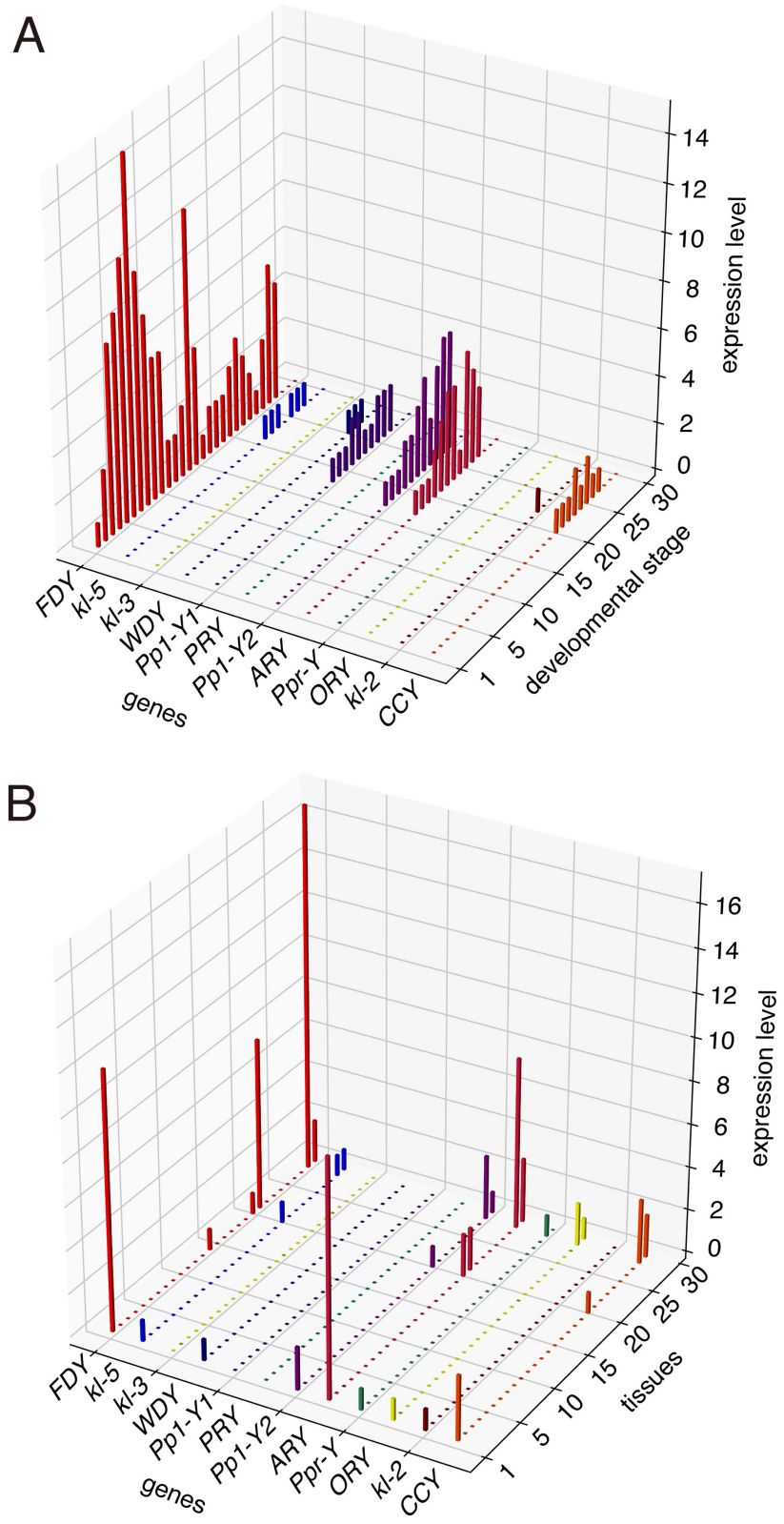
A
expression level
0
2
4
6
8
10
12
14
developmental stage
1
5
10
15
20
25
30
genes
FDY
kl-5
kl-3
WDY
Pp1-Y1
PRY
Pp1-Y2
ARY
Ppr-Y
ORY
kl-2
CCY
B
expression level
0
2
4
6
8
10
12
14
16
tissues
1
5
10
15
20
25
30
genes
FDY
kl-5
kl-3
WDY
Pp1-Y1
PRY
Pp1-Y2
ARY
Ppr-Y
ORY
kl-2
CCY

Figure S1 Expression of Y chromosome genes during different developmental stages and in different tissues. (A) Expression of the indicated genes from FlyBase. **X axis**, genes. **Y axis**, numbers from **1-30** indicate different development stages. **1-12** represent different 2 hour windows during embryogenesis (e.g. 1, 0-2 hr; 12, 22-24 hr). **13-18**, different larval stages: 13, L1; 14, L2; 15, L3 12 h; 16, L3 puffstage 1-2; 17, L3 puffstage 3-6; 18, L3 puffstage 7-9. **19-24**, different pupal stages: 19, white prepupae; 20, prepupae 12 hr; 21, pupae 1 day; 22, pupae 2 day; 23, pupae 3 day; 24, pupae 4 day. **25-30,** adults: 25, 1-day old males; 26, 5-day old males; 27, 30-day old males; 28, 1-day old females; 29, 5-day old females; 30, 30-day old females. Z axis, relative expression levels (arbitrary units). (B) Expression of the indicated genes in different tissues from FlyBase. **X axis**, genes. **Y axis**, numbers from **1-30** indicate different tissues. **1**, imaginal disc, larvae L3 wandering. **2**, central nervous system of larvae L3. **3**, central nervous system of pupae P8. **4-12**, head of adults: 4, virgin female 1 day; 5, virgin female 4 day; 6, virgin female 20 day; 7, mated female 1 day; 8, mated female 4 day; 9, mated female 20 day; 10, mated male 1 day; 11, mated male 4 day; 12, mated male 20 day. **13**, salivary gland of larvae L3 wandering. **14**, salivary gland of white prepupae. **15**, digestive system of larvae L3. **16-18**, digestive system of adults: 16, 1-day old; 17, 4-day old; 18, 20-day old. **19**, fat body of larvae L3. **20**, fat body of white prepupae. **21**, fat body of pupae P8. **22**, carcass of larvae L3 wandering. **23-25**, carcass of adults: 23, 1-day old; 24, 4-day old; 25, 20-day old. **26**, ovary of virgin 4-day female. **27**, ovary of mated 4-day female. **28**, testis of mated 4-day male. **29**, accessory gland of mated 4-day male. Z axis, relative expression levels (arbitrary units).

kl-2

Targeting site DNA

5‘-TGTTAAGATTCCTAGAAATTTATCCAGGGACGTACAGAAA-3’

gRNA

kl-3

Targeting site DNA

5‘-TGGTTTGGGTCGTATATACAAGGGACTGGCCCAATCCGGA-3’

gRNA

kl-5

Targeting site DNA

5‘-GAAAAGAAAACCGCATTATAACGACCTTAACCCAAAAGCT-3’

gRNA

CCY

Targeting site DNA

5‘-TAAAAAT GGATAACGCGGAGATTAAGAGGATTATAAATCA -3’

gRNA

Pp1-Y2

Targeting site DNA

5‘-GATAATGGGATGGAGCGACAACGATCGAGGCGTCAGTGTT-3’

gRNA

Ppr-Y

Targeting site DNA

5‘-TGGTGAGGGAAGACGTTTACATCAACTGGAGCCTGTTGTA-3’

gRNA

FDY

Targeting site DNA

5‘-GCTCTTTGGCACCTTTGGCGCCTTTGCCGGTTCCGGCTTC-3’

gRNA

PRY

Targeting site DNA

5‘-ATACTTTAATGGATATTTATTATGAAACGATGGTAAATGA-3’

gRNA

ORY

Targeting site DNA

5‘-TGAAGACAATGGAAGAACAAAAAGAAGCACATGGAAAGAC-3

gRNA

ARY

Targeting site DNA

5‘-ATACACAATGTGGAGAAAAGGCTTTACTAATGGCCCCAAA-3’

gRNA

Figure S2 Targeted indel mutations generated by CRISPR/Cas9. The genes are indicated. The underlined regions represent the gRNA sequences. The sequencing chromatograms from F_0 males are shown on the right.

CCY

GGAAGACAGCTTAATAAATGCGGCCGATGACGCAATCGATTGGTTACAAGATCTTATGAAAAGAAACATCCTTAACAA
CCAGCTACCTGAATCAGACATGAAGCTATTATCGATAAAAATGGATAACGCGGAGATTAAGAGGATTATAA w^{1118}

GGAAGACAGCTTAATAAATGCGGCCGATGACGCAATCGATTGGTTACAAGATCTTATGAAAAGAAACATCCTTAACAA
CCAGCTACCTGAATCAGACATGAAGCTATTATCGATAAAAATGGATAACGCGGAGATTA----------TAA [-9]

Pp1-Y2

CTATGGTCAGACCCCGATCCAAAGATAATGGGATGGAGCGACAACGATCGAGGCGTCAGT w^{1118}

CTATGGTCAGACCCCGATCC----------GAGGCGTCAGT [-29]
CTATGGTCAGACCCCGATCCAAAGATAATGGGATGGAGCGA----------GGCGTCAGT [-10]
CTATGGTCAGACCCCGATCCAAAGA----------GGCGTCAGT [-26]

Ppr-Y

AAACACGTTCATCGTGGTGAGGGAAGACGTTTACATCAACTGGAGCCTGTTGTATTGGAA w^{1118}

AAACACGTTCATCGTGGTGAGGGAAGACGTTTAC------TGGAGCCTGTTGTATTGGAA [-6]
AAACACGTTCATCGTGGTGAGGGAAGACGTT-ACATCAACTGGAGCCTGTTGTATTGGAA [-1]
AAACACGTTCATCGTGGTGAGGGAAGACGTTTA----------GCCTGTTGTATTGGAA [-11]
AAACACGTTCATCGTGGTGAGG----------AGCCTGTTGTATTGGAA [-21]

FDY

GTCGCGGCGCCCAAGAAGCCGGAACCGGCAAAGGCGCCAAAGGTGCCAAAGAGCAAGTCG w^{1118}

GTCGCGGCGCCCAAGAAGCCGGAACCGGCAAAGGCGCCAAAG----------AGCAAGTCG [-9]

Figure S3 Targeted indel mutations identified in each of the indicated genes. The top sequences in each panel are the w^{1118} DNA sequences, with the PAM sequences highlighted in red, and the gRNA sequences underlined. Shown below are the indels in F_1 males. The missing nucleotides are represented by red dashes. The number of deleted nucleotides is indicated to the right.

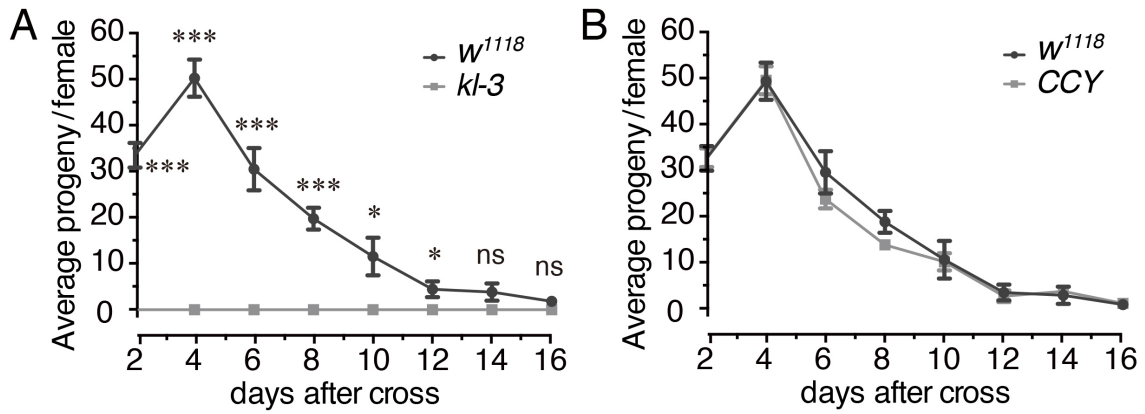

Figure S4 Male fertility tests performed with flies with mutations in Y chromosome genes. (A) *kl-3.* (B) *CCY.* w^{1118} males were used as the control. Means ±SEMs. Unpaired Student's *t*-tests. n=20 crosses each. * $p<0.05$, *** $p<0.001$, ns: not significant.

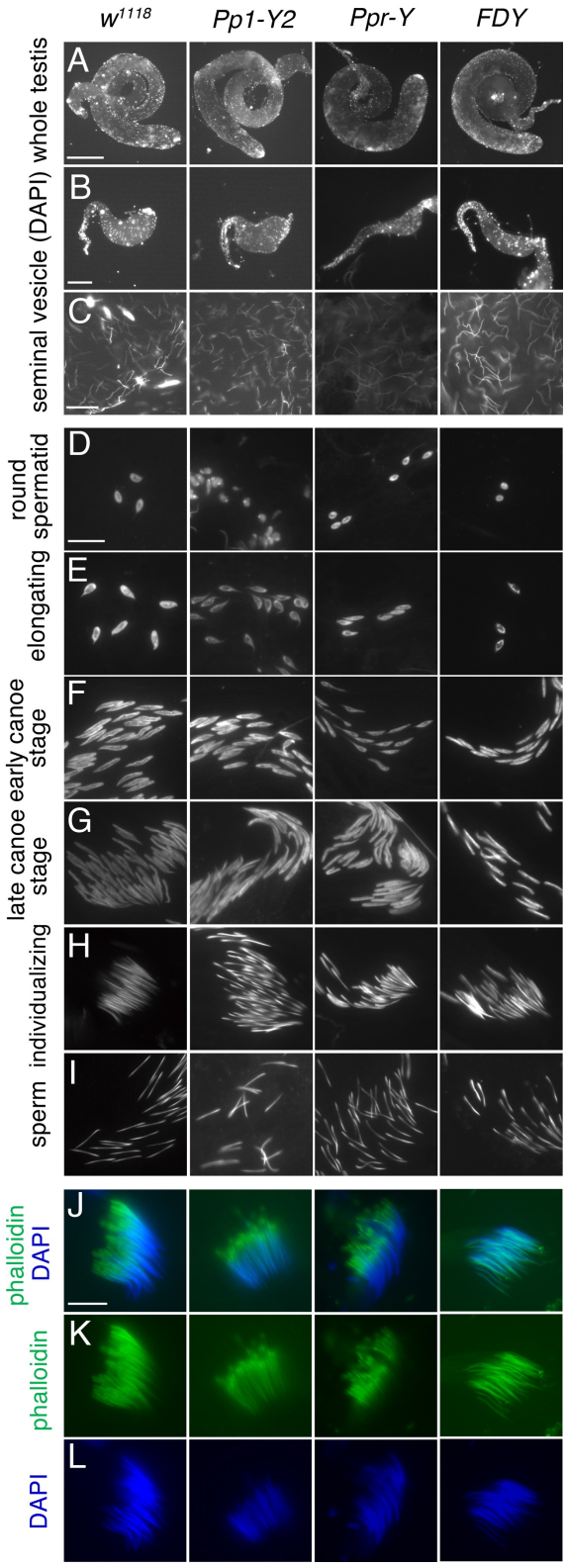
w1118
Pp1-Y2
Ppr-Y
FDY
A
B
C
D
E
F
G
H
I
J
K
L
whole testis
seminal vesicle (DAPI)
round spermatid
elongating
early canoe stage
late canoe stage
individualizing
sperm
phalloidin DAPI
phalloidin
DAPI

Figure S5 Testing effects of mutations in Y chromosome genes on spermatogenesis. Testes were isolated from mutant males and w^{1118} (control) males and stained with: (A—L) DAPI, and (J—K) and phalloidin. (A-C) Morphology of the testes. (A) Whole testes. (B) Seminal vesicles. (C) Mature sperm in the seminal vesicles. (D—I) Different stages during spermatogenesis. (J—L) Actin and nuclei in individualization complexes (ICs) were stained with phalloidin (green) and DAPI (blue), respectively. Scale bars: (A) 200 μm, (B) 100 μm, (C) 20 μm, (D—L) 10 μm.

Gene	Target site (5' to 3') (PAM is underlined)	oligonucleotide-forward (5' to 3')	oligonucleotide-reverse (5' to 3')	on-target scores	off targets	off-target scores
kl-2	GATTCCTAGAAATTTATCCAGGG	TAATACGACTCACTATAGATTCCTAGAAATTTATCCAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	59.7	3	96.5
kl-3	GGGTCGTATATACAAGGGACTGG	TAATACGACTCACTATAGGGTCGTATATACAAGGGACGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	55.6	0	99.1
kl-5	GGGTTAAGGTCGTTATAATGCGG	TAATACGACTCACTATAGGGTTAAGGTCGTTTATAATGGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	65.8	0	49.5
CCY	GGATAACGCGGAGATTAAGAGG	TAATACGACTCACTATAGGATAACGCGGAGATTAAGGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	53.0	3	99.1
Pp1-Y2	GGATGGAGCGACAACGATCGAGG	TAATACGACTCACTATAGGATGGAGCGACAACGATCGGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	67.7	0	49.3
Ppr-Y	GGGAAGACGTTTACATCAACTGG	TAATACGACTCACTATAGGGAAGACGTTTACATCAACGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	41.3	0	99.0
FDY	GGAACCGGCAAAGGCGCCAAAGG	TAATACGACTCACTATAGGAACCGGCAAAGGCGCCAAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	56.1	3	48.9
ORY	GGAAGAACAAAAAGAAGCACATGG	TAATACGACTCACTATAGGAAGAACAAAAAGAAGCACAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	63.6	4	81.4
PRY	GGATATTTATTATGAAACGATGG	TAATACGACTCACTATAGGATATTTATTATGAAACGAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	64.6	0	48.4
ARY	GGAGAAAAGGCTTTACTAATGG	TAATACGACTCACTATAGGAGAAAAGGCTTTACTAAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	55.7	0	95.6

Table S1 Target sites and oligonucleotides used to transcribe gRNAs. The underlined nucleotides are the PAM regions. The sequences of the forward and reverse oligonucleotides used to synthesize the gRNAs are shown. The numbers of off-target sites are indicated. The on-target and off-target scores range from 0-100 (Hsu *et al.* 2013; Doench *et al.* 2016). The on-target score represents the cleavage efficiency of Cas9, with a higher score indicating greater potential effectiveness. The score indicates the probability that a given gRNA will be in top 20% of cleavage activity. The scoring system is not linear, and only

5% of gRNAs receive a score of 60 or higher. The off-target score means the inverse probability of Cas9 having off-target activity. A higher score means the gRNA has less chance to bind to an unintended sequence in the genome.

Gene	Target site (5' to 3') (PAM is underlined)	oligonucleotide-forward (5' to 3')	oligonucleotide- reverse (5' to 3')	off targets
ARY	GAATATATTTCTAACGACCAAGG	TAATACGACTCACTATAGAATATATTTCTAACGACCAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
	GAAGATAATTCTCAAGTTGCGG	TAATACGACTCACTATAGAAGATAATTCTCAAGTTGGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
	TCTCAAGTTGCGGTGATACTTGG	TAATACGACTCACTATATCTCAAGTTGCGGTGATACTGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
	CTTAGATACTTGGCGAGCAATGG	TAATACGACTCACTATACTTAGATACTTGGCGAGCAAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
ORY	GGACAGAAAGAAAGCCAAAAAGG	TAATACGACTCACTATAGGACAGAAAGAAAGCCAAAAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	4
	GAAGATGCTATGAGCAAAATGG	TAATACGACTCACTATAGAAGATGCTATGAGCAAAAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
	AAAACTTAAAGATTTGTTGGGGG	TAATACGACTCACTATAAAAACTTAAAGATTTGTTGGGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
	TGATGCATCTGATTCTTTCCAGG	TAATACGACTCACTATATGATGCATCTGATTCTTTCCGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
PRY	AGAGGTACGTCGATTGGAAATGG	TAATACGACTCACTATAAGAGGTACGTCGATTGGAAAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	2
	GATTCACAGCCTCTACATTAAGG	TAATACGACTCACTATAGATTCACAGCCTCTACATTAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
	CCTCGTTCGCCTTACAAAGAGGG	TAATACGACTCACTATACCTCGTTCGCCTTACAAAGAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	0
Pp1-Y1	GGAAGATCGATAGAAAAATGATGG	TAATACGACTCACTATAGGAAGATCGATAGAAAAATGAGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	1
	GGATAGAGGAAAATACTCGGTGG	TAATACGACTCACTATAGGATAGAGGAAAATACTCGGGTTTTAGAGCTAGAAATAGC	AAAAAAAGCACCGACTCGGTGCCAC	1

Table S2 Target sites and oligonucleotides used to transcribe gRNAs to edit *ARY*, *ORY*, *PRY* and *Pp1-Y1*. The underlined nucleotides are the PAM sequences. The sequences of the forward and reverse oligonucleotides used to synthesize the gRNAs are shown. The numbers of off target sites are listed. Note that these gRNAs did not work effectively, so we did not test the fertility of the injected flies.

Gene	Primer name	Primer sequence (5' to 3')
kl-2	*kl-2*-F	CGAACAAAGCAGGCATTGAAACC
	kl-2-R	GCCCACGACACCGCAATAC
kl-3	*kl-3*-F	CGATGTCATAGTTGGGATAACTGATG
	kl-3-R	ATTATTATTGTTTACTTACTATATTTGTTGAGCAGCC
kl-5	*kl-5*-F	CGCGACGATAGACAGCGG
	kl-5-R	GAGAGCAATGCGCTCGTTGC
CCY	*CCY*-F	CGCGTTTTGTTGCCGATTTAGTG
	CCY-R	GCACTCGCTTCTACACATTTGTGC
Pp1-Y2	*PP1-Y2*-F	CTCCTCGAGCTTTCCGCACC
	PP1-Y2-R	GAATTCTCCGCAGTAATTTGGTGCAG
Ppr-Y	*Ppr-Y*-F	CCGAAGTACAGAAGCCCCTTG
	Ppr-Y-R	CCCTTTCCACACTTAAAATGCTTGG
FDY	*FDY*-F	CCGCTACGAGCTGTTGTTCATG
	FDY-R	CGAATTCTCCGATCGACTCCTT
PRY	*PRY*-F	GCGATCATTTTTCTGTATGTGACGACG
	PRY-R	GGCAGCTACTTAACTTCCAATGAGC
ORY	*ORY*-F	GAGAACGCGACCGGTTAGC
	ORY-R	CCGTTCGGCCATTTGCATTC
ARY	*ARY*-F	ATGATCCCAGCGGACTTTTTGAC
	ARY-R	GACATGGGCATCAGAATTTCATCGC

Table S3 List of primers used to amplify the indicated regions to identify indels by DNA sequencing. F, forward primer. R, reverse primer.

Gene	Primer name	Primer sequence (5' to 3')
kl-2	*kl-2*-RT-F	GATGTTCTCACTGGTTGCCTTG
	kl-2-RT-R	CACATTGTCTCCCCATTCCATA
kl-3	*kl-3*-RT-F	GAAGTTCGATGCCTGTTGAACG
	kl-3-RT-R	TGTTGCTGGGCCTAATAAACGA
kl-5	*kl-5*-RT-F	CCGAACCCATTAGCAAATTTATCA
	kl-5-RT-R	CGTCCATGCAAATAGGAAGAGG
CCY	*CCY*-RT-F	GGTGCAATCAAGTTATGCAGGA
	CCY-RT-R	CATTAGTTGCTGGTATTGACAATCAT
Pp1-Y2	*Pp1-Y2*-RT-F	TTTTTCTCCTTCGTGGAAACCA
	Pp1-Y2-RT-R	ACCCTTGTCAGGAACGTCACAT
Ppr-Y	*Ppr-Y*-RT-F	GAAGTACAGAAGCCCCTTGTGG
	Ppr-Y-RT-R	TGTTCCAATACAACAGGCTCCA
FDY	*FDY*-RT-F	GCCAAAAACGCTAGTCCAGTGA
	FDY-RT-R	CGAATTCTCCGATCGACTCCTT
WDY	*WDY*-RT-F	GACCCCCAATGGTTAGGAAATC
	WDY-RT-R	AGATGTCCAGAAGCGCATCATT
PRY	*PRY*-RT-F	TGCGTTTCATCGTCAAATCTGT
	PRY-RT-R	AACGCTTCTTTTCGCTCACTTG
ORY	*ORY*-RT-F	ACGTCCCCAAATATTGTCATCG
	ORY-RT-R	TGCGAGGCCTTCACACTTTATT
ARY	*ARY*-RT-F	TAGATACTTGGCGAGCAATGGA
	ARY-RT-R	ACCAAGAGGTGAAAAGGCTGTC
Pp1-Y1	*Pp1-Y1*-RT-F	CATCGCTGCTTAGCTGGAAGAT
	Pp1-Y1-RT-R	GCCCAGATGTCTCGAAATAACG

Table S4 List of primers used for RT-PCR. F, forward primer. R, reverse primer.