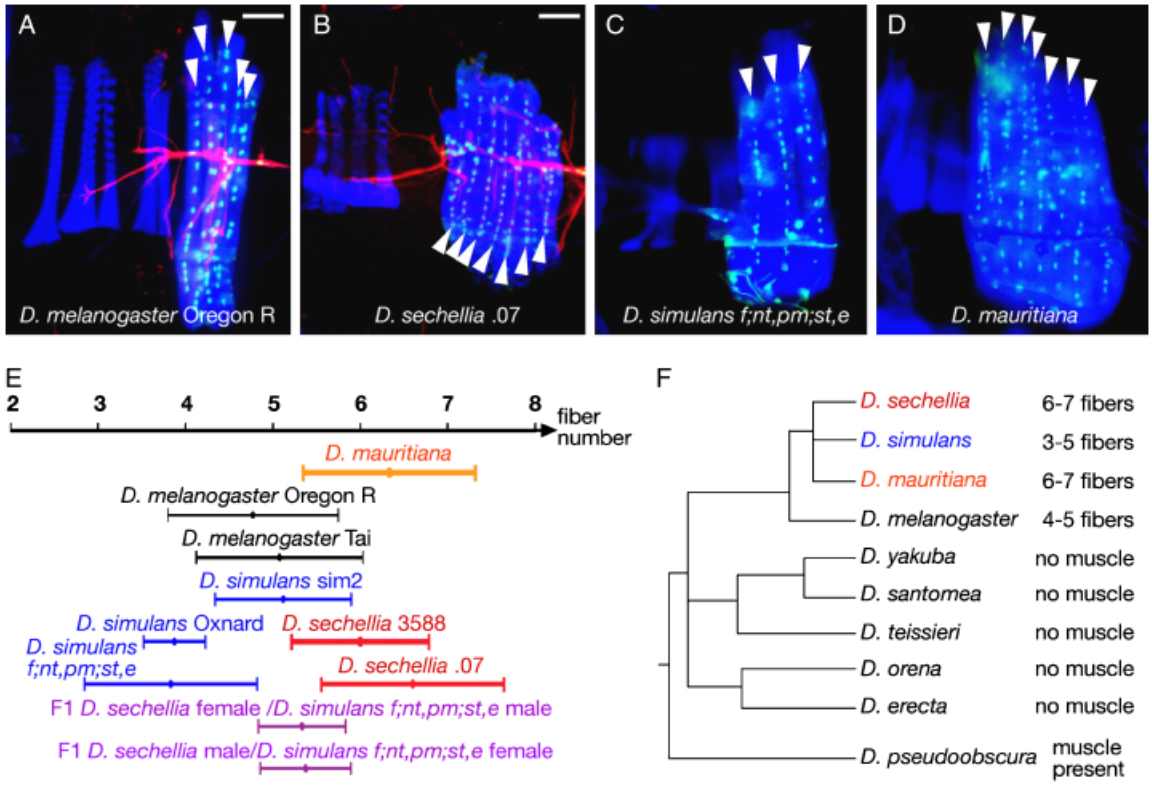

Fig. 1. Variation in muscle of Lawrence (MOL) fiber number in the *Drosophila melanogaster* species subgroup. (A–C) MOL morphology in *D. melanogaster* Oregon R (A), *Drosophila sechellia* .07 (B), *Drosophila simulans f; nt, pm; st, e* (C) and *Drosophila mauritiana* (D) stained for actin in blue, DNA in green, and Futsch in red in A–B. Anterior is up and the dorsal midline is left. Scale bar equals 50 µm. Arrowheads point to the MOL fibers. (E) Mean fiber number and standard deviation for *D. mauritiana* ($n = 12$ flies), *D. melanogaster* (Oregon R, $n = 22$; Tai, $n = 19$), *D. simulans* (sim2, $n = 17$; Oxnard, $n = 8$; *f; nt, pm; st, e*, $n = 18$), *D. sechellia* (3588, $n = 14$; 0.07, $n = 20$), F1 progeny from *D. sechellia* .07 females crossed with *D. simulans f; nt, pm; st, e* males ($n = 9$) and F1 progeny from *D. simulans f; nt, pm; st, e* females crossed with *D. sechellia* .07 males ($n = 8$). (F) Phylogeny (Powell 1997; Harr et al. 1998; Kliman et al. 2000; Ting et al. 2000; Malik and Henikoff 2005) showing the number of MOL fibers for *D. melanogaster*, *D. sechellia*, *D. simulans*, and *D. mauritiana*. No MOL is detected in *Drosophila santomea* (data not shown) and in the other species of the *D. melanogaster* subgroup (Gailey et al. 1997).

Quantitative trait loci (QTL) mapping

Female *D. simulans f;nt,pm;st,e* flies were crossed to male *D. sechellia* .07 flies and the female progeny were backcrossed to *D. simulans f;nt,pm;st,e* males. Thirty-two (2^5) phenotypically different classes of males result from this cross. Progeny flies from this *D. simulans* backcross were stored in acetone in different tubes based on the five visible markers. At least five males of each phenotypic class were dissected and stained as above. Only flies for which fiber number could be accurately scored for both left and right MOL were included in the QTL analysis. Genomic DNA was isolated with the Puregene purification kit (Gentra Systems). Molecular markers were polymerase chain reaction (PCR) amplified and separated on 2% agarose or 4.5% agarose SFR (Amresco). All PCR reactions followed this format: 95°C for 15 min, 94°C for 30 sec, annealing temperature as indicated in Table 1 for 30 sec, 72°C for the elongation time indicated in Table 1, 35 cycles, 72°C for 8 min. We scored natural variation in sequence length or differences in restriction enzyme sites (Table 1).

Statistical analysis was performed using QTL cartographer (Basten et al. 1997). For each genetic marker, the data were fitted to a linear regression model, $y_i = b_0 + b_1 x_i + e_i$ where y_i = mean of MOL fiber number of individual i, $x_i = 0$ if individual i is *D. simulans*/*D. simulans* for the analyzed marker and 1 if individual i is *D. sechellia*/*D. simulans* for the analyzed marker, b_1 and b_0 are the estimated parameters, and the error term e_i represents the unexplained variation in fiber number for individual i. The estimated effect b_1 corresponds to the number of fibers that are added on average when one *D. simulans* allele is virtually replaced by a *D. sechellia* allele at the analyzed marker. The F statistics compare two hypotheses: $b_1 = 0$ (no QTL) versus $b_1 \neq 0$ (presence of a QTL).

RESULTS

Intra- and interspecific variation in muscle fiber number

The MOL is a bundle of muscle fibers located in the dorsal region of the fifth abdominal segment in *Drosophila* adult males. Compared with the neighboring abdominal dorsal

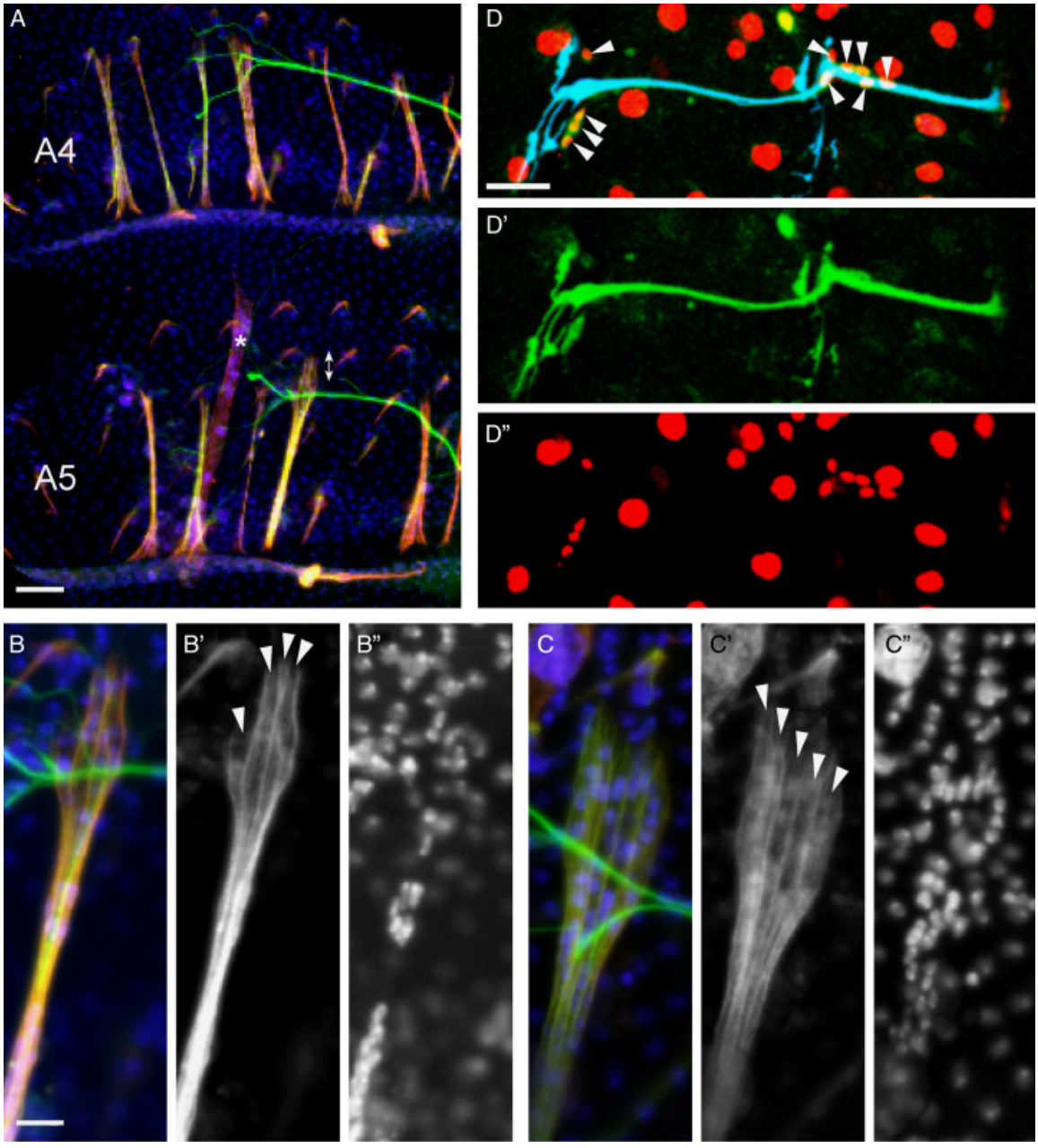

Fig. 2. Development of the muscle of Lawrence (MOL) in *Drosophila melanogaster* Oregon R. (A–C) 47 h after puparium formation (APF) abdomen stained for β3-tubulin in red, Futsch in green, and DNA in blue. (A) In hemisegment A5, a bundle of muscle fibers extends more anteriorly than adjacent abdominal dorsal muscle fibers (see double arrow) and is interpreted as the developing MOL. The asterisk indicates a persistent larval abdominal muscle. (B–B″) High magnification of the developing MOL shown in A. (C–C″) Picture of another developing MOL at 47 h APF. (B–C″) The β3-tubulin and DNA stainings are shown individually in panels B′/C′ and B″/C″, respectively. Four elongating fibers are seen in (B) and five in (C) (arrowheads). (D–D″) Dorsal part of hemisegment A5 at 0 h APF stained for Twist in green, Futsch in blue, and DNA in red. The Twist and DNA stainings are shown individually in panels D′ and D″, respectively. Ten Twist-positive myoblast cells are observed in this hemisegment in the region where the future MOL will form (arrowheads). Note that this batch of anti-Twist antibodies also gives a non-specific staining in motoneurons. Anterior is up and the dorsal midline is left in all panels. Scale bar equals 50 μm.

muscles, the MOL is longer and consists of a bundle of several muscle fibers (Lawrence and Johnston 1984, Fig. 1). Each MOL fiber contains multiple nuclei that are aligned in a single row along the fiber axis. It is therefore easy to count MOL fiber number based on a DNA staining (Fig. 1, A–D). The MOL is composed of a mean of 5.7 ± 0.2 (SE) fibers in the *D.*

Table 1. Molecular markers used in the quantitative trait loci (QTL) mapping

Marker	Primers	PCR conditions	Approximate PCR fragment size[1]	Source
spalt (*sal*)	GCC ACG ATG AAA CTA CTG GTG C ACT CCT CCC TGG CCA ATT C	55°C 1 min	sim:290 bp se:230 bp	Gleason and Ritchie (2004)
acc004516	TCG TCG CCC GTT AAT ATA ACC GTT CGT GGG TCA AAT AG	55°C 1 min	sim:295 bp se:250 bp	Colson et al. (1999)
temperature-induced-paralytic-E (*tipE*)	TCA CCA GCT GAA AGT CCA GA ACT CGT CGT CAT CGT CTT GC	55°C 1 min	sim:490 bp se:475 bp	Gleason and Ritchie (2004)
3R:9.6	CAK CAG GCT GGG ATA CAC GGA ATG AGC CGA CTT CTC TGC ACA	52°C 30 sec	sim:305 bp se:220 bp	This article
cubitus interruptus (*ci*)	GCG AGT ATC CGG GAT GTA GC CCG TTG CCT AGC CAA AAC AG	55°C 1 min	SacI cuts se	Orgogozo et al. (2006)

[1]sim, *Drosophila simulans*; se, *Drosophila sechellia*; PCR, polymerase chain reaction.

melanogaster Canton S strain (Taylor 1992). We observed a mean of 5.1 ± 0.2 and 4.8 ± 0.2 fibers in two other *D. melanogaster* strains, suggesting that there is little intraspecific variation in MOL fiber number for *D. melanogaster* (Fig. 1E). Because MOL fiber number has been observed to vary with temperature (B. Taylor, personal communication), it is possible that the difference between the measurements of Taylor (1992) and ours was caused by a divergence in the experimental procedures.

A survey of other species of the *D. melanogaster* subgroup revealed interspecific differences in MOL fiber number that are larger than intraspecific differences, with *D. sechellia* and *D. mauritiana* exhibiting the largest number of fibers and *D. simulans* the smallest fiber number (Fig. 1, B–E). In agreement with our observations, it can be noted that in Fig. 1 of Gailey et al. (1997), D. *sechellia* and *D. mauritiana* MOL appear larger than *D. melanogaster* and *D. simulans* MOL.

We found significantly different fiber numbers between strains of *D. simulans* (Fig. 1, two-tailed *t*-test between *D. simulans* sim2 and *D. simulans* Oxnard, $P < 0.0003$; between *D. simulans* sim2 and *D. simulans f;nt,pm;st,e*, $P < 0.0002$) but the two *D. sechellia* strains that we analyzed were not significantly different (two-tailed *t*-test, $P > 0.07$).

Change in the total number of MOL nuclei

From our current understanding of abdominal muscle development in *Drosophila*, each adult muscle fiber develops from a single founder cell and the number of founder cells equals the number of fibers in a muscle (Dutta et al. 2004). Our results thus suggest that there has been a change in the number of MOL founder cells during evolution.

During development of *Drosophila* adult muscles in general, founder cells are selected from a pool of equivalent myoblast cells during metamorphosis (Dutta et al. 2004, 2005). The remaining myoblasts fuse with founders to create multinucleate fibers (Rushton et al. 1995; Baylies et al. 1998; Paululat et al. 1999; Dutta et al. 2004). Therefore, the total number of nuclei observed within an adult muscle is a good approximation of the total number of myoblasts that were present before myofiber formation (Broadie and Bate 1991; Taylor and Knittel 1995).

To test whether the total number of MOL myoblasts has changed during evolution, we counted the total number of MOL nuclei and the number of nuclei per fiber in the four *D. melanogaster* subgroup species. If the total number of MOL myoblasts has not changed, then the total number of MOL nuclei is expected to be the same in each species and the number of nuclei per fiber should vary. In *D. sechellia* and *D. mauritiana*, which possess about twice as many MOL fibers as *D. melanogaster* and *D. simulans*, we found about twice as many MOL nuclei than in *D. melanogaster* and *D. simulans* (Table 2). The number of nuclei per fiber was not significantly different between species (Table 2, one-tailed *t*-test, $P > 0.09$ for the species pair exhibiting the largest difference). We therefore conclude that the total number of MOL myoblasts is likely to have changed during evolution.

MOL development

To investigate further the developmental events responsible for the variation in muscle fiber number between species, we next examined the development of the MOL. During metamorphosis, at 47 h APF, we observed a bundle of muscle fibers that extends more anteriorly than adjacent abdominal dorsal muscle fibers at the presumptive position of the MOL (double arrow in Fig. 2A). This bundle is thus likely to be the developing MOL. We observed that this bundle contains a mean of 4.8 ± 0.2 elongating fibers (Fig. 2, B–C″, $n = 6$ hemisegments) in *D. melanogaster* Oregon R, which is approximately equal to the number of muscle fibers in the adult MOL (Fig. 1E). Based on these observations, we conclude

Table 2. Number of nuclei in adult muscle of Lawrence (MOL) of the *Drosophila melanogaster* subgroup

Strain	Total number of nuclei (SD)[1]	Number of nuclei per fiber (SD)[1]	Number of MOL analyzed
D. melanogaster Oregon R	65.1 (13.2)	14.0 (1.8)[2]	28
Drosophila mauritiana	93.1 (14.1)	14.5 (0.8)	11
Drosophila simulans f; nt, pm; st, e	54.1 (10.6)	14.7 (2.2)	17
D. simulans Oxnard	54.5 (6.0)	14.1 (0.8)	8
Drosophila sechellia 0.07	99.8 (11.1)	13.8 (1.0)	11

[1]Mean and standard deviation (SD) are indicated.
[2]Note that Taylor and Knittel (1995) reported a mean of 24 nuclei per fiber for the MOL in *D. melanogaster* Canton S. The discrepancy with our measurements might be due to a divergence in experimental procedures, because number of nuclei per fiber varies with temperature (B. Taylor, personal communication).

that the number of MOL fibers is determined before 47 h APF.

We next examined MOL development at the pupariation stage (0 h APF), which lasts about 1 h, when the animal becomes immobile and its white larval cuticle hardens (Ashburner et al. 2004). At 0 h APF, the precursor cells for abdominal dorsal muscles, including the MOL, are myoblast cells associated with the intersegmental nerves (Bate et al. 1991; Currie and Bate 1991). They accumulate the transcription factor Twist and can be distinguished from the polyploid larval cells by their smaller nuclei (Currie and Bate 1991). If variation in MOL fiber number is due entirely to a change in the total number of myoblast cells before 0 h APF, then we expected to observe about twice as many myoblast cells in *D. sechellia* than in *D. melanogaster* (see Table 2). At 0 h APF in *D. melanogaster* Oregon R, we counted a mean of 10.2 ± 0.9 (SE) Twist-positive myoblast cells associated with the intersegmental nerve in segment A5 in the region where the future MOL will form, i.e., in the dorsal part of the intersegmental nerve that spans its two dorsal-most branches ($n = 6$ hemisegments). Approximately the same number of myoblasts was observed in *D. sechellia* (10.7 ± 0.6, $n = 8$, one-tailed *t*-test, $P > 0.29$). In both species, myoblasts were either distributed along the intersegmental nerve or merged into two nests (Fig. 2, D–D″).

In conclusion, our results suggest that the change(s) in MOL development between species appear(s) between 0 and 47 h APF.

Identification of the genomic regions responsible for the change in MOL fiber number

To identify the genomic regions, so-called QTL, responsible for the evolutionary change in MOL fiber number, we chose to focus on the species pair exhibiting the largest phenotypic difference: *D. sechellia* and *D. simulans*. F1 male hybrids have a mean of 5.3 ± 0.2 (SE) fibers (progeny from *D. sechellia* females crossed with *D. simulans* males) and 5.4 ± 0.2 fibers (progeny from *D. simulans* females crossed with *D. sechellia* males, Fig. 1E). These values are intermediate between the parental values, suggesting that the fiber number difference involves alleles with additive effects or several alleles with opposite dominant effects in each species. Because the number of MOL fibers is not significantly different between reciprocal F1 hybrids (two-tailed *t*-test, $P > 0.86$) (and reciprocal hybrids carry either a chromosome X from *D. sechellia* or from *D. simulans*), we infer that there is no maternal effect and no major gene on chromosome X regulating MOL fiber number.

To map the dominant and additive alleles from *D. sechellia* and the recessive and additive alleles from *D. simulans* that are responsible for the difference in muscle fiber number, we performed QTL mapping using a *D. simulans* backcross. We counted the mean number of MOL fibers of 105 progeny flies and we genotyped them for five visible markers and five molecular markers. Markers were chosen such that any genetic locus would be at most 27 cM away from a marker. For each marker, we tested whether there is likely to be a QTL at the marker position (see "Materials and Methods"). The results are shown in Table 3. No significant QTL was found on chromosome X (in agreement with the number of muscle fibers in reciprocal hybrids) or on chromosomes 3 or 4. For two markers located at both extremities of chromosome 2, test for a QTL was significant. Because these two markers are separated by more than 100 cM and because no significant QTL was found for other markers located in the middle of chromosome 2, the difference in MOL fiber number cannot be caused by a single genomic region in the middle of chromosome 2. In conclusion, our QTL mapping identified two genomic regions responsible for the difference in MOL fiber number that are located at the extremities of chromosome 2.

For each QTL, the estimated effect of substituting a *D. simulans* allele with a *D. sechellia* allele is 0.6 fibers (Table 3). This means that for each QTL region, a *D. simulans*/*D. sechellia* heterozygote contains on average 0.6 additional MOL fibers compared with a *D. simulans* homozygote. The total estimated effect of both QTL, which is 1.2 fibers, corresponds to the expected total difference between *D. simulans* and *D. simulans*/*D. sechellia* hybrids (Fig. 1E). This suggests that we

Table 3. Quantitative trait loci (QTL) mapping results

Marker	Cytological location[1]	Genetic position (cM)[2]	n	Estimated effect (# fibers)	$F(1, n-2)$	pr(F)
Chromosome X						
f	15F4-7	39	105	−0.11	0.44	0.509
Chromosome 2						
nt	21B1	0	105	**0.61**	15.053	**<0.001**
sal	32E4-F1	41	46	0.53	6.216	0.014
acc004516	52D12	89	55	0.71	6.309	0.014
pm	57C7-8	115	105	**0.62**	15.436	**<0.001**
Chromosome 3						
tipE	64A10	13	55	0.25	2.209	0.140
st	73A3	55	105	0.36	4.855	0.030
e	93C7-D1	77	105	0.24	2.018	0.158
3R:9.6	88D10	104	56	−0.20	1.291	0.259
Chromosome 4						
ci	102A1-3	1	54	−0.22	2.683	0.104

[1]Cytological locations were obtained from Flybase (http://flybase.bio.indiana.edu/) and are given in *Drosophila melanogaster* cytological units. *Drosophila sechellia* and *Drosophila simulans* contain a large inversion on chromosome 3 relative to *D. melanogaster* (compare cytological location of markers *e* and 3R:9.6).

[2]Note that the *D. simulans*/*D. sechellia* hybrid genetic map is larger than the *D. melanogaster* map.

For each marker, we indicate its cytological position, its genetic position in centiMorgans (cM) from the left telomere, the number of flies that were genotyped for this marker (n), the estimated effect of substituting a *D. simulans* allele with a *D. sechellia* allele for this marker, the F statistics that test whether there is a QTL at this marker position, and its associated probability.

have identified all of the major genomic regions responsible for variation in muscle fiber number in the *D. simulans* backcross.

DISCUSSION

Rapid evolution and role of the MOL

We have characterized a new morphological difference between fly species within the *D. melanogaster* subgroup: whereas the *D. melanogaster* and *D. simulans* MOL possess about four to five fibers, the *D. mauritiana* and *D. sechellia* MOL contain six to seven fibers. However, we could not reconstruct the ancestral character states for MOL fiber numbers because MOL fiber number exhibited marked intra- and interspecific variability. Thus, in addition to the multiple losses of the MOL that have occurred during evolution of the *Drosophila* genus (Gailey et al. 1997), the size of the MOL itself appears to have evolved rapidly. The reasons for the rapid evolution of this male-specific muscle might be linked to its function. However, its utility is still unclear. *D. melanogaster* males deprived of the MOL due to a mutation in the *fruitless* gene can still bend their abdomen, can copulate, and are fertile (Gailey et al. 1991, 1997). It has been proposed that the MOL could be required for unbending the abdomen as copulation terminates (Lee et al. 2001). This possibility is supported by the location of the MOL on the dorsal region of the abdomen and by the longer mating durations observed with *fruitless* mutant males without MOL (Lee et al. 2001). Interestingly, copulation time has been shown to vary between species of the *D. melanogaster* subgroup (Spieth and Hsu 1950; Coyne and Kreitman 1986; Cobb et al. 1988; Jagadeeshan and Singh 2006). However, the species differences in copulation time (*D. sechellia* $\geq$ *D. simulans* = *D. melanogaster* $\geq$ *D. mauritiana*) do not correlate with the differences that we observed in MOL fiber number (*D. mauritiana* $\geq$ *D. sechellia* $\geq$ *D. melanogaster* $\geq$ *D. simulans*). This suggests that if the number of MOL fibers has any influence on copulation time, other factors must also influence copulation duration.

Developmental change responsible for the variation in MOL fiber number

Adult abdominal muscles, including the MOL, are produced during metamophosis from myoblast cells that are set aside during embryogenesis (Bate et al. 1991; Broadie and Bate 1991; Currie and Bate 1991). Because we did not detect any difference between *D. sechellia* and *D. melanogaster* at 0 h APF, we conclude that the change in MOL development between these species must appear after 0 h APF. We also found that the total number of MOL nuclei has changed during evolution of the *D. melanogaster* subgroup but that species exhibit the same number of nuclei per muscle fiber. Based on all these observations, we suggest that this difference in MOL fiber number might have occurred through evolution of one or more of the following mechanisms. (A) The total number of myoblasts in the MOL region might have increased in *D. sechellia* relative to *D. melanogaster*. (B) The number of founder cells selected from a given number of myoblasts might have increased in *D. sechellia* relative to *D. melano-*

gaster. (C) The assembly of the MOL into a bundle of fibers from a given number of developing muscle fibers might have changed. (D) The number of cell divisions within MOL polynucleate fibers might have increased in *D. sechellia* relative to *D. melanogaster*.

A change in the number of cell divisions within MOL polynucleate fibers (scenario D) is unlikely because no cell division in MOL myotubes has been detected in pupae (D. A. Currie, unpublished observations; B. J. Taylor, unpublished observations, cited in Taylor and Knittel 1995).

A change in the total number of myoblasts (scenario A) would have to occur after 0 h APF because we observed the same number of myoblasts at 0 h APF in *D. sechellia* and *D. melanogaster*. Reduction of the number of myoblasts by application of a DNA-synthesis inhibitor leads to a decrease in the total number of adult muscle nuclei (Broadie and Bate 1991). Because we observed a change in the total number of MOL nuclei between *Drosophila* species, scenario (A) is likely to have occurred during evolution. Candidate genes responsible for this change are genes regulating myoblast cell proliferation, cell death, or cell determination, such as the *Drosophila myostatin* homolog *myoglianin* (Lo and Frasch 1999).

The precise pattern of adult founder cells in the abdomen of the fly (scenario B) is determined around 26 h APF in *D. melanogaster* (Dutta et al. 2005). Unfortunately, several technical problems hampered our ability to compare the number of MOL founder cells between *D. sechellia* and *D. melanogaster*. First, *D. sechellia* metamorphosis is slower than in *D. melanogaster* and occurs over a longer time span (data not shown). Second, no clear developmental markers for small periods of time during metamorphosis are available. The number of founder cells is determined by the interplay of several components of the FGF signaling pathway: Heartless, Heartbroken, and Sprouty (Dutta et al. 2005). Mutations in these genes, as well as in the determinant of founder cell identity *dumbfounded* (Ruiz-Gomez et al. 2000; Kozopas and Nusse 2002; Dutta et al. 2004), could thus be responsible for the evolutionary change in MOL founder cell number (scenario B).

A change in the assembly of the MOL into a bundle of fibers (scenario C) would have to occur before 47 h APF in *D. melanogaster* because we found that the number of MOL fibers is already determined at 47 h APF in *D. melanogaster*. Several experiments (nuclei transplantations, muscle nuclei counts, and treatment with a DNA-synthesis inhibitor) have indicated that MOL fibers form from the same pool of myoblasts as the other dorsal muscle fibers located between the dorsal midline and the MOL (Lawrence and Johnston 1986; Broadie and Bate 1991; Gailey et al. 1991; Taylor and Knittel 1995). We can thus envisage that the change in MOL fiber number has occurred at the expense of the number of these medial muscle fibers during evolution. However, this is unlikely because similar numbers of medial fibers were observed in the four *Drosophila* species we examined (data not shown).

In vertebrates, extra muscle fibers can develop during postnatal growth from satellite cells, a group of undifferentiated cells that surrounds muscle fibers and appear at late fetal and postnatal stages (Zammit et al. 2006). For example, the sex difference in muscle fiber number of both the vocal organ in adult African frogs *Xenopus laevis* and the levator ani muscle in rats is the result of the addition of more fibers in males than in females after metamorphosis and after birth, respectively (Marin et al. 1990; Joubert and Tobin 1995). It is not known whether satellite cells exist and whether addition of muscle fibers occurs after metamorphosis in *Drosophila*, in particular during MOL formation.

Genes responsible for the change in MOL fiber number

The difference in MOL fiber number between *D. sechellia* and *D. simulans* seems to have a simple genetic basis. Because we did not find any QTL on chromosomes X, 3, and 4, our data eliminate the candidate genes *myoglianin*, *heartless*, heartbroken, *sprouty*, and *dumbfounded*, at least for an intermediate or large effect on MOL fiber number (Fig. 3). Furthermore, our

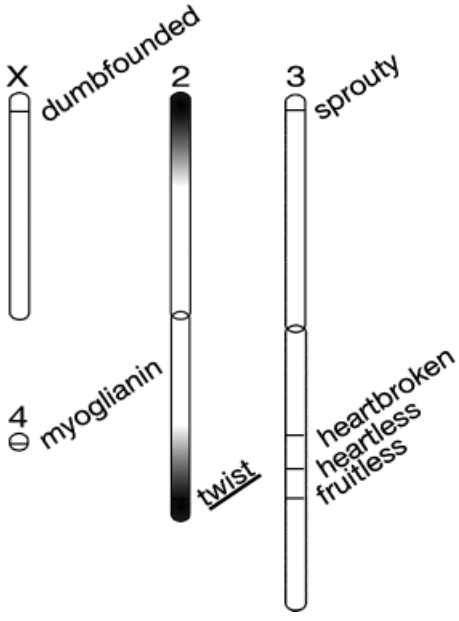

Fig. 3. Genomic regions and candidate genes responsible for the variation in muscle of Lawrence (MOL) fiber number. The position of the candidate genes is indicated on the schematic representation of the four chromosomes of a *Drosophila melanogaster* subgroup species. The genomic regions that we have identified to be responsible for the difference in MOL fiber number between *Drosophila simulans* and *Drosophila sechellia* are shaded in black. *Twist* is the only candidate gene included in these regions.

results rule out another candidate gene located on chromosome 3, the *fruitless* gene, which is expressed in the motor neuron innervating the MOL (Billeter and Goodwin 2004) and that controls MOL development (Gailey et al. 1991; Taylor and Knittel 1995; Yamamoto et al. 1998; Usui-Aoki et al. 2000).

We identified two QTL located at the extremities of chromosome 2. One candidate gene for the right end of chromosome 2 is *twist* (Fig. 3). *Twist* is expressed in adult myoblast cells during larval and pupal stages (Bate et al. 1991; Currie and Bate 1991). In *twist* hypomorphic mutants, the dorsal longitudinal muscles located in the thorax contain fewer fibers (Anant et al. 1998), indicating that *twist* might control the number of myoblasts (Fig. 3). As muscle differentiation begins, founder cells are the first cells in the myoblast pool to show declining levels of *twist* expression (Dutta et al. 2004), and this decrease in *Twist* levels is a requirement for founder cell differentiation (Anant et al. 1998; Dutta et al. 2004). This suggests that *twist cis*-regulation might also be part of the molecular mechanism involved in the selection of founder cells (Fig. 3). Based on these observations, it is tempting to propose that a change in the *twist cis*-regulatory region might have caused part of the evolutionary change in MOL fiber number. To test this hypothesis, we tried to generate introgression lines containing the right end of *D. sechellia* chromosome 2 into *D. simulans*. Unfortunately, we were not successful, probably because of sterility or inviability factors located nearby (Hollocher and Wu 1996).

No obvious candidate gene can be proposed for the left end of chromosome 2, highlighting the currently limited understanding of muscle fiber number determination. Interactions between motor neurons and muscles are known to play an important role during development. The close association of the developing MOL with motor neurons (see, e.g., Fig. 2) suggests that MOL fiber number might be controlled by the innervating neuron. Using sexually mosaic flies, MOL formation was found to depend on the sex of the innervating neuron but not of the myoblasts or epidermis (Lawrence and Johnston 1984, 1986). Analogous experiments using mosaic flies made with different species of the *D. melanogaster* subgroup could be used to determine whether the species-specific MOL fiber number is determined by myoblasts themselves, or by other tissues such as the epidermis or the innervating neuron.

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