Supporting Information

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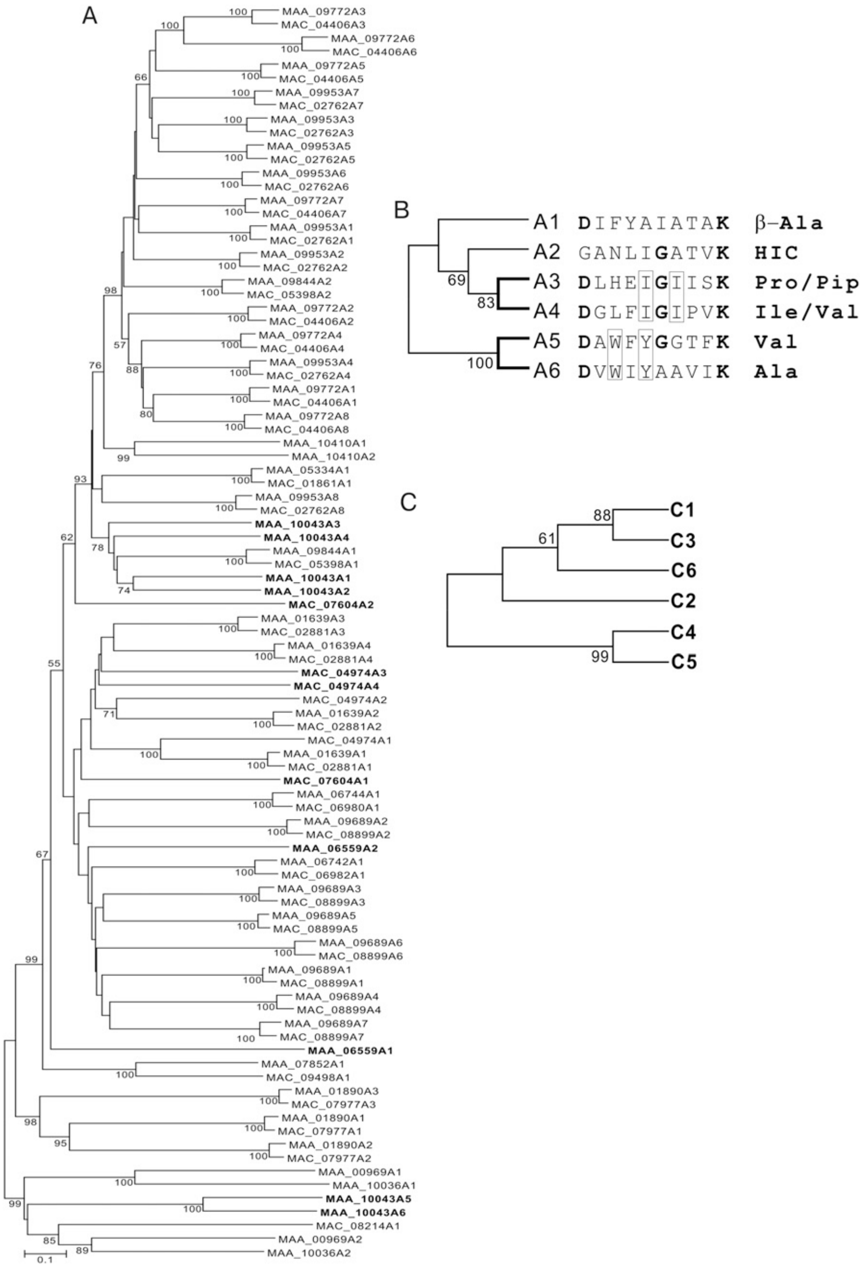

Fig. S1. Phylogenetic analysis. (*A*) Analysis of *M. robertsii* (loci tagged as MAA) and *M. acridum* (loci tagged as MAC) NRPSs based on their adenlylation domain sequences. The domains highlighted in bold indicate that the corresponding NRPS gene is present in one species but absent in the other. (*B*) Phylogenetic and signature analysis of DtxS1 A domains. (*C*) Analysis of DtxS1 C domains.

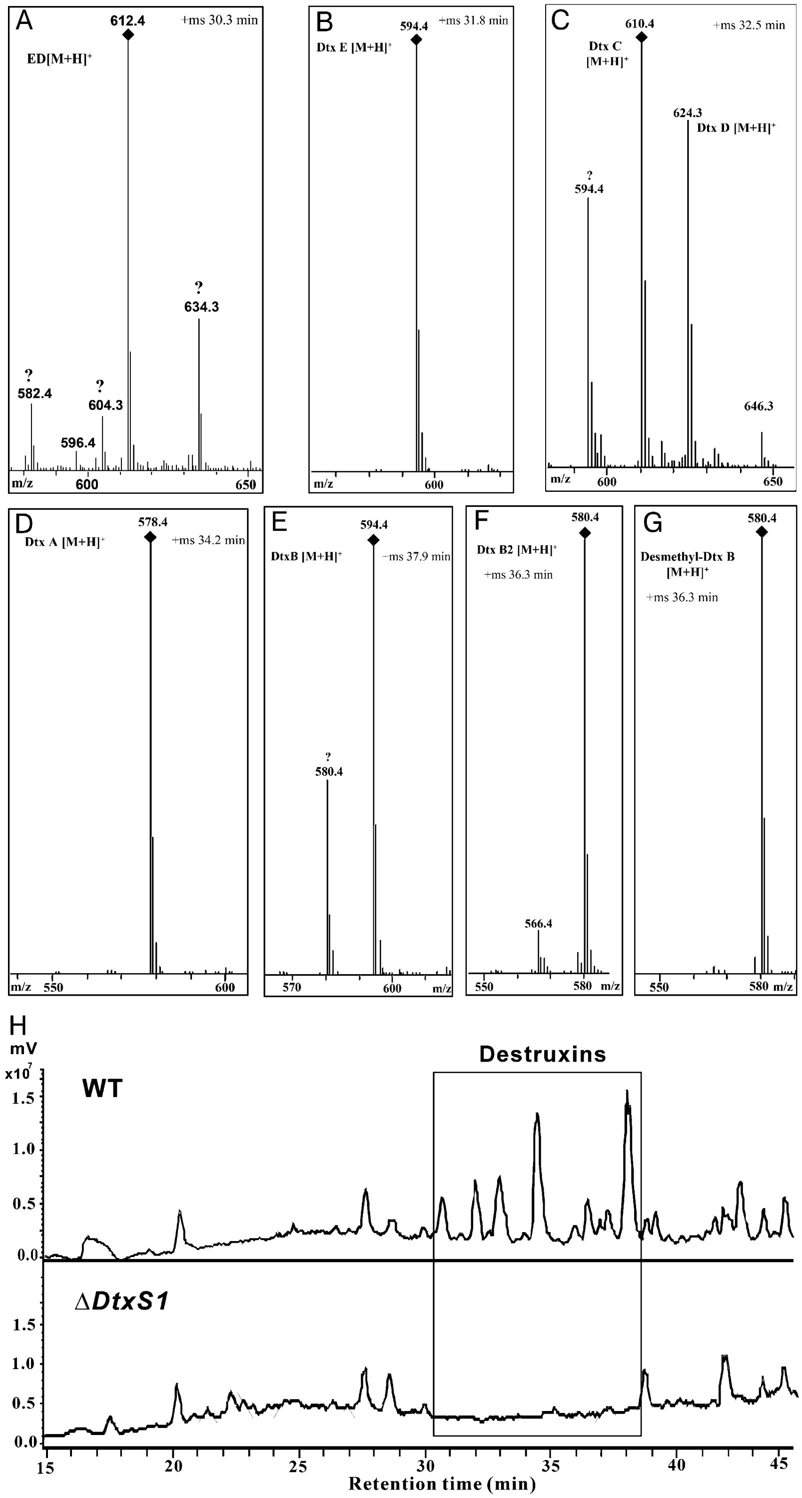

Fig. S2. Mass spectrometric and high-performance ion chromatography analysis. (*A*) Mass spectrum of dtx E-diol in the cultures of *M. robertsii* strain ARSEF 23 after incubation in CD medium for 7 d. (*B*) Mass spectrum of dtx E. (*C*) Mass spectra of dtx C and dtx D. (*D*) Mass spectrum of dtx A. (*E*) Mass spectrum of dtx B. (*F*) Mass spectrum of dtx B_2. (*G*) Mass spectrum of desmethyl-dtx B. (*H*) High-performance ion chromatography analysis of dtxs produced *in insecta* by the WT and Δ*dtxS1* on mycosed silkworm larvae.

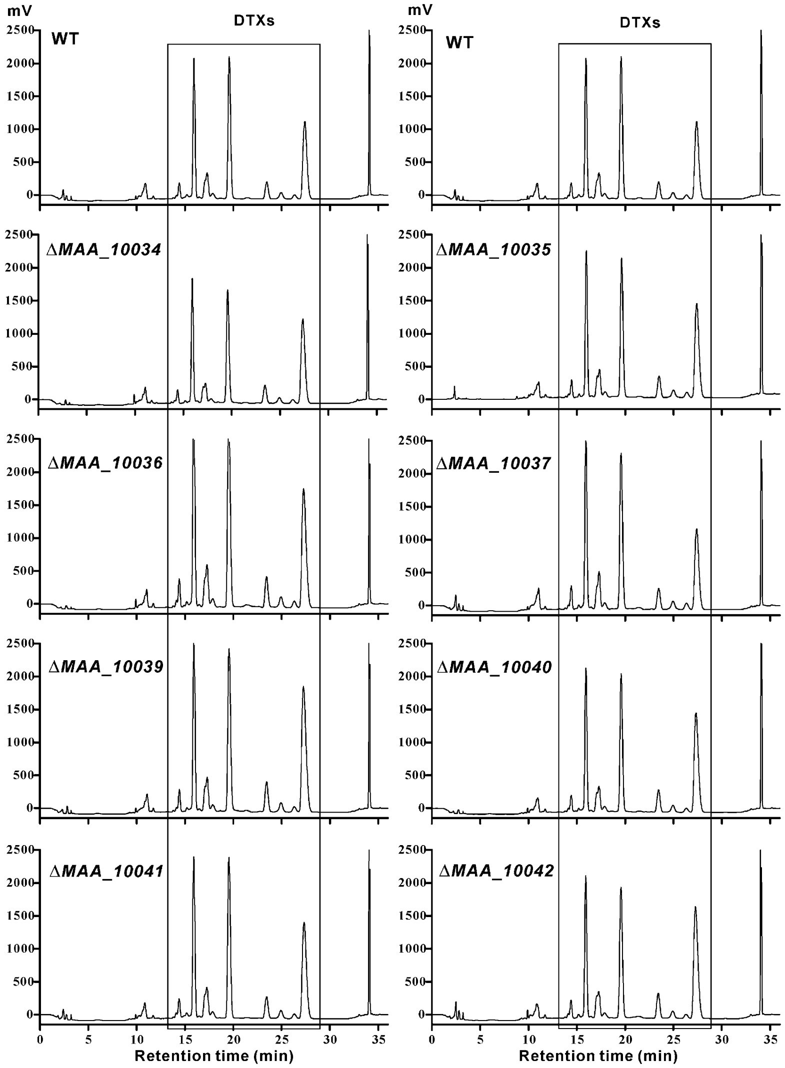

Fig. S3. HPLC profiling of the WT and *dtxS1* upstream gene deletion mutants of *M. robertsii*. The profiles of the WT and mutants are similar, indicating that these genes are not involved in dtx biosynthesis. Gene locus numbers are labeled in each panel and the peaks for dtxs are framed.

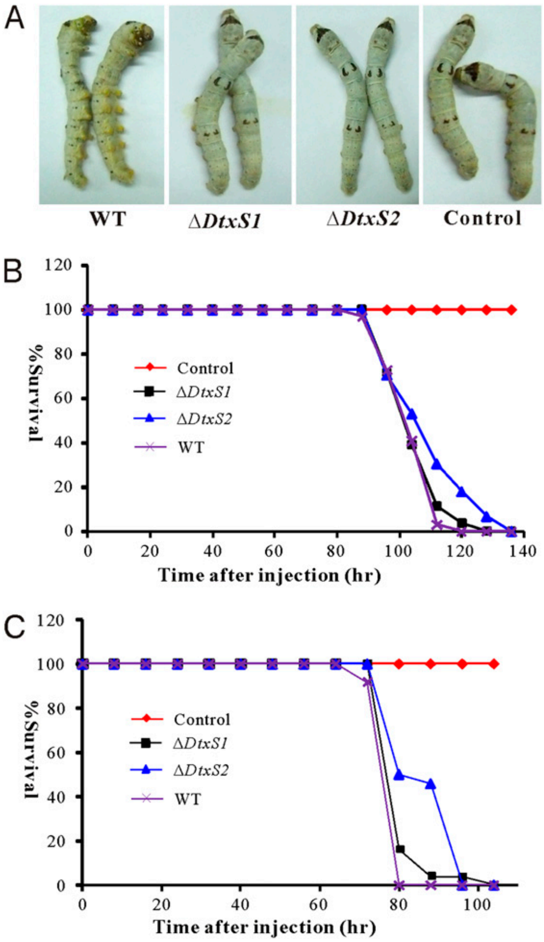

Fig. S4. Insect bioassays. (*A*) The WT and mutant strains of *M. robertsii* were grown in the CD medium for 7 d. The culture filtrates were used for injection of the fifth instar larvae of silkworms (50 μL each insect). The insects injected with the WT sample demonstrated an instant paralysis symptom and died within a few minutes. In contrast, no apparent symptoms were observed for those injected with the mutant culture filtrates or CD medium (control). (*B*) Survival curves of locusts after injection of a spore suspension (1×10^6 spores/mL; 10 μL per insect) of the WT, *ΔdtxS1*, and *ΔdtxS2* mutants. (*C*) Survival curves of silkworm larvae after injection of a spore suspension (5×10^5 spores/mL; 10 μL each) of the WT, *ΔdtxS1*, and *ΔdtxS2* mutants. Insects injected with water were included as controls.

Table S1. Modulation analysis of NRPS proteins encoded in the *M. robertsii* and *M. acridum* genomes

Species and locus	No. of amino acids	No. of A domains	No. of C domains	Modulation analysis*
M. robertsii				
MAA_00969	1,983	2	2	ATC-ATC
MAA_01639	5,433	4	4	ATEC-ATC-ATC-ATEC
MAA_01890	4,845	3	4	AC-ATC-CACC
MAA_05334	1,800	1	2	ATC-TC
MAA_06559	1,975	2	1	ATC-A
MAA_06742	1,301	1	1	ATC
MAA_06744	1,854	1	1	ATC
MAA_07852	1,647	1	2	C-ATC
MAA_09689	9,413	7	7	ATEC-ATC-ATC-ATEC-ATEC-ATEC-ATC
MAA_09772	10,277	8	8	ATC-ATC-ATEC-ATC-ATEC-ATEC-ATC-ATC-T
MAA_09844	3,552	2	3	ATC-ATEC-TC
MAA_09953	10,188	8	8	ATC-ATC-ATEC-ATC-AEC-ATC-ATEC-ATC
MAA_10036	2,139	2	2	ATC-ATC
MAA_10043	**7,898**	**6**	**6**	**ATC-ATEC-ATC-ATC-A(NM)TC-A(NM)TC**
MAA_10410	3,183	2	2	TC-ATEC-AT
M. acridum				
MAC_01861	1,644	1	2	ATC-TC
MAC_02762	10,186	8	8	ATC-ATC-ATEC-ATC-AEC-ATC-ATEC-ATC
MAC_02881	5,435	4	4	ATEC-ATC-ATC-ATEC
MAC_04406	10,285	8	8	ATC-ATC-ATEC-ATC-ATEC-ATEC-ATC-ATC-T
MAC_04974	5,358	4	4	ATEC-ATC-ATC-ATEC
MAC_05398	3,530	2	3	ATC-ATEC-TC
MAC_06980	1,793	1	1	ATC
MAC_06982	889	1	1	ATC
MAC_07604	2,607	2	2	ATC-ATEC
MAC_07977	4,838	3	4	AC-ACC-ACC
MAC_08214	1,693	1	1	T-ATTC
MAC_08899	9,390	7	6	ATEC-ATC-ATC-ATEC-ATEC-ATEC-AT
MAC_09498	1,616	1	2	C-ATC

For modulation analysis: A, adenylation domain; C, condensation domain; E, epimerization domain; and NM, *N*-methylation domain; T, thiolation (or peptidyl carrier protein, PCP) domain.

*The locus MAA_10043 highlighted in bold is verified for the biosynthesis of dtxs.

Table S2. H and C NMR data for structural verifications of dtx B, B_2 and desmethyl-B produced by *M. robertsii*

	Dtx B*		Dtx B_2†		Desmethyl-Dtx B†	
No.	δ (H)	δ (C)	δ (H)	δ (C)	δ (H)	δ (C)
1α	4.91 m	71.96 d	4.96 m	71.9 d	4.89 dd, 10.0, 2.8	71.53 d
1β	1.38 m	38.96 t	1.39 m	39.0 d	1.28 m	38.97 t
	1.96 m		1.87 m		1.97 m	
1γ	1.87 m	24.42 d	1.88 m	24.4 d	1.43 m	24.47 d
$1\delta_1$	1.00 d, 6.6	23.37 q	1.02 d, 6.4	24.0 q	1.01 d, 6.8	21.44 q
$1\delta_2$	0.87 d, 8.5	15.40 q	0.90 d, 6.0	21.5 q	0.96 d, 6.8	23.32 q
1C=O	—	173.58 s	—	173.2 s	—	171.85 s
2α	4.68 d, 7.0	60.74 d	4.69 d, 6.8	60.7 d	4.23 dd, 9.6, 6.0	60.81 d
2β	1.93 m	28.89 t	1.93 m	28.9 t	1.96 m	30.47 t
	2.51 m		2.55 m		2.16 m	
2γ	1.32 m	24.48 t	1.95 m	24.1 t	2.06 m	24.18 t
	1.45 m		2.09 m			
2δ	3.44 m	46.48 t	3.45 m	46.5 t	3.46 m	46.61 t
	3.92 t, 17.3, 9.0		3.92 m		3.93 m	
2C=O	—	170.97 s	—	171.1 s	—	171.67 s
$3N\underline{H}$	7.20 d, 9.1	—	7.25 d, 9.2	—	7.08 d, 8.8	—
3α	4.89 d, 7.7	53.69 d	4.91 m	54.1 d	4.55 dd, 9.2, 6.4	55.65 d
3β	1.94 m	37.52 d	2.17 m	30.3 d	1.93 m	38.43 d
$3\gamma_1$	1.93 m	24.13 t	0.95 d, 7.2	17.3 q	1.43 m	25.09 t
	2.08 m					
$3\gamma_2$	0.96 d, 6.9	21.49 q	0.98 d, 6.4	19.4 q	0.92 d, 6.4	15.33 q
3δ	0.85 d, 7.2	11.37 q	—	—	0.86 t, 6.4	11.36 q
3C=O	—	169.72 s	—	169.9 s	—	169.69 s
$4N\underline{CH_3}$	3.23 s	30.86 q	3.25 s	30.8 q	6.49(4NH), 6.4	—
4α	4.94 d, 11.0	58.14 d	4.93 m	58.2 d	4.61 d, 6.4	56.31 d
4β	2.34 m	27.27 d	2.33 m	27.4 d	2.43 m	28.45 d
$4\gamma_1$	0.90 d, 6.3	19.66 q	0.90 d, 6.0	19.8 q	0.85 d, 7.2	19.67 q
$4\gamma_2$	0.95 d, 7.5	20.04 q	0.97 d, 6.4	20.0 q	0.83 d, 7.2	19.67 q
4C=O	—	171.12 s	—	171.4 s	—	170.83 s
$5N\underline{CH_3}$	2.74 s	28.10 q	2.75 s	28.1 q	2.73 s	29.15 q
5α	5.20 q, 14.6, 6.8	55.49 d	5.21 q, 14.8, 6.8	55.5 d	5.06 dd, 10.0, 6.8	55.66 d
5β	1.32 d, 6.6	15.26 q	1.32 d, 6.8	15.1 q	1.32 d, 6.8	14.84 q
5C=O	—	169.73 s	—	169.7 s	—	169.87 s
$6N\underline{H}$	8.22 d, 9.8	—	8.14 d, 8.0	—	8.29 d, 6.8	—
6α	2.59 m	34.44 t	2.56 m	34.4 t	2.54 m	34.26 t
	2.71 m		2.69 m		2.69 m	
6β	3.11 t, 24.6, 12.7	33.25 t	3.10 m	33.3 t	3.16 m	33.58 t
	4.08 m		4.06 m		3.98 m	
6C=O	—	173.78 s	—	174.0 s	—	173.31 s

The compounds were dissolved in $CDCl_3$. δ in ppm, *J* in Hz.

*600/150 MHz.

†400/100 MHz.

Table S3. Quantification of dtxs E, A, B, and B$_2$ produced by the WT, mutants, and different species of *Metarhizium* in Czapek Dox broth for 7 d

Strains	Dtx E (mg/L)	Dtx A (mg/L)	Dtx B (mg/L)	Dtx B2 (mg/L)
ARSEF 23	63.1 ± 4.5Cd	80.6 ± 4.6Bb	43.1 ± 7.1Bb	7.8 ± 1.1Bb
ΔDtxS1	0	0	0	0
ΔDtxS1+Dtx B	15.1 ± 2.3Df	5.6 ± 0.6Dd	43.7 ± 11.2Bb	0
ΔDtxS2	0	0	232.2 ± 31.1Aa	32.9 ± 4.4Aa
ΔDtxS1ΔDtxS2 +Dtx B	0	0	56.8 ± 13.1Bb	0
ΔDtxS3	0	0	0	0
ΔDtxS3+HIC	19.2 ± 5.1Df	2.8 ± 0.3Dd	1.6 ± 0.8Dd	0.1 ± 0Cd
ΔDtxS4	0	0	0	0
ΔDtxS4+β-Ala	92.6 ± 12.7Bb	105.1 ± 18.6Bb	58.1 ± 10.3Bb	10.9 ± 2.7Bb
ARSEF 2575	41.6 ± 3.5Ce	68.4 ± 8.0BCb	36.3 ± 1.9ABb	5.5 ± 0.4BCc
CQMa 102	0	0	0	0
ARSEF 324	0	0	0	0
ARSEF 549	63.1 ± 6.2Cd	37.2 ± 1.95Cc	7.6 ± 2.1Cc	0.3 ± 0Cd
V275	25.1 ± 1.3De	42.4 ± 5.8Cbc	14.5 ± 2.5Cc	0.7 ± 0.25Cd
ARSEF 576	93.6 ± 8.8Bb	42.2 ± 1.1Cbc	12.5 ± 0.4Cc	0.3 ± 0Cd
ARSEF 1009	38.6 ± 4.9Ce	47.9 ± 14.5BCbc	11.5 ± 2.4Cc	2.5 ± 0.1Cc
ARSEF 297	0	0	0	0
ARSEF 3297	190.1 ± 10.8Aa	180.4 ± 22.4Aa	57.3 ± 5.4Bb	8.5 ± 0.5Bb
ARSEF 1941	0	0	0	0
ARSEF 4124	0	0	0	0

The data are presented as averages ± SD (SD). Capital and lower letters in the same column show the difference levels at αα = 0.01 and αα = 0.05, respectively, between species/strains by ANOVA Duncan's tests. The values tagged with the same letter mean no significant differences between each other.

Table S4. Insect hosts and geographic origins of the *Metarhizium* species included in this study

Strains	Species	Original insect hosts	Geographic origin
ARSEF 23	*M. robertsii*	Coleoptera: Elateridae	USA: North Carolina
ARSEF 2575	*M. robertsii*	*Curculio caryae* [Coleoptera: Curculionidae]	USA: North Carolina
CQMa 102	*M. acridum*	*Ceracris kiangsu* [Orthoptera: Acrididae]	China: Chongqing
ARSEF 324	*M. acridum*	*Austracris guttulosa* [Orthoptera: Acrididae]	Australia
ARSEF 549	*M. anisopliae*	Not available	Brazil
ARSEF 977	*M. guizhouense*	*Melolontha melolontha* [Coleoptera: Scarabaeidae]	France
ARSEF 576	*M. pingshaense*	*Nilaparvata lugens* [Homoptera: Delphacidae]	Indonesia
ARSEF 1009	*M. pingshaense*	*Ornebius kanetataki* [Orthoptera: Gryllidae]	Japan: Tokyo
ARSEF 297	*M. majus*	*Xyloryctes jamaicensis* [Coleoptera: Scarabaeidae]	Western Samoa
ARSEF 3297	*M. brunneum*	*Boophilus* sp. [Acari: Ixodidae]	Mexico
ARSEF 1941	*M. album*	*Nephotettix virescens* [Hemiptera: Cicadellidae]	Philippines: Roxas
ARSEF 4124	*M. frigidum*	*Adoryphorus* sp. [Coleoptera: Scarabaeidae]	Australia: Ballarat

Table S5. Summary of the original insect host orders for different *Metarhizium* species cataloged in the USDA-ARS culture collection

Species	Total strain no.*	Original host order (no. of strains isolated from which)
M. robertsii	35	Coleoptera (25), Hemiptera (1), Homoptera (1), Hymenoptera (2), Isoptera (2), Lepidoptera (3), Orthoptera (1)
M. anisopliae[†]	38	Coleoptera (5), Diptera (6), Hemiptera (1), Homoptera (18), Isoptera (2), Lepidoptera (4), Orthoptera (2)
M. pingshaense	38	Coleoptera (11), Diptera (5), Hemiptera (2), Homoptera (6), Isoptera (2), Lepidoptera (2), Orthoptera (10)
M. brunneum	33	Acari: Ixodiae (1), Coleoptera (18), Diptera (2), Homoptera (1), Hymenoptera (1), Isoptera (1), Lepidoptera (9), Orthoptera (1)
M. guizhouense	10	Coleoptera (5), Diptera (1), Lepidoptera (3), Orthoptera (1)
M. majus[‡]	14	Coleoptera (12), Lepidoptera (2)
M. acridum	13	Orthoptera (13)
M. frigidum	15	Isoptera (14), Coleoptera (1)
M. album	9	Homoptera (9)

US Department of Agriculture Agricultural Research Service collection of entomopathogenic fungal cultures (http://www.ars.usda.gov/is/np/systematics/fungibact.htm).

[*] Only those with clear records to show isolation and purification from insects were summarized.

[†] The total number only includes the strains have been determined as *M. anisopliae* sensu stricto.

[‡] The number includes the records of the species of *M. majus* and *M. anisopliae* var. *majus*.

Table S6. Primers used for gene deletions and mutant verifications

Genes		Primers*	Restriction enzymes	Length (bp)†
MAA_10034	U1	CGCGGATCCCATTCGCTCTTTACGCTGTG	BamHI	937
	U2	CGCGGATCCGGCCTGATATCCTGCGTATT	BamHI	
	L1	TGCTCTAGAGCAGGCACCAGAATTGACTT	XbaI	866
	L2	TGCTCTAGACTTGTGGCCGGTTACATGAT	XbaI	
	F	CAGGAGGACTTCGTGGTGAG		790 (WT)
	R	AATGAATGTTGCCGTCTCG		1,336 (KO)
MAA_10035	U1	CGCGGATCCTAATGCTGCGTCTCCATTTGC	BamHI	844
	U2	CGCGGATCCGTGAAGTTTGCACTCCACGA	BamHI	
	L1	TGCTCTAGAATTGGGCGACGAGATAGATG	XbaI	1,226
	L2	TGCTCTAGAAGCCCCTTTAGGGATTGTTG	XbaI	
	F	GCAACGTATATCGCGTTTGG		1,486 (WT)
	R	GTGGAAGTGTGGAAGGTTGG		1,658 (KO)
MAA_10037	U1	CCGGAATTCCGGGCATTACTTCCATGACT	EcoRI	1,211
	U2	CCGGAATTCAATATCCGCAAAGTCGACCA	EcoRI	
	L1	TGCTCTAGAACCGCTGTGGTAGTATGCAG	XbaI	821
	L2	TGCTCTAGAGCTTCGGTGATCCTAATCCA	XbaI	
	F	CGTACACCTTGGGCTGAAAA		1,507 (WT)
	R	AGCTCGACAGTCCTTTGCAT		1,856 (KO)
MAA_10039	U1	CGCGGATCCCTATCCGATGCACGTGTGAC	BamHI	1,196
	U2	CGCGGATCCGCAAGTGTATCGGGAAAAGG	BamHI	
	L1	TGCTCTAGACATGTGCCTTGTTGTTGGTC	XbaI	530
	L2	TGCTCTAGAGGCCGAGAATTAGGATGGTT	XbaI	
	F	CGTACAGTCCAGCACCAATC		1,057 (WT)
	R	CGTCGAGGTGCACTATTCAA		1,445 (KO)
MAA_10041	U1	CCGGAATTCGTGAAATGCGTGTTGCGATA	EcoRI	1,514
	U2	CGCGGATCCCACAGCAACGTCCATGATTT	BamHI	
	L1	TGCTCTAGATCACAACTCCCTACCGGAAT	XbaI	1,802
	L2	TGCTCTAGAGACAAGTTTTGCGTTGTGTTG	XbaI	
	F	TGGTCTCCATTACTGCTACCC		1,675 (WT)
	R	TCAACGCATCTCGTCACCTA		1,082 (KO)
MAA_10042	U1	CCGGAATTCGTGAAATGCGTGTTGCGATA	EcoRI	1,949
	U2	CGCGGATCCCACAGCAACGTCCATGATTT	BamHI	
	L1	TGCTCTAGATCACAACTCCCTACCGGAAT	XbaI	617
	L2	TGCTCTAGA GACAAGTTTTGCGTTGTGTTG	XbaI	
	F	TGGTCTCCATTACTGCTACCC		1,020 (WT)
	R	TCAACGCATCTCGTCACCTA		1,584 (KO)
MAA_10043 *DtxS1*	U1	CCGGAATTCCATGACTCCGTTTGAGCAGA	EcoRI	2,280
	U2	CGCGGATCCCGCCATGTCTTGTATCAACG	EcoRI	
	L1	CGGACTAGTTTTGCATCGTACAGCTTTGG	SpeI	2,058
	L2	TGCTCTAGATGAAGATGCGACTGAACGAG	XbaI	
	F	GGCGTACGACAAATGGATCT		2,030 (WT)
	R	CCGCAAAGGCATTATTGAGT		1,111 (KO)
	dtxF	AGATTTGCCGCAGCTACCTA		424
	dtxR	CACCAGATGCGAGTTCTCAA		
MAA_10044 *DtxS2*	U1	CCGGAATTCCAACCACCAGTTTTGGCTTT	EcoRI	749
	U2	CCGGAATTCGCGAGGATCAAGGAGTAACG	EcoRI	
	L1	CGGACTAGTCCAGGTATGCGGAGCATATT	SpeI	679
	L2	TGCTCTAGAGGGTTGAGTGAAAGGGTCAA	XbaI	
	F	TGCTTGAATCCCCAATCTTC		1,611 (WT)
	R	TGCCATCTGGTCCTTTCCTA		1,731 (KO)
MAA_10043, MAA_10044 *DtxS1*, *DtxS2*	U1	CGCTGCAGCAACGCCCAAATCCAAGAGC	PstI	2,236
	U2	CGCGTCAGATTCAAGCAGCGCACCTTCC	PstI	
	L1	CGGACTAGTATGCAATTTCGAGGGCCGTA	SpeI	1,513
	L2	CGGACTAGTCGAGCTTGCCATCTGGTCCT	SpeI	
	F	TCGCAGGCACAATGACAAGA		426 (WT)
	R	GCGGCCGTATTCAGTTGAGC		1,367 (KO)
MAA_10045 *DtxS3*	U1	TGCTCTAGAAACCCTGCAGTCCAGTGAAT	Xba I	711
	U2	TGCTCTAGAGAGATGGTGGAATTCGAAGC	Xba I	
	L1	CGCGGATCCCCGTTGGCATCTGGTAGACT	BamHI	922
	L2	CGCGGATCCATGGAACGGTCAAAGGCATA	BamHI	
	F	TGGCCAAAGTATCTCTTGAAA		1,470 (WT)
	R	GTTGTCCCCGATTTAGCGTA		1,428 (KO)

Table S6. Cont.

Genes		Primers*	Restriction enzymes	Length (bp)[†]
MAA_10046 *DtxS4*	U1	CGCGGATCCCCAATGGAGAGTCCGAGAGA	BamHI	1,820
	U2	CGCGGATCCCTTTGCCGTTTCCCTCATTA	BamHI	
	L1	CGGACTAGTGGAACGGTTGAGGGACTTTT	SpeI	2,142
	L2	CGGACTAGTGGAAGGACGAGGTTAGTCCA	SpeI	
	F	CGAGCAGATGAACTTGACGA		1,459 (WT)
	R	ACCGCTTCCAATCCCTTG		1,228 (KO)
18S	F	CAGGGCTCTTTTGGGTCTTG		652
	R	AGTTTCAGCCTTGCGACCAT		

*The introduced enzyme restriction sites are underlined.

[†] The PCR product sizes are provided for the wild-type (WT) and corresponding gene knockout (KO) mutants.