Supporting Online Material

Materials and Methods

Parasites, hybrid selections. and phenotype analyses. The *L. major* lines and clones were grown in M199 medium as described (*1*), and containing 25 ug/ml hygromycin B (Sigma, St. Louis) and/or 100 µg/ml nourseothricin (Jena Bioscience, Jena, Germany), as necessary. For selection of double drug resistant lines *in vitro*, cultures were initiated by seeding log-phase cells of each parent at $1x10^6$/ml, and the two antibiotics were added at days 3, 7, or 11. All cultures were static by 2-4 days, and all parasites were dead by 10-24 days following addition of the antibiotics. For selection of double drug resistant lines during co-infection in the mammalian host, BALB/c mice were co-inoculated in the ear dermis with 10^4 metacyclic promastigotes of each parental strain. Four weeks after infection, ear dermal cells were prepared and aliquots serially diluted without antibiotics to quantify the number of amastigotes per lesion, as described (*1*). The remaining cells from each ear were cultured in 2 ml media containing both antibiotics. For selection of double drug resistant lines during co-infection in the sand fly vector, *P. duboscqi* sand flies were infected and used for transmission of *L. major* by bite as described (*1*). Briefly, 2 to 4-day-old *P. duboscqi* females were infected by feeding through a chick skin membrane on heparinized mouse blood containing 3 and 1 x 10^6 / ml logarithmic phase promastigotes of the LV39c5(HYG) and FV1(SAT) lines, respectively. Midguts were dissected 13-16 days post-infection, and individual midguts were transferred directly to 96 well plates containing 0.1 ml media without antibiotics. After 2 days of growth, the parasites were transferred to 2 ml media containing both antibiotics. For selection of hybrid lines and clones from mouse lesions initiated by infected sand fly bites, 13 day co-infected flies were permitted to feed on the ear pinnae of BALB/c mice, 10 flies per ear. After 4-6 weeks, the ear tissue homogenates were prepared as described (*1*), and cultured in M199 medium containing both antibiotics. Hybrid clones were

generated by distribution in 96 well blood agar plates in 0.1 ml M199 containing both antibiotics. Poisson analysis was used to determine the percentage probability of clonality, and was in each case >95%. Animal virulence tests were performed in BALB/c mice by s.c footpad inoculation of 10^4 metacyclic promastigotes, purified as described (*2*) from stationary phase promastigotes freshly transformed from lesion amastigotes. Agglutination of metacyclic promastigotes with monoclonal antibody 3F12 was performed as previously described (*3*).

DNA content analysis. DNA contents were determined by flow cytometry following staining of 10^6 permeabilized RNAse-treated cells with propidium iodide, as previsouly described (*4*)**.** *L. major* promastigotes (5×10^6) were washed with PBS and fixed with 90% methanol–10% PBS for 15 min at 4°C. Parasites were pelleted, washed twice with PBS, resuspended in 1 ml of PBS containing 20 μg/ml propidium iodide and 200 μg/ml RNase A, and incubated at 25 °C for 1 hour. Data were acquired on a FACS flow cytometer (Becton Dickinson), counting at least 10,000 cells per sample, and analyzed using CellQuest 3.1 (BD Bioscience) software.

Maxicircle sequencing. PCR primers (Supplemental Table 3) were designed using *L. major* FV1 maxicircle sequences found in a shotgun sequencing survey previously (*5*). Single pass sequencing was done using dye terminators and AmpliTaq DNA polymerase and analyzed using Lasergene software v.7.1.0 (DNASTAR, Inc, USA). Sequences for parental lines were submitted to the GenBank (NCBI) (**http://www.ncbi.nlm.nih.gov**) with accession numbers FJ349262-FJ349263 (12SrRNA) and FJ349264- FJ349265 (Divergent region). SNP positions, insertions and deletions are listed in Supplementary Table 1.

LV39 sequencing and SNP identification. A cosmid library of LV39c5 DNA was prepared in the vector cLHYG (*6*). *SCG*-bearing cosmids were identified by colony hybridization using a generic *SCG* universal coding region probe (*7*), and restriction mapping and comparisons to the

SCG loci of the sequenced *L. major* Friedlin genome were used to assign individual cosmids as *SCG*1-7. Representative cosmids encompassing the intact *SCG* genes and extending ~25-35 kb inward were subjected to random shotgun sequencing, and assembled independently and with the Friedlin genome as a scaffold. Sequence comparisons revealed numerous SNPs, and several from each *SCG*-containing chromosome were studied further in the parental and progeny lines generated in this work (Supplementary Table 1).

Comparative SNP-CAPS analysis of parental and progeny lines. The parental lines were screened for polymorphisms on the basis of single nucleotide differences between the lines, and SNP markers on seven chromosomes at nine different loci were used in this work. The parental sequences were compared by BLAST analysis (http://www.genedb.org/genedb/leish/index.jsp). Blast output then was uploaded in its entirety to the BlastDigester website (*8*) (http://www.bar.utoronto.ca/ntools/cgi-bin/ntools_blast_digester.cgi) in order to identify suitable cleaved amplified polymorphic sequence markers (CAPS). SNP comparison for parental genomes is presented in Supplementary Table 2. PCR primers for SNP-CAPS analysis were generated manually (Supplementary Table 3). PCR amplification of specific fragments was carried in 100 μl volume using 20 ng genomic DNA (*9*). KlentaqLA polymerase and 40 pmoles of each primer. The reactions were performed in a thermocycler (PTC-200, MJ Research, MA) programmed for 35 cycles as follows: 93°C, 30 sec; 50°C, 45 sec and then 68°C, 2 min. After amplification, DNA products were ethanol precipitated, the pellets were washed with 75% ethanol, resuspended in 40 μl of sterile nuclease-free water, and 4 μl of product was electrophoresed on a 1.5% agarose gel to ensure homogeneity and yield. In all cases PCR amplification resulted in a homogeneous DNA fragment of the size expected (Supplementary Table 2). Usually 10 μl of product was digested with 5 to 10 U of restriction enzyme for 16

hours in the buffer recommended by the supplier (NE Biolabs, MA), electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining.

DNA sequencing and trace analysis. DNA sequencing was carried out as follows: 1 µl of purified PCR template (~50 ng DNA) was combined with 1 µl of 10 µM primer, 4 µl of 2.5X sequencing buffer (200 mM Tris, pH 9.0, 5 mM MgC12) and 10 µl with 10 mM Tris/0.01 mM EDTA, pH 8.0. This mixture was heat-denatured for 5 min at 98°C in a thermocycler (PTC-200, MJ Research, MA), placed on ice and 4 µl of the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit mix was added (v. 3.1; Applied Biosystems, CA). The amplification reactions were performed in a PTC-200 thermocycler for 40 cycles (96°C for 10 sec, 50°C for 5 sec, and 60°C for 2 min). Excess dye was removed by gel filtration on a 96-well filter plate filled with Sephadex G-50 beads. The samples were then heat-denatured for 2 min at 95°C and electrophoresed on an ABI3700 genetic analyzer (Applied Biosystems, CA) under conditions recommended by the manufacturer. Prior to sequencing reactions 1:1 mix of parental templates were prepared as a technical control. Trace peaks at SNP positions for 1:1 mix control sequencing reaction showed equal height. These data along with the parental traces were compared to all sequencing data generated for progeny clones and analyzed using Lasergene software (Supplementary Fig. S2).

References

1. N. Kimblin *et al.*, *Proc Natl Acad Sci U S A* **105**, 10125 (Jul 22, 2008).
2. G. F. Spath, S. M. Beverley, *Exp Parasitol* **99**, 97 (Oct, 2001).
3. D. L. Sacks, R. P. da Silva, *J Immunol* **139**, 3099 (1987).
4. A. K. Cruz, R. Titus, S. M. Beverley, *Proc Natl Acad Sci U S A* **90**, 1599 (1993).

5. N. S. Akopyants *et al.*, *Mol Biochem Parasitol* **113**, 337 (Apr 6, 2001).

6. K. A. Ryan, S. Dasgupta, S. M. Beverley, *Gene* **131**, 145 (Sep 6, 1993).

7. D. E. Dobson *et al.*, *J Biol Chem* **278**, 15523 (May 2, 2003).

8. K. Ilic, T. Berleth, N. J. Provart, *Trends Genet* **20**, 280 (Jul, 2004).

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Fig S1

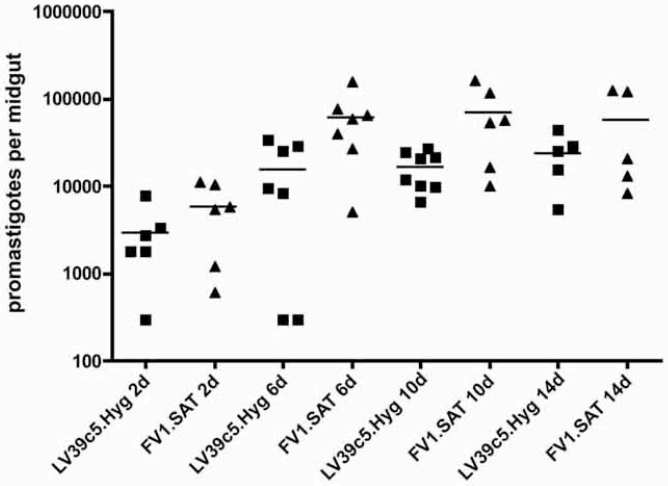


Fig. S1. Growth of parental lines in *Phlebotomus duboscqi* (Mali).

Flies were infected by membrane feeding on mouse blood containing 2 million logarithmic phase promastigotes/ml. Midguts were dissected on the indicated days post-feeding and scored for numbers of viable promastigotes.

Fig. S2

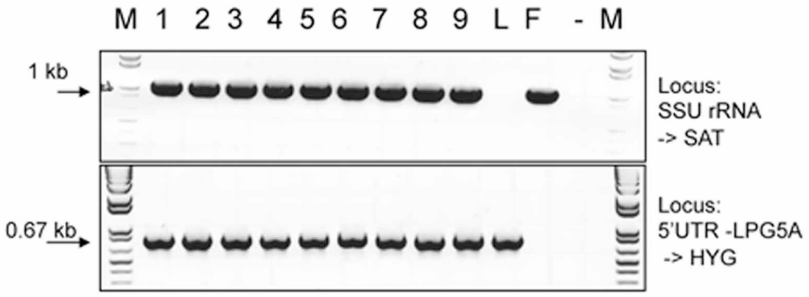


Fig S2. Analysis of drug marker loci: double drug resistant clones had both marker genes in their original locus without rearrangements. PCR test for locus confirmation. Samples are F, FV1(SAT); L, LV39c5(HYG); M, 1kb plus marker (Invitrogen, CA); -, no template control; 1, 1.10.B12; 2, 1.10.D9; 3, 4.3.G12; 4, 4.3.A12; 5, 1.14.E10; 6, 5.12.F11; 8, 1.14.B11; 9, 5.22.10.

Fig S3

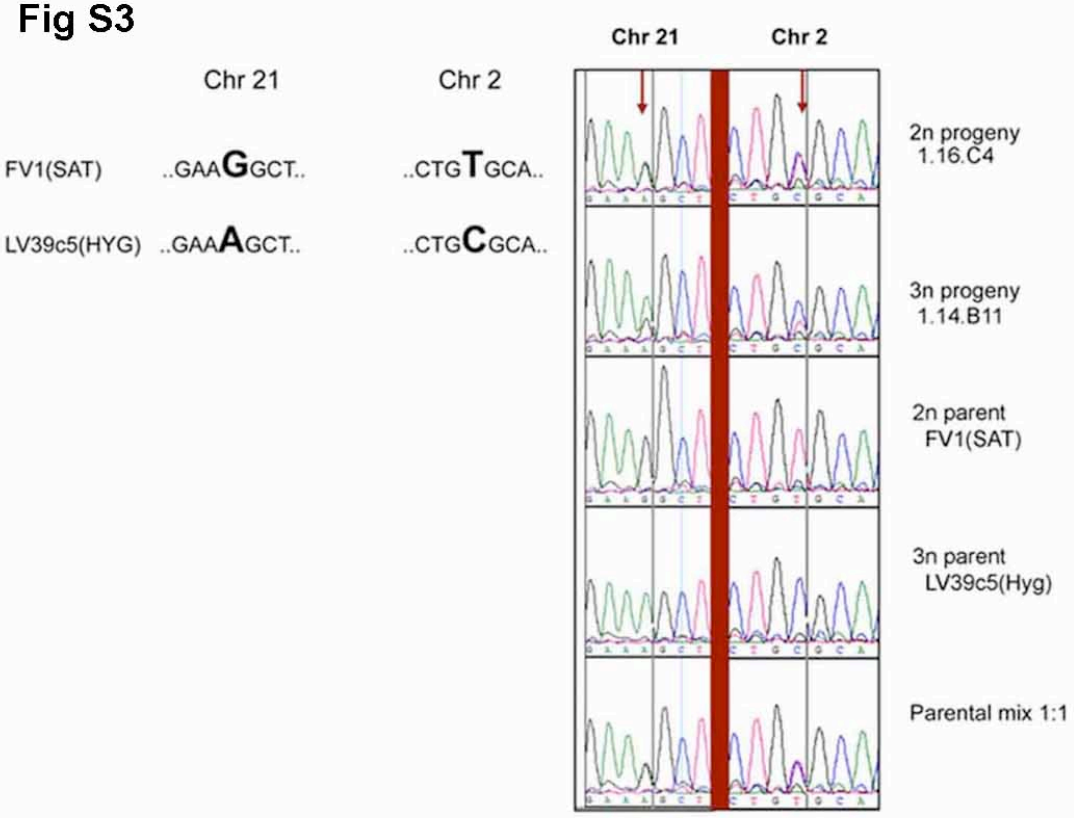
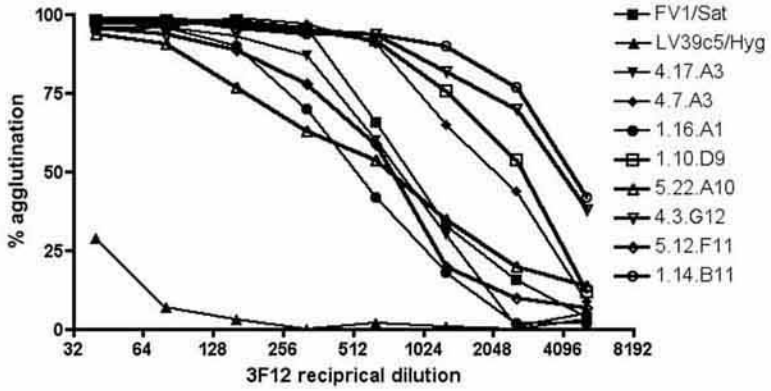


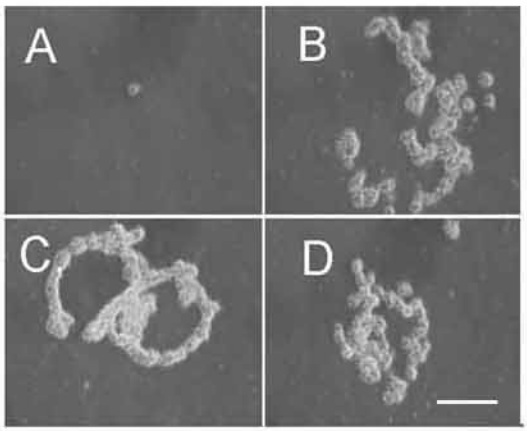
Fig. S3. DNA sequence traces for two loci from parental lines and 2n and 3n progeny. DNA sequencing traces are shown for two representative loci (chromosome 21.0040, chromosome 2.0085) and one representative 2n (1.16.C4) and 3n (1.14.B11) progeny clone.

Fig. S4

A. 3F12 agglutination
FV1 dominant allele



B. Clumpiness inheritance
LV39c5 dominant allele



Parental cells:
A. FV1
B. LV39c5

Progeny cells:
C. 4.9.C8.
D. 4.17.A3

Fig S4. LPG and clumpiness phenotypes of parental and hybrid progeny clones.

A. Titration of agglutination of metacyclic promastigotes with mAb3F12.

B. Parasites were photographed during logarithmic phase growth. At the magnification used individual cells are not clearly evident; thus only a small clump is evident in the middle of panel A. Scale bar, 100μm.

Fig. S5

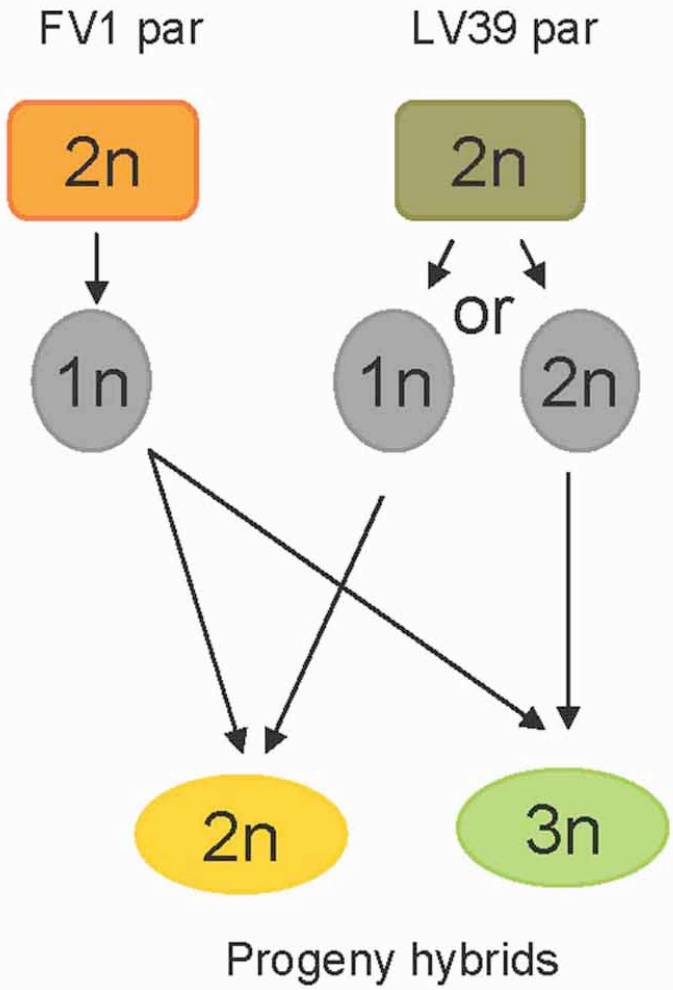


Fig S5. Model for genetic exchange in *Leishmania*

Supplementary Table 1.

SNPs in *L. major* FV1 (F) relative to LV39c5 (L) used in this work.

Chromosome	Locus/ORF	Number of analyzed SNPs /locus	SNP position/description[b]	Number of analyzed loci
2[a]	LmjF02.0060	4	197/F=C, L=T; 350/F=C, L=G; 356/F=G, L=C; 386/F=A, L=G	2
	LmjF02.0085	4	208/F=T, L=C; 294/F=C, L=A; 368/F=G, L=T; 388/F=C, L=T	
7	LmjF07.1150	2	2756/F=G, L=A; 3014/F=C, L=T	1
21	LmjF21.0040	1	284/F=G, L=A	1
25	LmjF25.2420	1	495/F=G, L=A	1
31[a]	LmjF31.3150	1	4051/F=G, L=T	2
	LmjF31.3110	3	2042/F=G, L=A; 2145/F=C, L=T; 2391/F=T, L=C	
35	LmjF35.0050	2	666/F=G, L=T; 755/F=T, L=C	1
36	LmjF36.0050	3	476/F=G, L=C; 554,833/F=T, L=C	1
maxicircle	12S rRNA	1	159/F=A, L=C	2
	Divergent region	11	30/F=A, L=T; 129, 270, 350/F=T, L=C; 313/F=C, L=A; 354, 370/F=C, L=T; 31, 248/insertion of A in F; 293/insertion of AATATGTTATA in F; 268/insertion of G in L	

[a] ~ 10 kb between two ORFs

[b] SNP location in GeneDB ver. 5.2 coordinates

Supplementary Table 2.

Comparative SNP-CAPS patterns for the two parental genomes.

Chromosome	ORF ID	PCR product (bp)	Enzyme	FV1 (SAT) Predicted fragment (bp)	LV39c5(HYG) Predicted fragment (bp)
2	LmjF02.0060	557	Not I	361; 196	557
	LmjF02.0085	922	Hae III	HaeIII: 399;178;136;99;97;13	HaeIII: 313;178;136;99;97;86;13
			Fsp I	Fsp I: 693; 229	Fsp I: 653;229;40
7	LmjF07.1150	984	Dde I	Dde I: 619;292;73	Dde I: 580;258;107;39
21	LmjF21.0040	711	Hind III	711	394;317
25	LmjF25.2420	786	Cla I	Cla I: 786	Cla I: 496;290
			Ahd I	Adh I: 463;294;29	Adh I: 757;29
31	LmjF31.3150	530	Pvu II	291;159;65;15	450;65;15
	LmjF31.3110	749	Dde I	451;148;95;55	308;148;143;95;55
35	LmjF35.0050	1098	Dde I	876; 223	726; 223; 150
36	LmjF36.0050	1018	Xho I	Xho I: 927;91	Xho I: 522;405;91
			Hind III	Hind III: 889;129	Hind III: 1018
Maxicircle	ND5-DIV-R	500	Bfa I	500	290; 210

Supplementary Table 3. PCR primers.

Locus	PCR product (bp)	Forward primer	Reverse primer	Oligonucleotide Number
Hygromycin B (HYG)	1026	GGTAACGGTGCGGGCTGACGCCACCATGAAAAGCCTGAACTC	CGAGATCCCACGTAAGGTGCCTATTCCTTTGCCCTCG	B2561, B2562
Nourseothricin (SAT)	524	CCACCATGAAGATTTCGGTGATCC	TTAGGCGTCATCCTGTGCTCC	B2630; B2631
LPG5A::ΔHYG	620	ACGAGCTCGCGACATCTACACTG	AATACGAGGTCGCCAACATC	B3266; B2566
SSU::SAT-LUC	1100	ACATCAGACGTAAT TGCCGC	GAGAGGCGGAAGGTGACGAAATGG	B2618; B2617

Chromosome	ORF ID	PCR product (bp)	Forward primer	Forward primer coordinates[a]	Reverse primer	Reverse primer coordinates[a]	Lab Database entry
2	LmjF02.0060	557	ATGCCGGCCCCGAGCACTGCTGC	1-23	CACCGCTGGGCAATCAGCCGGTAC	557-534	B3384; B3385
	LmjF02.0085	922	ATGCCGCGCCGATACAGACACATG	1-24	CTGCTTCAGCCATGATGCACGCA	922-900	B3386, B3387
7	LmjF07.1150	984	GCAGAGCTGTCACTGCTCGAGAT	2518-2540	CGACAAGTCCACGTCGACACTCC	3501-3479	B3368, B3369
21	LmjF21.0040	711	GCGCTAAAGTTGGCGCCGTCTAC	28-50	TCAGTCGCTTTCGTACCCTTCAC	738-713	B3380, B3381
25	LmjF25.2420	786	ATGGCGGAGCTCCTTAGCAAA	1-21	CTACAGGCCCATCTTTGCTGGGTC	786-763	B3406, B3407
31	LmjF31.3150	530	GAAAGCTGATGAAGCTGCTGGA	3881-3903	CTAGCGCGTCTGCTTCGACACAAC	4410-4387	B3400, B3401
	LmjF31.3110	749	GCTCTGTGCGGAAGCGTATCCCA	1793-1815	CTAGCAGCACGCGCTCTTCACCT	2541-2519	B3404, B3405
35	LmjF35.0050	1098	ATGCGTCGCCTCTTTGATGCCTC	1-23	GAAGTGCACCACGGACTTGATA	1098-1077	B3412, B3413
36	LmjF36.0050	1018	ATGTCGTCAGAGGAGAAGTTAAC	1-23	GATTGGTGTAGTTCGGTGCCGTG	1018-996	B3388, B3389

[a] GeneDB version 5.2

kDNA	ORF ID	PCR product (bp)	Forward primer	Forward primer coordinates	Reverse primer	Reverse primer coordinates	Lab Database entry
Maxicircle	12S rRNA	1180	AGTTTAATGTTTAAATATTTAACTAGTGA	54-82[b]	CCGCAACGGCTGGCATCCATTTCTGAC	65-39[c]	B3422; B6-9
Maxicircle	ND5-DIV-R	500	GTTTGTGTGAAATATTGTTATATA	220-243[d]	CTCGTTCATAAATTTGTTGTGGC	301-323[e]	B3431; B3433

GenBank Accession: [b]BH017736; [c]AQ851364; [d]AQ850432; [e]BH017653