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>CYP125MRCA

MTTTTMAPTDIDLTDPDVYNRGVPHEQFAWLRRNEPVYWHPEPPPDTDGEGYWAVTRHADVVAVSRDPEIFSSQQGGTMIQDADA APEELEKQRMMMLNMDPPQHTRLRKLVSKGFTPRMIAKLEDKIRERAKQIVDEAIEKGECDFVADIAAELPLQVIAELIGVPQEDRQRL FDWSNRMIGYDDPEYHSSEADGEQAAAEMFAYAQELAAERRKNPRDDIVTALVQAEVDGQKLSDLEFNMFFLLLVVAGNETTRNAIS HGMLALLEHPDQWERLRADPSLAPTAVDEILRWASPVMSFRRTATRDTELGGQQIKAGDKVVMFYASANRDEEVFDDPYTFDITRSP NPHLAFGGGGGPHYCLGANLARLEIRVMFEELAERMPDIELTGPPERLRSNFINGIKHMPVRFTPARAVGGHHHHHH

>CYP125MRCAAlt

MTTTTMAPTDIDLTDPDVYNRGVPHEQFAWLRRNEPVYWHPEPPPDTDGEGYWAVTRHADVVAVSRDPEIFSSQQGGTMIQDADA APEELEKQRMMMLNMDPPQHTRLRKLVSKGFTPRMIAKLEDKIRERAKQIVDEAIEKGECDFVADIAAELPLQVIAELIGVPQEDRQRL FDWSNRMIGYDDPEYHSSEADGEQAAAEMFAYAQELAAERRKNPRDDIVTALVQAEVDGQKLSDLEFNMFFLLLVVAGNETTRNAIS HGMLALLEHPDQWERLRADPSLAPTAVDEILRWASPVMSFRRTATRDTELGGQQIKAGDKVVMFYASANRDEEVFDDPYTFDITRSP NPHLAFGGGGGPHYCLGANLARLEIRVMFEELAERMPDIELTGPPERLRSNFINGIKHMPVRFTPARAVGGHHHHHH

Figure S1: Ancestral sequence reconstruction of CYP125MRCA. Top: Phylogenetic tree used to create the ancestral sequence reconstruction CYP125MRCA (position highlighted by the blue circle). Unique P450 clades are shown in different coloured ranges. Bottom: Constructed CYP125MRCA and CYP125MRCAAlt fasta sequence.



Figure S2: UV-Vis difference spectra of the binding of CO to CYP125MRCA (top) and CYP125MRCAAlt (bottom) after baselining of the ferric resting state of the protein and subsequent reduction with sodium dithionite.



Figure S3: Zoomed in phylogenetic tree of the CYP125 clade used for ancestral sequence reconstruction. The CYP125MRCA node is shown in blue and the CYP125A1 search sequence highlighted as 'query' in blue. Extant sequence identifiers correspond to the IDs given in the alignment in Figure S3.

CYP125A1Mtb_CDC1551							. 17	
CYP125A1Mtb_CDC1551 CYP125A1Mtb_CDC1551 WP_150227942.1 WP_094057280.1 CYP125_Anc298 CYP125_Anc299 WP_088412999.1 WP_136170149.1 WP_085255059.1 WP_123027926.1 WP_0125_Anc292 CYP125_Anc297 CYP125_Anc297 CYP125_Anc296 CYP125_Anc294 CYP125_Anc295 WP_138918105.1	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	MSARSECWESHR	TKVSESLT	PPRDNC SJ	MSWN.HQS		TT TVPSPNLPPG .MQCPALPEG .MQCPALPEG .MTCPALPEG .MTCPALPEG .MTCPALPEG .MTCPALPEG .MTCPALPEG .MTCPALPEG .MTATPLPEG .MTATPLPEG .MTATPLPEG .MTATPLPEG .MTACPFPEG .MTACPFPEG .MTACPFPEG .MTACPFPEG .MTACPFPEG	
WP_106669128.1							<mark>.</mark>	<mark></mark>
CYP125_And291 CYP125MRCA	1						MTTTTMAPTD MTTTTMAPTD	IDLTD
CYP125_Anc290	1						MTTTMAPTD	IDLTD
CVP1 2531M+b CDC1 551		α1 0000 000	a2	β1	TT TT	β2	a3 000000000	β3
CYP125A1Mtb CDC1551	31	PATYAERLEVAE	RAELESAA		PGKGGGEH	GERATTA	LNDVKETSRH	SDVES
CYP125AIMEP_CDC1551 WP_12027942.1 WP_020140074.1 WP_094057280.1 CYP125_Anc298 CYP125_Anc299 WP_088412999.1 WP_136170149.1 WP_085255059.1 WP_12027926.1 WP_017593510.1 CYP125_Anc292 CYP125_Anc297 CYP125_Anc297 CYP125_Anc296 CYP125_Anc296 CYP125_Anc295 WP_138918105.1 WP_143910045.1 WP_106669128.1 CYP125_Anc291 CYP125_Anc291 CYP125_Anc290	31 15 15 15 15 15 15 15 15 15 15 15 15 15	PALYAEKPVAE		PIWWNGQ PVWWCPQ PVWWCPQ PVWWCPQ PVWWNPQ PVWNPQ PV PVWNPQ PV PV PV PV PV PV PV PV PV PV PV PV PV	DPGKGGGPH PRGIAGPQL PGGIAGPQL PGGIAGPQL PGGIAGPQL PGGIAGPQL PGGIAGPQL PGGIAGPQL PGGVGGPEL PGGVGGPEL PGGAGGPEL PGGAGGPEL PGGAGGPEL PGGAGGPEL PQ		HADVKYVSTH HADVKYVSTH HADVKYVSTH HADVKYVSTH HADVKYVSTH HADVKYVSTH HADVKYVSTH HADVKEVSRD HADVKEVSRD HADVREJSRD HADVKEVSRD HADVKEVSRH HADVKEVSRH HADVKEVSRH HADVKEVSRH HADVKEVSRH HADVKEVSRD HADVKEVSRD HADVKEVSRD HADVKEVSRD HADVKEVSRD HADVKEVSRD	S D V P S P E L P S P E L P S P E L P S P E L P S S D U Y S S D U Y S S S D U Y S S S D U Y S S E L P S S E L P S S E V P S S E V P S P E I P S P E I P S P E I P S P E I P S
CYP125A1Mtb_CDC1551		TTT . T	τ <u></u>	ι η1 <u>εξεεε</u> ε	η2 222 . 22	a5	η3 α6 222 22222	مەمە
CYP125A1Mtb_CDC1551 WP 150227942.1	91 74	SYENGVIPRFKN SHENTAVIRFNR	D IARED	IEVOR. F	VMLNMDAPH IMLNMDPPF	TRURKIIS	RGFTPRAVGR RGFTPRATEG	LHDEL
WP_020140074.1	74	SYLNTA IIRFNE	H <mark>I</mark> QR D Ă	IDAQR. L	ILLNMD PPEH	TRVRGIVO	RV FT PRA I RA	LEQRL
WP_094057280.1 CYP125 Anc298	74	STLNTAIIRFNE SHENTAIIRFNE		IDAQR. L	ILLNMDPPEH	TRVROIVO	RGFTPRAIRA RGFTPRAIRA	LEERL LEERL
CYP125_Anc299	74	SHLNTA IIRFNE	AAH <mark>I</mark> QR <mark>E</mark> A	IDAQR. L	ILLNMD PPEH	TRVRQIVO	RGFTPRAIRA	LEERL
WP_088412999.1 WP_136170149.1	74	SWENTALARYSD	H. VPRAA	IDANE G	IMLNMDAPF	TALEKIIVS	RGPTPRATAK	LRDAL
WP_085255059.1	82	SHDNGCVMRYSN	D VPPEE	LEAAK. V	LHNSDPPVH	TRLRKLIS	RMPTPRNVTA	LEASL
WP_123027926.1 WP_017593510_1	78	TWDNTVNIRFTD	D. ASPEQ	IEMSK. A	LLVNHDAPOH	TRLRKLIS	RMFTPRAIEA RCFTPRAICR	LRPRL MEEAT
CYP125_Anc292	74	SHENTAIVRYND	AADIPPEA	IEVOK. A	IMLNMDPPE	TRLRKIVS	RGFTPRAIAR	LEDRL
CYP125_Anc297 CYP125_Anc295	74	SHENTA IVRYND.	AADIGPEA	IEVOK. L	IMLNMDPPEH	TRLRKIVO	RGFTPRAIGR	LEERL
CYP125_Anc294	74	SHENTVIVRYND	AADIAPEE	IEVOK. A	IMLNMDPPOR	TRLRKIIS	RGFTPRAIGR	LHDRL
CYP125_Anc295	74	SHENTVIVRYND	AAD <mark>I</mark> SP <mark>E</mark> Q	IEVOK. A	LLLNMDPPOH	TRLRKIIS	RMFTPRAVNA	LHERL
WP_143910045.1	69	SALGTSOLODFD		ROKOA. AI	MLLNLDPPEH	TRORLLVS	RGPTPRVIAR	LESDI
WP_106669128.1	40	SRERLSLVEPE	<mark>.</mark> .EDV	LATOR. L	MMLNMD PPEH	SRLRNIVN	KGFTPRTTLT	LEDKV
CYP125_And291 CYP125MRCA	67 73	SRERGSMLODPD SOOGGTMIODAD	AAEET AAPEE	LAKOR. LI	MMLNMDPPEE	TRLERKLVS	KGPTPRIIAT	LEDKI
CYP125_Anc290	70	SOOGGSMLODAD	A AE <mark>E</mark> T	LEKOR. L	MMLNMDPPEH	TRLRKLVS	KGFTPRTIAR	LEDKI

Figure S4 Sequence alignment of extant sequences and reconstructed ancestral nodes in the CYP125 clade (continued overleaf).

		α7		β4 α8	α9	η4 α10
CYP125A1Mtb_CDC1551		222222222222	عفع		*00000000000	000000000000
CYP125A1Mtb_CDC1551	148	QERAQKIAAEA	AAAG	SGDEVEQ	SCELPLOAIAGLLG	VPQEDRGKLFHWSNEMT
WP 020140074.1	131	HDRALAIVETARI	ILPGD	SFDEVIO	ACELPLOAIAELIG	IPODDRAKIFD SNKMI
WP_094057280.1	131	RA <mark>RA</mark> HA <mark>IV</mark> EH <mark>A</mark> A.	AQD <mark>G</mark>	PFDFVTQ	ACELPLOAIAELIG	VPQEDR D KIFDWSN K MI
CYP125_Anc298	133	RARARAIVEEAAA	AOPDG	SFDFVTQ	ACELPLOAIAELIG	VPOEDRSKIFD SNKMI
WP 088412999.1	132	VARARDIVDAA	AEKS	GGNEVSDI	ASVIENHATADIVG	IPESDROOVLD. TNOME
WP_136170149.1	128	SA <mark>RA</mark> NK <mark>IV</mark> AD <mark>A</mark>	LTD <mark>G</mark>	TGEFVADI	IAAELPLQAITELIG	IPQEORH KVFEWSN IMT
WP_085255059.1	139	IDSARLIVAEA	AAKK	EGDEVEDI	ISRRIPMKAIADLVG	FPAEDHDRLFA SDAMM
WP_12302/926.1 WP_017593510.1	135	RERAARIASRA	AEKG	GGDEVAD	AMELPLOAIAELMG	VEOKDRAKLFOUSNEML
CYP125_Anc292	133	RE <mark>RA</mark> RK <mark>IV</mark> AE <mark>A</mark>	AEKG	SGDFVAD	IAAELPLQAIAELIG	VPQEDRHKLFD SNKMI
CYP125_Anc297	133	RERARKIVAEA	AEKG	SGDEVAD	ACELPLOAIAELIG	VPOEDRAKLFD SNKMI
CYP125 Anc296	130	RERARKIVAEA	AEKG	SGDEVADI	ACELPLOAIADLIG	VPOEDROKLFD SNEMI
CYP125_Anc295	133	VE <mark>RA</mark> RK <mark>IV</mark> AE <mark>A</mark>	. <mark>a</mark> ek <mark>g</mark>	SG <mark>DFV</mark> AD	IACELPLOAIADLIG	VPEEDROKLFD <mark>WSNO</mark> MM
WP_138918105.1	169	DEECRRIVDQA	FTET	EFDEVQEI	IAAKLPIAIIAELMG	VPESHRDQLLTWSKLIA
WP 106669128.1	94	RDACERIVASA	LDRG	EGDEWAMO	AAELPLVVIADLMG	VPOIDRHRLFE SNKLV
CYP125_Anc291	123	RE <mark>AC</mark> ER <mark>IV</mark> DE <mark>A</mark>	. IEK <mark>G</mark>	E C DEV A E I	IAAELPLA <mark>VIAELM</mark> G	VPQEDRHRLFDWSNRMV
CYP125MRCA	129	RERAKQIVDEA	IEK <mark>G</mark>	ECDEVADI	IAAELPLQVIAELIG	VPQEDRORLFD SNRMI
CIP125_And290	126	RE RA RQ IV DE A	. VENG	ECDEMEDI	IA AEDEDAVUAEDMG	VPOEDRHRLFD_SNRMI
		η5	a	11	α12	
CIPIZALINED_COCIDSI	202	CHERREN AUTOR VA				
WP_150227942.1	191	AYDDPEYAITEEVGVE	AAMEL	IGYAMNMA	AARKECPAODIVSO	VAAEGOG.NISDDEEG
WP_020140074.1	187	SYDDPEYAITEEVGQES	SAMEL	IA YAMNMA	A DRKQCPAQDIVTR	LVSAEDEG. SLNSDEFG
WP_094057280.1 CVP125 Apg298	186	AYDDPEYALTEEVGAES	SATEI	IAYAMNMA IGYAMNMA	ADREQCEANDIVIQ	VAAEDEG. NISSDARG
CYP125_Anc299	189	AYDDPEYAITEEVGAE	AMEL	IAYAMNMA	ADRKOCPAODIVTO	VAAEDEG. NISSDEFG
WP_088412999.1	186	AYDDPAVGKDTATQ	ATVAM	LGYAYTMA	AEEROLNPRDDILTG	LVQGAYEDRPLTPLEFA
WP_136170149.1 WP_085255059_1	182	GRDDPDIIGDPVA	AIGQV MUPT	MQYSMGLA TCYSVVL	ADRRECPAEDIATA	VRAODEDGALTDLISEG
WP_123027926.1	189	RFDDPDV STQRAAE	TAEL	LGYSYQLA	EKRKSCPTGDIIST	VQADVDGQSLTEIEFG
WP_017593510.1	185	GYDEPEFGMDPAV	ASTEI	LG FA MALA	AG <mark>ERR</mark> AD <mark>P</mark> RG <mark>DIVS</mark> K	VQADVDGRGLTDDEFG
CYP125_Anc292 CYP125_Anc297	187	GYDDPEYATDEA AQ	SART	LGYAMALA	AERKKNPADDIVIA	VOADVDGOKUSDDEGG
CYP125_Anc296	190	GYDDPEYATDEAAQ	TAEL	LGYSYTL	AEERKRNPADDIITQ	VQADVDGQQLSEMEPG
CYP125_Anc294	187	GYDDPEYATDEA AQ	TAEL	LGYAMTMA	EERKKNPADDIVTQ	VQADVDGQKISDDEFG
WP 138918105.1	223	GESDHOHNG.VDGTRO	VEEM	AVYAAELE	ADRAAHPRODVATA	TSADADGORISEEPEH
WP_143910045.1	177	G FEDPDF HTTEADGEM	AAAEI	FLYANELA	AAQRRANPRD DIIT A	LVQPDEDGHMLSEVEPN
WP_106669128.1	148	GDADPDLRQDAGEAEQ	OMEM	FGYADALO	AARRECPVDDIVSK	LV CPDRDGQEL TAIEPD
CYP125_ARCA	183	GYDDPEYHSSEADGEO	AAEM	FAYAOEL	AERRKNPRDDIVTA	VOAEVDGOKISDLEEN
CYP125_Anc290	180	G FDDPEY HSSEADGEQ	AAAEM	FAYANELA	A ERRKNPRD DIVT A	LVOPDVDGOKLSEIEPN
CYP125A1Mtb_CDC1551		α13 20000000 000004	α14 ε0000	000000	α15 200000000	al6 200000000
CYP125A1Mtb_CDC1551	260	FFVVMLAVAGNETTRNS	ITQG	MMAFAEH	DOWELYKKVRP	TAADEIVRWATEVTAE
WP_150227942.1	250	FFVLLLAVAGNETTRN2	AISHG	MHAFLTH	DOWELYKRERP	ATTAEZIVRWATPVV SP
WP_020140074.1 WP_094057280_1	246	FEVENLAVAGNETTRNA FEVENLAVAGNETTRNA	ALTHG ALTHC	MHAFLTHE	GOORLYKAERP	ATAAEBIVRWATPVNAF
CYP125_Anc298	248	FFVLLLAVAGNETTRNA	AITHG	MHAFLTH	DOWELYKRERP	ATAAEE IVRWATP VVAF
CYP125_Anc299	248	FFVLMLAVAGNETTRN	AITHG	MHAFLTH	DOMELYKQERP	ATAAEBIVRWATPVAAF
WP_088412999.1	244	YEVIQLMVAGNETSENZ VEVUTIMUAGSETTENZ	AITHG	MAFADNE	AQCELYRRHRP	PTTADEIIRWASPIIAF KTATDETTEKSCOVESE
WP_085255059.1	250	YFIVLLV TAGNET TRN	ISIG	MQALLNN	AQWELYKTARP	VTAADE IIRWASPVNAF
WP_123027926.1	247	FFVLMLAVAGNETTRN	ATTLG	LMALLQN	DOWEIFKRORP	ATAINE IVRWSTPVNVF
WP_017593510.1	242	FFVILLAVAGNETTRN/	LTHG	MAFHSDE	EQUELYKRERP	RTAADE IVRWATPVIAF
CYP125_Anc297	245	FFVILLAVAGNETTRNA	AITHG	MMAFLSH	DOWELYKRERP	ATAADEIVEWATEVIAE
CYP125_Anc296	248	FFVIMLAVAGNETTRN	AITLG	MMALLDN	DOWELYKRORP	ATAADE IVRWATPVNAF
CYP125_Anc294 CYP125_Anc295	245	FFVIMLAVAGNETTRN/	AITHG	MMAFLDH	DO RUYKRERP	ATAADE IVRWATPVIAF
WP_138918105.1	282	AFFILMTVAGNETTRYZ	LSGA	IEAFDEYE	DEALRLRESPD.IA	KTATDEVLENVSPTEVE
WP_143910045.1	237	MFFVLLV IAGNETTRNS	SATGG	MLALIDH	GOWDRLRADPS.LA	PT AVDEVL RWITPVMDF
WP_106669128.1	208	LFFMLLAVAGNET TRNA MRETILLYVACNET TRNA	AISGG	MLALIEH	EQUERLRADPAGLA	GTAADE IVRWVSPVNAF
CYP125MRCA	243	MFFLLLVVAGNETTRN	ISHG	MLALLEH	DOWERLRADPS. LA	PTAVDEILEWASPVMSP
CYP125_Anc290	240	MFFLLLVVAGNETTRN	AISGG	MLALIEH	DOWERLRADPS.LA	PTAVDEILRWVSPVMAF

Figure S4 Sequence alignment of extant sequences and reconstructed ancestral nodes in the CYP125 clade (continued overleaf).

		β5	β6	β7	β8	α17					
CYP125A1Mtb_CDC1551					,	-2222		TT	TŢ	TT.	TT
CYP125A1Mtb_CDC1551	317	ORTALRD	YELSO	VQIKK	QRVVM	FYRSANF	DEEVEO	PFTF	NILRI	PNPHVGPG.	GTGAH
WP_150227942.1	307	QRTATOD	TELGO	GOK IKKG	DRIGL	FYS <mark>San</mark> n	DPEVEG	NPEVF	DITRI	PNPHLGPGO	GGPH
WP_020140074.1	303	ORTATOD	TELGO	SKQ IRKG	DRVGI	FYAAANH	DPDVFE	NPDVF	DITRI	PNPHLGFGG	GGPH
WP_094057280.1	302	ORTATED	TELGO	KRIREG	DRLGL	FYA SANH	DPEVED	DPDTF	DITRI	PNPHLGPGC	GGPH
CYP125_And298	305	ORTATOD	TELGO	SOR INKS	DRVGL	FYA SANH	DPEVED	NEDTE		PNPHLGFGG	GGGPH
WD 088412000 1	301	OPTAL OD	VPLG		OPUCM	TA SA NE	DPPVPD			PN PHI GROU	CUCTU
WP 136170149.1	296	ORTATED	TELGO	OSIGKG	DRLLM	LYASANY	DETVEE	PHTF	DIGRI	PNPHLGPG	GTGAR
WP_085255059.1	307	ORTARRD	TELGO	VV IRRG	QRVGL	FYGSANY	DEDVED	DPFAF	DIERN	PNPHLGPG.	GTGPH
WP_123027926.1	304	ORTARCD	LELGO	SVR IAK G	QRAGMI	FYG <mark>SAN</mark> F	DEDVED	PFSF	NILRI	PNPHVGFG.	GHGAH
WP_017593510.1	299	QRTA TAD	TEIGO	GQA IAEG	ERVGL	YYS <mark>SA</mark> NF	DEEVED	DPFTF	DITRI	PNPHLGFG.	GTGAH
CYP125_Anc292	302	ORTATED	TELGO	300 1K KG	DRVVM	FYA SANY	DEEVED	DPYTF	DITRI	PNPHLGFGG	GIGAH
CYP125_Anc297	302	ORTATED	TELGO	JOQ IKKE	ORVGL	FYA SANF	DEEVED	PEFTE		PNPHLGPGG	GIGAH
CVD125_And296	305	OPTALPD	TELGO	VALKAG	OPUCM	FYA SAND	PPVDD			PNPHLGPGG	CTCAN
CYP125 Ang295	302	ORTALED	TELGO	VRIRK	ORVGM	FYASANF	DEEVED	PFTF	NTERI	PNPHLGPGC	GTGAR
WP_138918105.1	341	RRTARVD	GEIGO	VV IRGG	EKVVA	HLTSGNR	DERVFE	PDSF	DIGRS	PNPHVAFGO	GGPH
WP_143910045.1	296	RRTA TRD	CMIGI	OP VA AG	DKVVMI	FYA <mark>sa</mark> nr	DEAVED	DPFAF	DITRI	ONAQLAFGO	GGAH
WP_106669128.1	268	KRTA VRD	TELSO	SQP IA AG	DKVVV	YYA SANH	DEDVED	PYRL	DIGRI	PNPMLGPGG	. GGPH
CYP125_Anc291	297	RRTATRD	TELGO	QP IKAG	DKVVM	YYA SANR	DEEVED	DPYTF	DIGRS	PNPHLAFGO	GGPH
CYP125MRCA	302	RRTATED	TELGO	OQ IKAG	DKVVM	FYA SANR	DEEVED	DPYTF	DITRE	PNPHLAPGO	GGGPH
CYP125_And290	299	RETATED	TELGO	OPIKAC	DKVVM	YA SANK	DEEVED	PITE	DI TRE	PNPHLAFGO	GGPH
				a18		80	810		B11	B12	
CVP1 25A1M+b CDC1 551		000	00000	00000000	0000	199			P11		
	0.0									OND WEER CO.	
CYP125A1Mtb_CDC1551	376	YCIGANL	RMTI	INLIPNA	VADHMI	PDLKPIS	APERLR	GWLN	GIKHV	OVDYTGRCP	VAH
WP_150227942.1	360	YOLCKST	VLE		TADAM	PDLILAG		A NUT N	GVKET	OWTLC	
WP 094057280.1	361	YCLGKSL	VLET	DLIFTA	TADAM	PGLTLVG	DPRBLB	SAWIN	GVKEI	PVSAG	
CYP125_Anc298	365	YCLGKSL	AVLEI	IDLIFNA	IADAM	PDLTLAG	DPRRLR	SAWIN	GVKEI	PVRAA	
CYP125_Anc299	365	YCLGKSL	AVLEI	IDLIFNA	IADAM	PDLTLAG	DPRRLR	SAWIN	GVKEI	PVSAG	
WP_088412999.1	360	YCLGANL	ARLEI	IGIMFDA	MADRLI	PDLVPTG	EPTRFR	S G W I N	GVVEI	PA NYVGRGO	RDQPA
WP_136170149.1	355	YCIGANL	RMEI	IELIYDA	INEQMI	PDISVIG	PPSRLN	SFIN	SVKSI	PVSYG. TCP	VQPR.
WP_085255059.1	366	YCIGANL	RKEI	LAVMLDA	IADRL	PDIEMIG	EPTRAQ	GWIN	GIATE	PVRFTAG	
WP_12302/926.1	363	YOLGAND	REGI	LELVENA		PDIIMIG	CORVERSE	SAWLH	CURUI	PUDIGTAPO	
CVD125 Apc202	362	YOLGANT	DMP		TADOM		DDDDTD	S S P T N	CVRUT	DUDYT ACT	VAU
CYP125 Apc297	362	YCLGANL	RMEI	DLIFNA	TADOM	PDIEVIG	EPERLE	SWIN	GVKHI	PVRYT	
CYP125 Anc296	365	YCIGANL	RMEI	IELIFNA	IADRL	PDIEMTG	EPTRLR	S G W I N	GIAEI	PVNYTAACO	
CYP125_Anc294	362	YCIGANL	A RME I	IDLIFNA	IADOM	PDIEVIG	EPERLR	S G <mark>W I N</mark>	GIKHI	PV RYTGRCP	VAH
CYP125_Anc295	362	YCIGANL	A RME I	IDLIFN <mark>A</mark>	IADRM	PDIEMTG	EPTRLR	S G <mark>W I N</mark>	GIVEI	PV NYTGRCG	VDH
WP_138918105.1	400	FCLGKHL	LME	IESMLRE	LASRAI	DRIEVIR	KPRRLL	SYHFN	GLVDI	EVRVTRS	
WP_143910045.1	355	YCLGTHL	RLEI	LRVLFET	LAARVI	ELVERIG	PARRER	SNFIN	GIKEN	RVRLHPAWG	APS
CVD125 Apg201	327	PCLGRHL	DIPI	PUMPRI	LERVI	ERVEPLG PDTPUTC	PARMA		GINDE	PVRIHKA	
CYP125MRCA	362	YCLGANL	RLEI	RVMPEE	LAERMI	PDIELTG	PPERLE	SNFIN	GIKHN	PVRFTPARA	VGG.
CYP125 Anc290	359	YCLGAHL	RLEI	RVMFET	LAERVI	ERIELTG	PPRRLR	SNFIN	GIKDN	PV RLHPARG	AGG
_							_				
CYP125A1Mtb_CDC1551											
CYP125A1Mtb_CDC1551											
WP_150227942.1											
WP_020140074.1											
WP_094057280.1											
CIP125_And298											
CYP125 Apc200											
CYP125_Anc299 WP 088412999.1	420	WGLTRAF	RP								
CYP125_Anc299 WP_088412999.1 WP_136170149.1	420	WGLTRAE	RP								
CYP125_Anc299 WP_088412999.1 WP_136170149.1 WP_085255059.1	420	WGLTRAE	RP								
CYP125_Anc299 WP_088412999.1 WP_136170149.1 WP_085255059.1 WP_123027926.1	420	WGLTRAE	RP								
CYP125_Anc299 WP_088412999.1 WP_136170149.1 WP_085255059.1 WP_123027926.1 WP_017593510.1	420	WGLTRAE	RP								
CYP125_Anc299 WP_088412999.1 WP_136170149.1 WP_085255059.1 WP_0123027926.1 WP_017593510.1 CYP125_Anc292 CYP125_Anc292	420	WGLTRAE	RP 								
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Figure S4: Sequence alignment of extant sequences and reconstructed ancestral nodes in the CYP125 clade. Positions of each sequence in the phylogenetic tree are shown in Figure S2.

CYP125A1_Mycobacterium_tuberculosis CYP125A1_Mycobacterium_tuberculosis CYP125_MRCA CYP125A69_Mycobacterium_abscessus WP_136170149.1 CYP125A13_Streptomyces_peucetius WP_094057280.1 CYP125A65_Mycobacterium_abscessus CYP125A68_Mycobacterium_abscessus CYP125A68_Mycobacterium_abscessus CYP125A67_Mycobacterium_atinum CYP125A67_Mycobacterium_atinum CYP125A7_Mycobacterium_atinum CYP125A7_Mycobacterium_atinum	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	MGSFPCPQKIEQVLLSGQGLNELSFASRPACAS	MSWNHQSVEIAVRRTTVPSPNLP MTTTTMAP MTTTCPT MSCPHLP MSCPHLP MSCPHLP MYQAQHPHLP MLVERVPHHGVVYGLGQETAVAQPNLP MPAAEPTATSVPNLP MPCPNLP
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Figure S5: Multiple sequence alignment of CYP125MRCA using ClustalW (continued overleaf).

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CYP125A6_Mycobacterium_marinum CYP125A67_Mycobacterium_abscessus CYP125A7_Mycobacterium_marinum	424 415 413	CPVAH. CPVLQ. CPVSH
CYP125A7_Mycobacterium_ulcerans	413	CPVSH.

Figure S5: Multiple sequence alignment of CYP125MRCA using ClustalW. Alignment is against known CYP125 enzymes from pathogenic descendants of CYP125MRCA, along with other CYP125 enzymes representing different clades of the family's phylogenetic tree.



Figure S6: Overall structure of CYP125MRCA showing characteristic P450 features. Left: Cartoon depiction of the entire CYP125MRCA structure. The F/G helices are highlighted in blue, the B' helix in red and the I helix in teal. The heme centre is shown in grey and the substrate (in this case vitamin D_3) in orange. Right: A zoomed in view of the heme binding groove.



Figure S7: Sequence alignment of CYP125MRCA and CYP125MRCAAlt using ClustalW, with key regions labelled and highlighted in purple.



Figure S8 UV-Vis absorption spectra of the resting states of CYP125MRCA (black) and CYP125MRCAAlt (red).



Figure S9: Selected UV-Vis spin-state shift assays used to screen the binding of various C-27 steroids to CYP125MRCA. In each case, the substrate free spectrum is shown in black while the substrate bound spectrum is shown in red. Substrate stocks were dissolved in either 40% EtOH or 40% hydroxypropyl-β-cyclodextrin in 50 mM Tris (pH 7.4).



Figure S9 Continued: Selected UV-Vis spin-state shift assays used to screen the binding of various C-27 steroids to CYP125MRCA. In each case, the substrate free spectrum is shown in black while the substrate bound spectrum is shown in **red**. Substrate stocks were dissolved in either 40% EtOH or 40% hydroxypropyl-β-cyclodextrin in 50 mM Tris (pH 7.4).



Figure S10: Selected UV-Vis spin-state shift assays used to screen the binding of various C-27 steroids to CYP125MRCAAlt. In each case, the substrate free spectrum after addition of imidazole (1.3 mM) is shown in black while the substrate bound spectrum is shown in **red**. Substrate stocks were dissolved in either 40% EtOH or 40% hydroxypropyl- β -cyclodextrin in 50 mM Tris (pH 7.4). Complete Type II shifts were avoided as saturating concentrations of imidazole interfered with the ability of substrates to cause spin-state shifts at reasonable concentrations.

Cholesterol, cholest-4-en-3-one and stigmast-4-en-3-one binding resulted in > 90% HS heme centres, while sitosterol resulted in > 80%. The shift induced by sitosterol marked a difference from CYP125MRCA in which the Soret band was shifted completely to HS, though direct comparisons of the degree of spin-state shift between the two ancestors was complicated by the addition of imidazole to CYP125MRCAAlt prior to analysis.



Figure S11: UV-Vis binding titration curves for various C-27 steroids to CYP125MRCA, using the Hill equation (Equation 1 in main text) in each case. Enzyme concentrations for the titrations were between 2.1-2.8 μ M (shown in Table S1). Substrate stocks were between 1 and 10 mM in 40% hydroxypropyl- β -cyclodextrin.

Table S1: Binding constants determined through analysis of UV-Vis difference spectra upon substrate titration for different binding models. The K_d values used to compare substrate efficiencies were from the highlighted Hill model column, given their lower relative error.

Substrate	K _d Michaelis Menten	K _d Morrison E fit (E)	K _d Morrison E set (E)	K _d Hill Equation (n)
Stigmasterol	1.5 ± 0.7	0.006 ± 0.013 (4.0)	0.6 ± 0.4 (2.3)	2.04 ± 0.12 (2.36)
Sitosterol	1.1 ± 0.3	0.004 ±0.011 (2.8)	0.01 ± 0.02 (2.6)	1.48 ± 0.03 (3.74)
Campesterol	3.1± 1.5	1.5 ± 3.6 (4.0)	2.3 ± 1.2 (2.1)	4.19 ± 0.05 (3.68)
Cholest-4-en-3-one	0.6 ± 0.1	0.04 ± 0.01 (1.7)	0.0002 ± 0.0082 (2.8)	0.85 ± 0.02 (2.15)
Cholecalciferol	1.4 ± 0.3	1.2 ± 0.8 (2.0)	0.8 ± 0.2 (2.6)	1.60 ± 0.22 (1.84)
Stigmast-4-en-3-one	1.1 ± 0.3	0.1 ± 0.09 (3.1)	0.4 ± 0.13 (2.1)	1.61 ± 0.11 (1.98)

The Hill equation gave the best fit evidenced by the lowest relative standard error of K_d compared to the Morrison and Michaelis-Menten models (see Table S1 for model comparisons). Given that n is greater than 1 for all substrates, some level of cooperativity in ligand binding is present. While differences are observed between the different fitting methods the overall trends are similar. Differences in affinity and in the values of n may reflect complex interactions between the enzyme, ligand and cyclodextrin.



Figure S12: GC-MS chromatograms of CYP125MRCA mediated oxidation of cholesterol (top), campesterol (middle) and a 1:1 mixture of cholesterol and sitosterol (bottom). In each case the substrate control is shown in black, and the CYP125MRCA mediated oxidation reaction in **red** and **blue**. Products were identified by their mass fragmentation pattern and relative retention times. * Indicates peaks arising from a campesterol impurity in the sitosterol stock.

Cholest-4-en-3-one and oxidation products



RT: 20.57 – 20.67 min, Cholest-4-en-3-one (parent ion mass: 384 m/z)



RT: 25.08 – 25.23 min, 26-hydroxycholest-4-en-3-one (parent ion mass: 472 m/z)

Figure S13: (a) Mass spectra of GC-MS separated cholest-4-en-3-one substrate and products of CYP125MRCAmediated *in vitro* oxidation reactions using a reconstituted spinach ferredoxin/reductase/NADPH electron transfer system.

Cholesterol and oxidation products



RT: 19.53 – 19.62 min, Cholesterol (parent ion mass: 458 m/z)



RT: 23.22 – 23.35 min, 26-hydroxycholesterol (parent ion mass: 546 m/z)



RT: 24.53 – 24.67 min, 26-cholestenoic acid (parent ion mass: 560 m/z)

Figure S13: (b) Mass spectra of GC-MS separated cholesterol substrate and products of CYP125MRCA-mediated *in vitro* oxidation reactions using a reconstituted spinach ferredoxin/reductase/NADPH electron transfer system.

Sitosterol and oxidation products



RT: 21.42 – 21.53 min, Sitosterol (parent ion mass: 486 m/z)



RT: 25.20 – 25.32 min, 26-hydroxysitosterol (parent ion mass: 574 m/z)



RT: 26.83 – 27.07 min, 26-sitostenoic acid (parent ion mass: 588 m/z)

Figure S14: (a) Mass spectra of GC-MS separated sitosterol substrate and products of CYP125MRCA-mediated *in vitro* oxidation reactions using a reconstituted spinach ferredoxin/reductase/NADPH electron transfer system.

Campesterol and oxidation products



RT: 20.48 – 20.63 min, Campesterol (parent ion mass: 472 m/z)



RT: 24.20 – 24.32 min, 26-hydroxycampesterol (parent ion mass: 560 m/z),



RT: 25.72 – 26.00 min, 26-campestenoic acid (parent ion mass: 574 m/z)

Figure S14: (b) Mass spectra of GC-MS separated campesterol substrate and products of CYP125MRCA-mediated *in vitro* oxidation reactions using a reconstituted spinach ferredoxin/reductase/NADPH electron transfer system.

Stigmast-4-en-3-one and oxidation products



RT: 22.73 – 22.87 min, Stigmast-4-en-3-one (parent ion mass: 412 m/z)



RT: 27.28 – 27.52 min, 26-hydroxystigmast-4-en-3-one (parent ion mass: 500 m/z)

Figure S14: (c) Mass spectra of GC-MS separated stigmast-4-en-3-one substrate and products of CYP125MRCAmediated *in vitro* oxidation reactions using a reconstituted spinach ferredoxin/reductase/NADPH electron transfer system.



Figure S15: Raw, normalised fluorescence data for protein thermal shift assays (PTS assays), conducted in triplicate, for CYP125A7 from *Mycobacterium ulcerans* and CYP125MRCA. The dye and protocol used were sourced from ThermoFischer Scientific (Protein Thermal Shift Dye Kit[™], catalog number 4461146).



Figure S16: GC-MS chromatograms of CYP125MRCA and CYP125MRCAAlt mediated oxidation of cholesterol. Products were identified by their mass fragmentation pattern and relative retention times. * indicates significant unidentified impurity peaks.* indicates the presence of unknown oxidation products.

Table S2: X-ray crystallographic data collection, processing, and refinement statistics for CYP125MRCA structures.

	CYP125MRCA					
	Sitosterol	Cholecalciferol (Vitamin D ₃)				
PDB ID	8VXI	8VXG				
Space Group	C2221	C2221				
Unit cell lengths a, b, c (Å)	72.96, 118.88, 94.76	72.59, 118.49, 94.82				
Unit cell angles α, β ,γ (°)	90, 90 ,90	90, 90 ,90				
Wavelength (Å)	0.9537	0.9537				
Number of obsevations	350972 (24050)	52492 (24760)				
Number of unique reflections	25931 (1950)	39392 (2063)				
Resolution (Å)	2.06	1.78				
R _{meas}	0.187 (1.721)	0.159 (1.870)				
R _{pim}	0.051 (0.482)	0.043 (0.525)				
<i o(i)=""></i>	9.8 (1.3)	10.4 (1.2)				
CC(1/2)	0.998 (0.640)	0.998 (0.640)				
Completeness (%)	99.8 (97.2)	99.6 (92.5)				
Multiplicity	13.5 (12.3)	13.3 (12.0)				
R _{work} /R _{free}	0.1665/0.2144	0.1734/0.2183				
Bond length R.M.S.D (Å)	0.0138	0.0072				
Bond angle R.M.S.D (°)	1.67	1.09				
Ramachandran Favoured (%)	96.71	98.19				
Ramachandran Allowed (%)	3.04	1.81				

Cholecalciferol oxidation products – Normalised to highest TIC peak



RT: 20.58 – 20.67 min, hydroxylated product (parent ion mass = 544 m/z)



RT: 21.22 – 21.30 min, hydroxylated product (parent ion mass = 544 m/z)



RT: 20.05 – 20.15 min, partially underivatised hydroxylated product (parent ion mass = 472 m/z)



RT: 22.21 – 22.40 min, hydroxylated product (parent mass = 544 m/z)

Figure S17: Mass spectra of GC-MS separated vitamin D₃ derived substrates and products of CYP125MRCA-mediated *in vitro* oxidation reactions using a reconstituted spinach ferredoxin/reductase/NADPH electron transfer system.



RT: 23.08 – 23.22 min, hydroxylated product (parent ion mass = 544 m/z)

Figure S17 Continued: Mass spectra of GC-MS separated vitamin D₃ derived substrates and products of CYP125MRCAmediated *in vitro* oxidation reactions using a reconstituted spinach ferredoxin/reductase/NADPH electron transfer system.



Figure S18: GC-MS chromatograms of CYP125MRCA and CYP125MRCAAlt mediated oxidation of Vitamin D_3 . The substrate control is shown in black, a 25-hydroxyvitamin D_3 in **red**, the CYP125MRCA mediated oxidation reaction in **blue** and the CYP125MRCAAlt mediated reaction in **magenta**. Products were identified by their mass fragmentation pattern and relative retention times. * indicates significant unidentified impurity peaks.



Figure S19: CYP125A7-mediated *in vitro* oxidation reactions using a reconstituted spinach ferredoxin/reductase/NADPH electron transfer system (blue), overlaid with a vitamin D_3 control (black) and CYP125MRCA vitamin D_3 positive control (red). The vitamin D_3 substrate peak is shown by a *, while the CYP125MRCA mediated oxidation products are highlighted with an*.





Figure 20: Active-site comparisons between cholecalciferol (orange) bound CYP125MRCA (green) and cholest-4-en-3-one (magenta) bound CYP125A1 (blue). Top: Active-site overlay highlighting difference in the active-site residue positions within 5Å of the heme centre. Bottom: Protein surface differences, highlighting the side-cavity opening of CYP125MRCA allowing for the binding of cholecalciferol (left) and the smaller equivalent cavity in CYP125A1, due to the steric gating by the F260 residue.





Figure S21: CYP125-MRCA surface hydrophobicity scale, with increasing intensity of red indicating higher hydrophobicity (top). CYP125 MRCA-sitosterol (purple) overall structure with solvent accessible channels produced by Caver shown in red (bottom left). Caver settings were min probe radius = 0.9, shell depth = 4, shell radius = 3, clustering threshold = 3.5, substrate emitted from tunnelling calculations. A zoom in of the position of sitosterol (green) within the solvent access channels of CYP125MRCA (bottom right).



Figure S22: CYP125A1 surface hydrophobicity scale, with increasing intensity of red indicating higher hydrophobicity (top). CYP125 MRCA-cholest-4-en-3-one (blue) overall structure with solvent accessible channels produced by Caver shown in red (left). Caver settings were min probe radius = 0.9, shell depth = 4, shell radius = 3, clustering threshold = 3.5, substrate emitted from tunnelling calculations. A zoom in on the position of cholest-4-en-3-one (magenta) within the solvent access channels of CYP125MRCA (right).



Figure S23: Left: CYP125MRCA-cholecalciferol (top) and CYP125MRCA-sitosterol (right) active-sites. Residues, substrate, and the heme centre are shown as sticks, while the electron density FEM map of the substrate is shown as a grey mesh (carve radius = 1.8 Å).