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Supplemental Information

A Beetle Flight Muscle Displays Leg Muscle Microstructure

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Supporting Figures

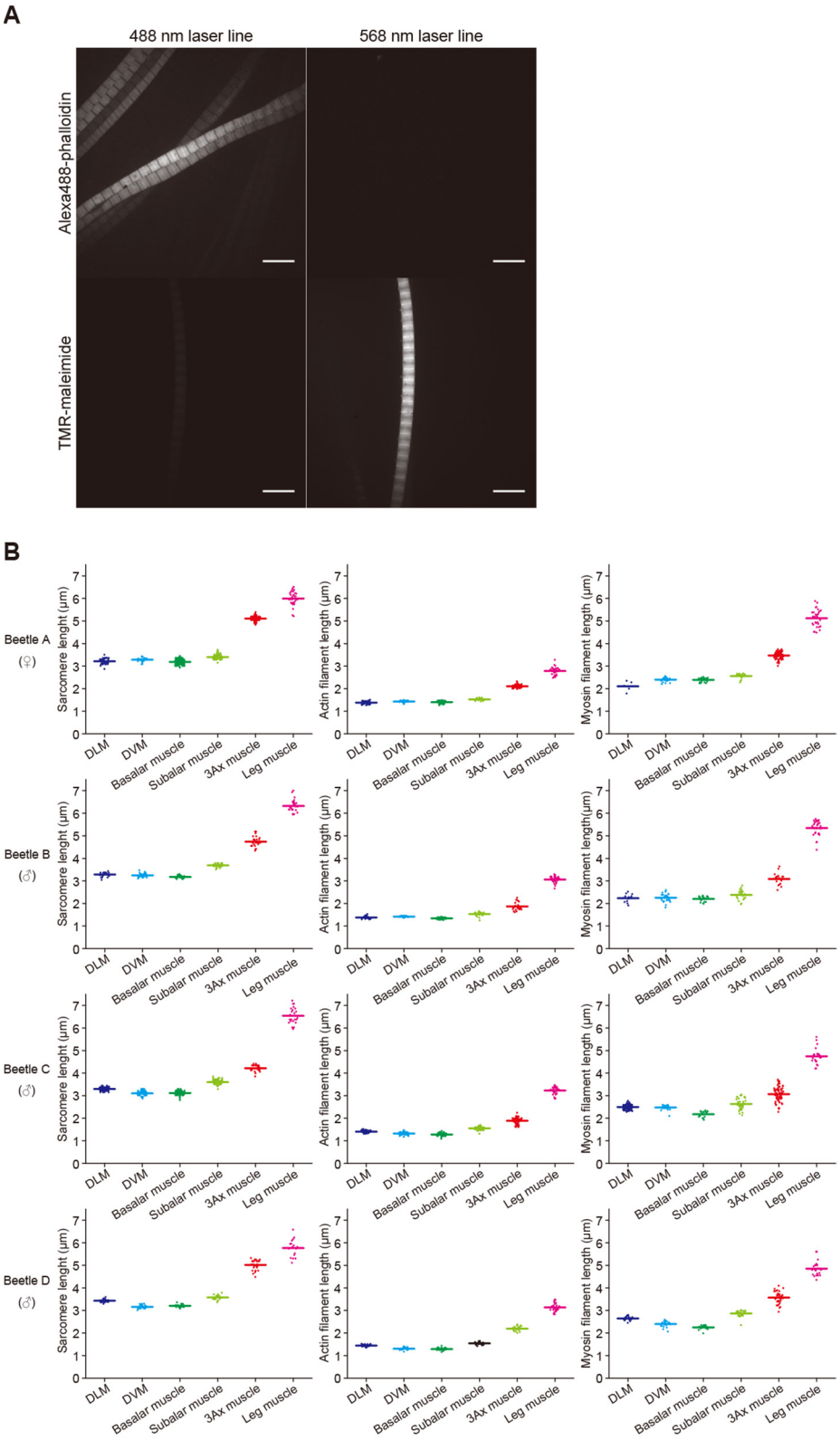

Figure S1 Confocal fluorescence microscopy of flight and leg muscles.

(*A*) Cross-talk between Alexa Fluor 488 and TMR in confocal microscopy setup. Fluorescence images of myofibrils of DLM co-labeled with Alexa488-phalloidin (top) and TMR-maleimide (bottom). Images were acquired with laser excitation of 488 nm (left) or 568 nm (right). Alexa Fluor 488 fluorescence was undetectable with 568 nm excitation (top, right). Although TMR fluorescence was observed with 488 nm excitation, it was negligibly weaker than Alexa Fluor 488 fluorescence. Scale bars, 10 μm. (*B*) Sarcomere, myosin filament and actin filament lengths in each animal determined from confocal fluorescence images.

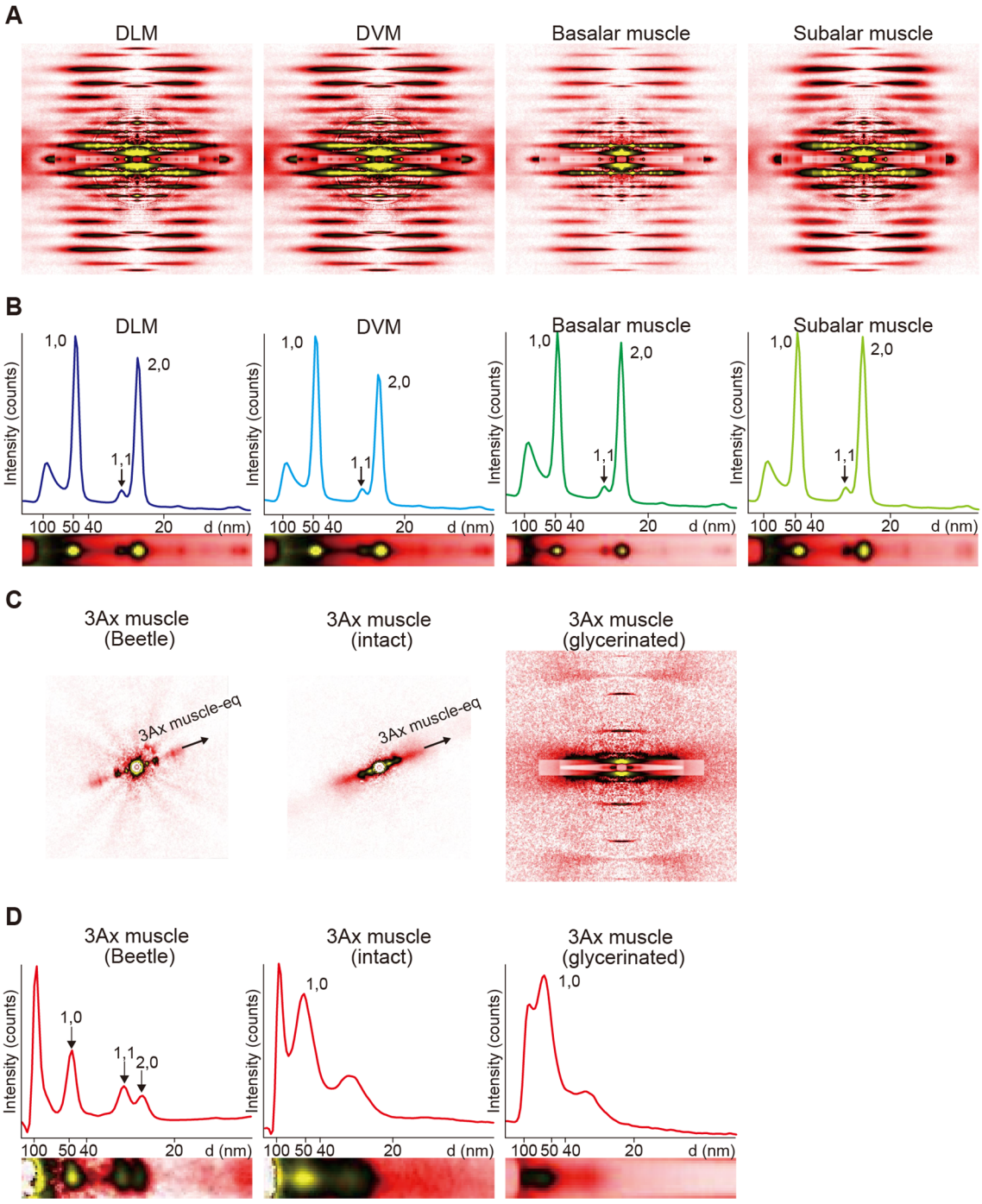

Figure S2 Synchrotron x-ray diffractions from flight muscles.

(*A* and *B*) Insect flight muscles in rigor condition. (*A*) The meridians of the patterns (the longitudinal axes of the muscle fibers) are oriented vertically. (*B*) Magnified views of a half of the equatorial reflections and their intensity profiles reproduced from (*A*). (*C* and *D*) The 3Ax muscle in the beetle (left), the 3Ax muscle dissected fresh from the beetle

(center, within half an hour from dissection) and the glycerinated fiber of the 3Ax muscle (right). (*C*) The orientation of the pattern is as recorded (left and center), or the meridian of the pattern from the glycerinated fiber (the longitudinal axes of the muscle fiber) is oriented vertically (right). (*D*) Magnified views of a half of the equatorial reflections and their intensity profiles reproduced from (*C*).

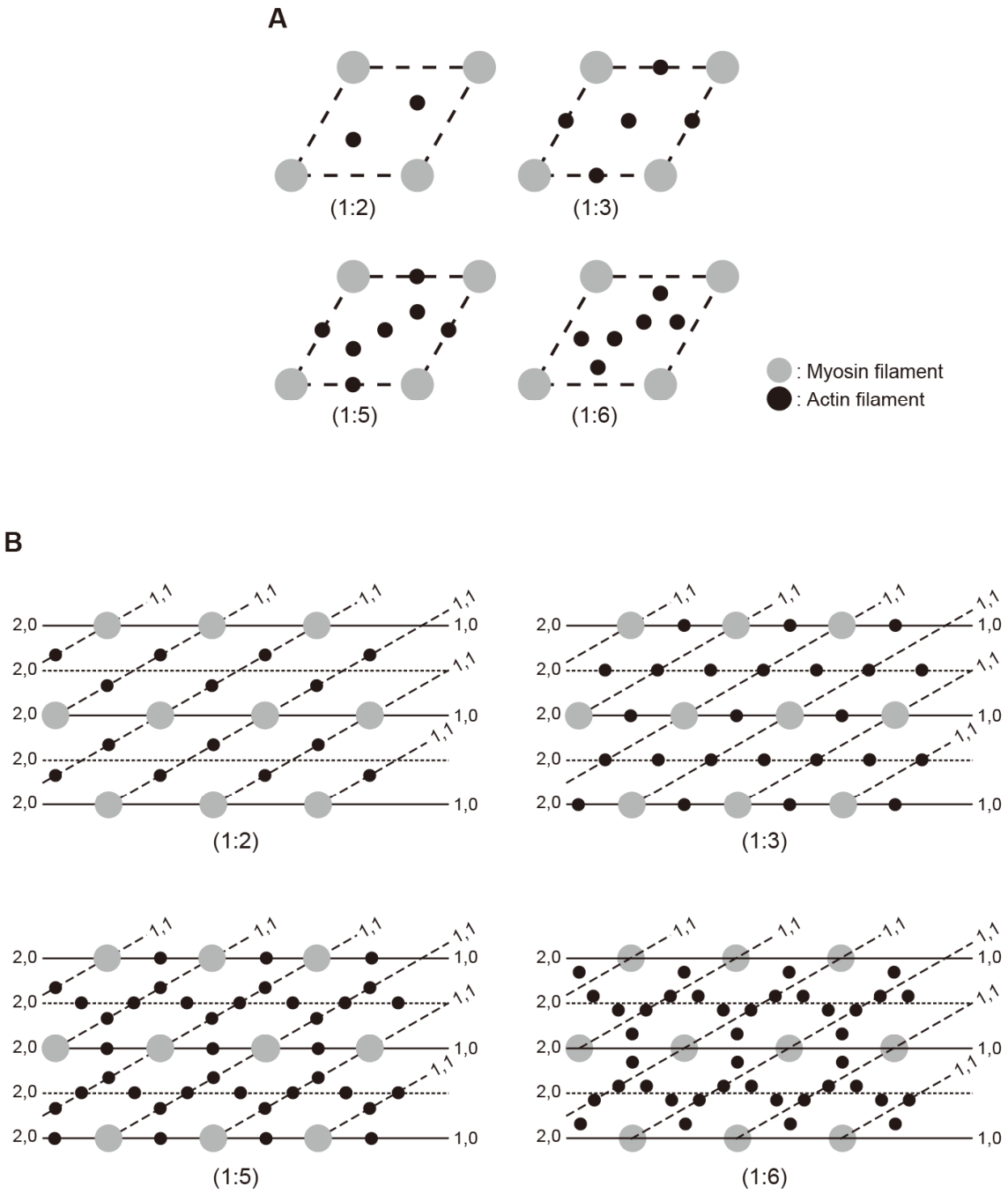

Figure S3 Schematic illustration of the various filament lattices.

(*A*) Representative arrangements of thick (myosin) and thin (actin) filaments. (*B*) The arrangement of thick and thin filaments in 1 : 2, 1 : 3, 1 : 5 and 1 : 6 lattices. The 1,0, 1,1 and 2,0 planes are shown by solid, broken and dotted lines, respectively. The thick-to-thin filament ratio can be inferred from the comparable intensities of 1,1 and 2,0 reflections in x-ray diffraction patterns. See Supplementary Discussion for details.

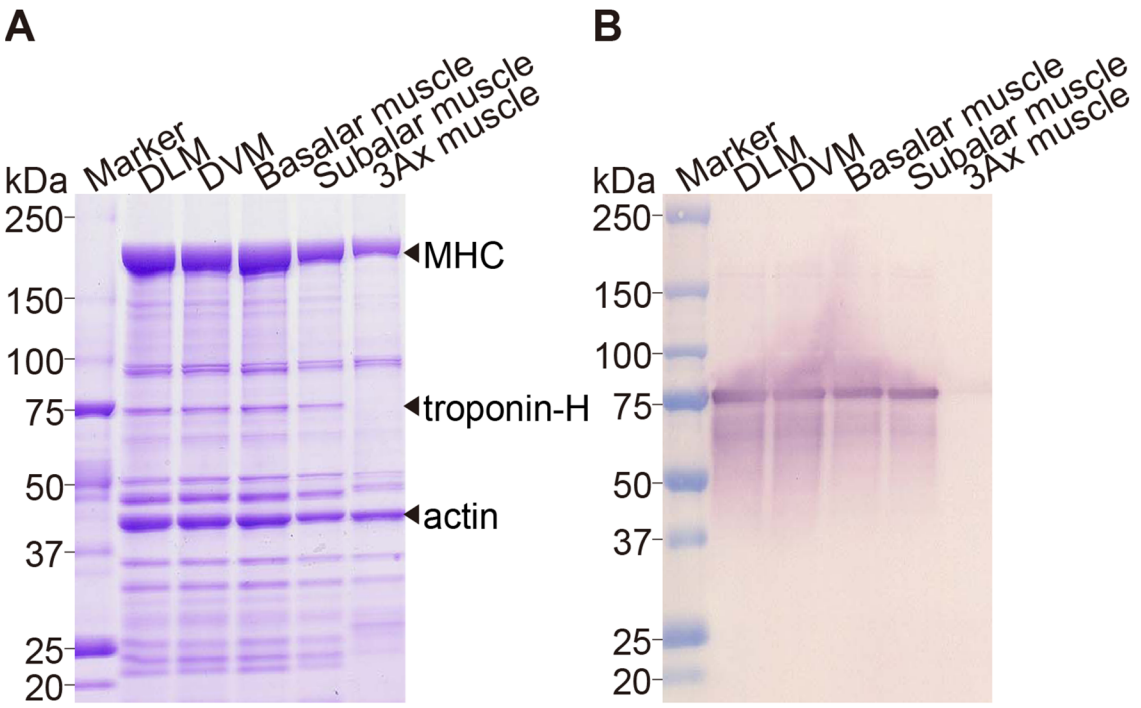

Figure S4 SDS-PAGE and immunoblotting of flight muscles

(*A*) SDS-PAGE of muscle fibers. Note that the bands corresponding to troponin-H are absent in the 3Ax muscle, and the lower mass regions show different expression patterns between the 3Ax muscle and other flight muscles. (*B*) Immunoblot analysis against troponin-H (MAC143).

Table S1 Sarcomere, myosin filament and actin filament lengths and A-band width determined by light microscopy.

Phase-contrast microscopy

Muscle	Sarcomere length (μm)	Myosin filament length (μm)
Dorsal longitudinal muscle (DLM)	3.40 ± 0.16 (n=31, N=3)	2.55 ± 0.08 (n=31, N=3)
Dorso-ventral muscle (DVM)	3.39 ± 0.11 (n=33, N=3)	2.50 ± 0.09 (n=33, N=3)
Basalar muscle	3.39 ± 0.14 (n=31, N=3)	2.48 ± 0.14 (n=33, N=3)
Subalar muscle	3.52 ± 0.12 (n=35, N=3)	2.70 ± 0.90 (n=32, N=3)
3Ax muscle	5.15 ± 0.26 (n=29, N=3)	3.27 ± 0.29 (n=30, N=3)
Leg muscle	6.34 ± 0.27 (n=28, N=3)	4.87 ± 0.19 (n=33, N=3)

n, the number of sarcomeres. N, the number of beetles. (mean ± SD)

Fluorescence microscopy

Muscle	Sarcomere length (μm)	Myosin filament length (μm)	Actin filament length (μm)
Dorsal longitudinal muscle (DLM)	3.29 ± 0.11 (n=301, N=5)	2.49 ± 0.14 (n=212, N=5)	1.42 ± 0.05 (n=292, N=5)
Dorso-ventral muscle (DVM)	3.21 ± 0.11 (n=273, N=5)	2.40 ± 0.15 (n=131, N=5)	1.38 ± 0.08 (n=204, N=5)
Basalar muscle	3.18 ± 0.09 (n=300, N=5)	2.33 ± 0.17 (n=128, N=5)	1.34 ± 0.07 (n=302, N=5)
Subalar muscle	3.50 ± 0.14 (n=301, N=5)	2.61 ± 0.25 (n=113, N=5)	1.54 ± 0.06 (n=152, N=5)
3Ax muscle	4.89 ± 0.33 (n=200, N=5)	3.27 ± 0.32 (n=139, N=5)	2.00 ± 0.15 (n=146, N=5)
Leg muscle	6.31 ± 0.42 (n=238, N=5)	5.12 ± 0.41 (n=136, N=5)	2.92 ± 0.32 (n=144, N=5)

n, the number of sarcomeres. N, the number of beetles. (mean ± SD)

Table S2 Summary of significance test results for optical microscopy analyses

S.L. by phase-contrast microscopy

	DLM	DVM	Basalar	Subalar	3Ax muscle	Leg
DLM		0.99939	0.99914	0.10298	9.22×10^{-8}	$< 1\times10^{-10}$
DVM	0.99939		1	0.03668	1.39×10^{-8}	$< 1\times10^{-10}$
Basalar	0.99914	1		0.03409	1.40×10^{-8}	$< 1\times10^{-10}$
Subalar	0.10298	0.03668	0.03409		7.69×10^{-7}	$< 1\times10^{-10}$
3Ax muscle	9.22×10^{-8}	1.39×10^{-8}	1.40×10^{-8}	7.69×10^{-7}		1.64×10^{-8}
Leg	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	1.64×10^{-8}	

A-band by phase-contrast microscopy

	DLM	DVM	Basalar	Subalar	3Ax muscle	Leg
DLM		0.76657	0.42506	0.00109	$< 1\times10^{-10}$	$< 1\times10^{-10}$
DVM	0.76657		0.99423	2.41×10^{-6}	$< 1\times10^{-10}$	$< 1\times10^{-10}$
Basalar	0.42506	0.99423		1.69×10^{-7}	1.33×10^{-8}	$< 1\times10^{-10}$
Subalar	0.00109	2.41×10^{-6}	1.69×10^{-7}		$< 1\times10^{-10}$	$< 1\times10^{-10}$
3Ax muscle	$< 1\times10^{-10}$	$< 1\times10^{-10}$	1.33×10^{-8}	$< 1\times10^{-10}$		$< 1\times10^{-10}$
Leg	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	

S.L. by fluorescence microscopy

	DLM	DVM	Basalar	Subalar	3Ax muscle	Leg
DLM		1.63×10^{-5}	3.89×10^{-9}	8.54×10^{-10}	$< 1\times10^{-10}$	$< 1\times10^{-10}$
DVM	1.63×10^{-5}		0.365	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$
Basalar	3.89×10^{-9}	0.365		$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$
Subalar	8.54×10^{-10}	$< 1\times10^{-10}$	$< 1\times10^{-10}$		$< 1\times10^{-10}$	$< 1\times10^{-10}$
3Ax muscle	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$		$< 1\times10^{-10}$
Leg	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	

Actin filament by fluorescence microscopy

	DLM	DVM	Basalar	Subalar	3Ax muscle	Leg
DLM		0.02735	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$
DVM	0.02735		0.0315	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$
Basalar	$< 1\times10^{-10}$	0.0315		$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$
Subalar	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$		1.17×10^{-8}	$< 1\times10^{-10}$
3Ax muscle	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	1.17×10^{-8}		$< 1\times10^{-10}$
Leg	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	

Myosin filament by fluorescence microscopy

	DLM	DVM	Basalar	Subalar	3Ax muscle	Leg
DLM		0.04182	7.37×10^{-7}	2.77×10^{-4}	4.67×10^{-9}	$< 1\times10^{-10}$
DVM	0.04182		0.1772	$< 1\times10^{-10}$	4.58×10^{-9}	$< 1\times10^{-10}$
Basalar	7.37×10^{-7}	0.1772		$< 1\times10^{-10}$	5.28×10^{-9}	$< 1\times10^{-10}$
Subalar	2.77×10^{-4}	$< 1\times10^{-10}$	$< 1\times10^{-10}$		$< 1\times10^{-10}$	$< 1\times10^{-10}$
3Ax muscle	4.67×10^{-9}	4.58×10^{-9}	5.28×10^{-9}	$< 1\times10^{-10}$		$< 1\times10^{-10}$
Leg	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	$< 1\times10^{-10}$	

Data were statistically compared by using one-way ANOVA with Tukey-Kramer's test. Values of significance tests were described in Figure 2 as: NS, not significant; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Table S3 The 1,0 lattice spacing (*d1,0*) and interfilament distance (the distance between the thick filaments) determined by x-ray diffraction study.

Muscle	*d1,0* (nm)	Interfilament distance (nm)
DLM	50.2 ± 0.2 (N=3, n=5)	58.0 ± 0.3 (N=3, n=5)
DVM	50.0 ± 0.4 (N=3, n=5)	57.7 ± 0.4 (N=3, n=5)
Basalar muscle	50.3 ± 0.3 (N=2, n=3)	58.1 ± 0.4 (N=2, n=3)
Subalar muscle	50.3 ± 0.3 (N=2, n=3)	58.1 ± 0.4 (N=2, n=3)
3Ax muscle	48.5 ± 1.2 (N=4, n=7)	56.0 ± 1.4 (N=4, n=7)
Leg muscle	50.1 ± 1.1 (N=2, n=2)	57.8 ± 1.2 (N=2, n=2)

n, the number of samples. N, the number of beetles.

The interfilament distance was determined by $d1,0 \times 2/\sqrt{3}$.

(mean ± SD)

Table S4 Summary of the length of muscle filaments and sarcomeres, the thick-to-thin filament ratio and the interfilament distance in previous studies from various species.

Muscle	Sarcomere length (μm)	Thick filament length (μm)	Thin filament length (μm)	Thick-to-thin filament ratio	Interfilament distance (nm)	References
Cockroach femur	6.4	4.5	2.3	5	57.0	(47,48)
Cockroach flight	NA	2.7	NA	3.8	NA	(47)
Cockroach intersegmental	7.8-8.3	3.6-4.1	4.1*	6	NA	(49)
Crab leg (long)	10.6*	5*	5*	6	59.4	(33,34)
Crab pink eye-raiser	4-12	NA	NA	7	50.0*	(35)
Crayfish leg	9.6	about 6	NA	6	62.1	(36,37)
Dog heart	1.90-1.98	1.5*	0.9*	2	NA	(38,39)
Dragonfly DVM	2.3	2.04*	1.08*	3*	NA	(40)
Frog sartorius	2.3	1.65	1*	2	42.1	(41)
Lethocerus femur	4.6*	3.1-3.8	1.8-2.2	4.8-5.2	NA	(16)
Lethocerus flight (indirect muscle)	2.6	2.3	1.2*	3	53.0	(43,44)
Lobster claw	12.4	6	4.5*	12	50.0	(18)
Lobster tail	4.0-5.2	3.4	1.7*	3	43.5	(18)
Rabbit psoas	1.8*	1.3*	0.8*	2	50.0	(16,29, 42)
Rhodnius prolixus ventral intersegmental abdominal muscles	8-10	6.6*	4.9*	6	NA	(45)
Scallop striated adductor	2.2-3.7	1.76	1.01	5-6	60.6	(46)
Tarantula femur	5	4.0-4.7	2.1*	about 5	NA	(17)

*, values were estimated based on the electron microscopy images provided.

Supporting Discussion

The calculated effect of lattice type on equatorial intensities

Different lattice types have different effects on the equatorial intensities. By assuming an ideal hexagonal lattice and that all the mass of the filament is only at the center of the filament, Elliott *et al.* (1) and Iwamoto (2) have calculated the intensities of equatorial reflections in 1 : 2 (vertebrate skeletal muscle type) and 1 : 3 (flight muscle type) lattices. Briefly, in 1 : 2 lattices, the phases of two thin filaments in a unit cell with respect to that of the thick filament are $2\pi/3$ and $4\pi/3$ for 1,0; 0 and 0 for 1,1; and $4\pi/3$ and $8\pi/3$ for 2,0 (Fig. S3). The amplitudes of equatorial intensities can be calculated by summing these structural factors; $F(1,0) = F(2,0) = \mathrm{FM} - \mathrm{FA}$, and $F(1,1) = \mathrm{FM} + 2\mathrm{FA}$, where FM and FA are the amplitudes of scattering factors for the thick (myosin) and thin (actin) filaments, respectively. The strong 1,1 and the weak 2,0 reflections are therefore expected. In 1 : 3 lattices, the amplitudes are $F(1,0) = F(1,1) = \mathrm{FM} - \mathrm{FA}$, and $F(2,0) = \mathrm{FM} + 3\mathrm{FA}$. In this lattice, the 1,1 reflection is expected to be substantially weaker than 2,0 (cf. Fig. 3 *B*)

The 1 : 5 lattice is the superposition of the 1 : 2 and 1 : 3 lattices (Fig. S3 *A*). Thus, under the same assumptions, the amplitudes in the 1 : 5 lattice can be calculated as the sum of the 1 : 2 and 1 : 3 lattices; i.e., $F(1,0) = \mathrm{FM} - 2\mathrm{FA}$, $F(1,1) = \mathrm{FM} + \mathrm{FA}$, and $F(2,0) = \mathrm{FM} + 2\mathrm{FA}$.

In the 1 : 6 lattice (Fig. S3 *A*), the phases of six thin filaments are $(2\pi\ (3 - \sqrt{3}))/3$, $(2\pi\ (2\sqrt{3} - 3))/3$, $(2\pi\ (3 - \sqrt{3}))/3$, $2\pi\sqrt{3}/3$, $2\pi\ (6 - 2\sqrt{3}))/3$ and $2\pi\sqrt{3}/3$ for 1,0; $2\pi\ (2 - \sqrt{3})$, $2\pi\ (\sqrt{3} - 1)$, 0, 0, $2\pi\ (2 - \sqrt{3})$ and $2\pi\ (\sqrt{3} - 1)$ for 1,1; and $(2\pi\ (6 - 2\sqrt{3}))/3$, $(2\pi\ (4\sqrt{3} - 6))/3$, $(2\pi\ (6 - 2\sqrt{3}))/3$, $4\pi\sqrt{3}/3$, $(2\pi\ (12 - 4\sqrt{3}))/3$ and $4\pi\sqrt{3}/3$ for 2,0. Thus, the amplitudes are calculated to be; $F(1,0) = \mathrm{FM} - 2.41\mathrm{FA}$, $F(1,1) = \mathrm{FM} + 1.55\mathrm{FA}$, and $F(2,0) = \mathrm{FM} + 1.53\mathrm{FA}$. Different from 1 : 2 and 1 : 3 lattices, the 1,1 and 2,0 reflections are estimated to be comparable in 1 : 5 and 1 : 6 lattices.

Supporting References

1. Elliott, G. F., J. Lowy, and C.R. Worthington. 1963. An X-ray and light-diffraction study of the filament lattice of striated muscle in the living state and in rigor. J. Mol. Biol. 6: 295–305.

2. Iwamoto, H. 2013. Flight muscle-specific Pro-Ala-rich extension of troponin is important for maintaining the insect-type myofilament lattice integrity. J. Struct. Biol. 183: 33–39.