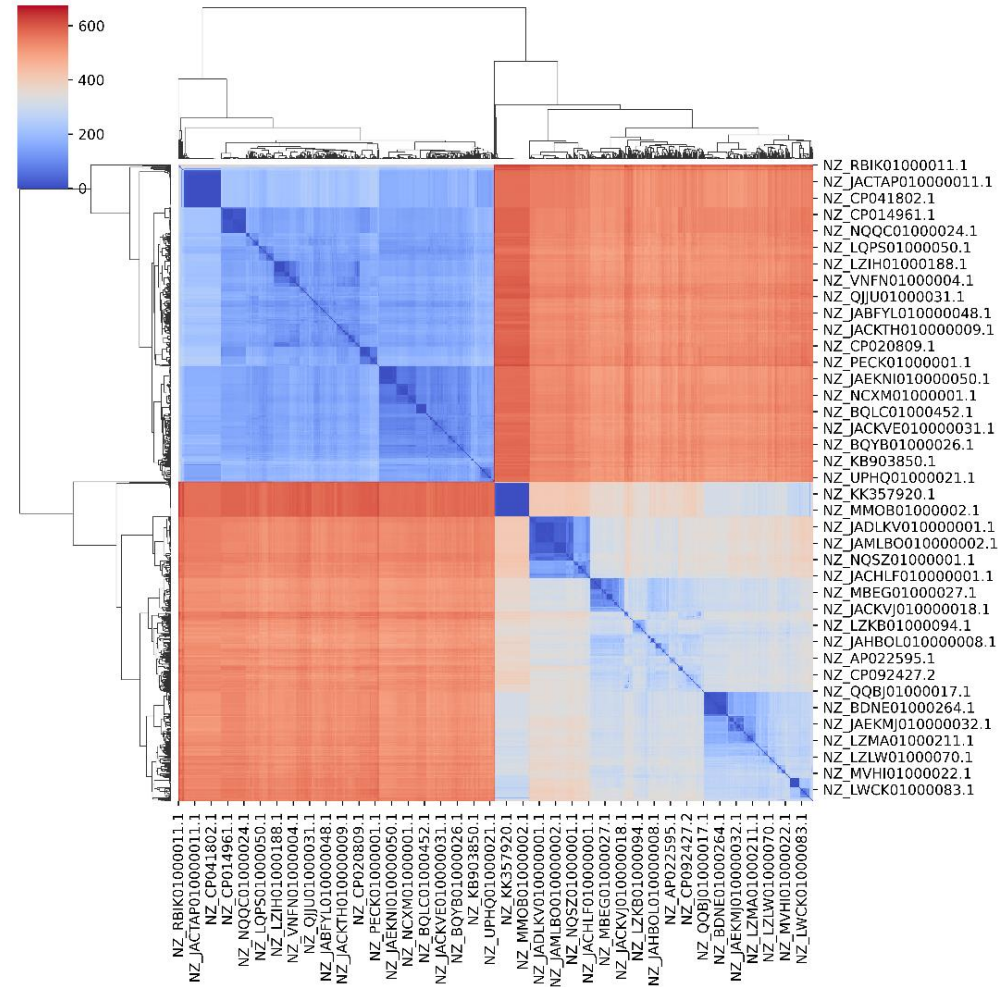


A tool to automatically design multiplex PCR primer pairs for specific targets using diverse templates

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Supplementary Figure S1. The heatmap of distance matrix among suspected *hsp65* sequences in Mycobacteriaceae.

Method	Template	Specific Application	Support unaligned sequence	Target region	Primer design	Primer	Primer selection	Primer evaluation	Reference
DECIPHER	Subgroup	All	Yes	Auto selection	Consensus sequence	Degenerate primer	Yes	Template coverage	DesignSignatures: a tool for designing primers that yields amplicons with distinct signatures
PrimerDesign-M	All	All	No	Assignment	Consensus sequence	Degenerate primer	Yes	No	PrimerDesign-M: a multiple-alignment based multiple-primer design tool for walking across variable genomes
openPrimeR	All	Immunoglobulin genes	Yes	Assignment	Consensus sequence	Primer pair	Yes	Template coverage	openPrimeR for multiplex amplification of highly diverse templates
PhyloPrimer	Subgroup /All	Prokaryote	Yes	Auto selection	Consensus sequence	Normal primer	Yes	Taxon specificity & Target specificity	PhyloPrimer: a taxon-specific oligonucleotide design platform
rprimer	All	Virus	No	Auto selection	Consensus sequence	Degenerate primer	No	Template coverage	rprimer: an R/bioconductor package for design of degenerate oligos for sequence variable viruses
PMPrimer	Subgroup /All	All	Yes	Auto selection	Haplotype sequence	Degenerate primer & Primer pair	Yes	Template coverage, Taxon specificity & Target specificity	This study

Supplementary Table S1. Methods for design of multiplex PCR primers for diverse templates.

Conserved region	Primer region	Direction	Degenerate primer	Haplotype primer: count	Coverage
519-533 ^a (7818-8175) ^{b1}	519-533(7818-8175)	forward	CAGCMGCCGCGGTAA	CAGCAGCCGCGGTAA : 225	94.3%
		reverse	TTACCGCGGCKGCTG	CAGCCGCCGCGGTAA : 10861	94.3%
781-806(10171-11015) ¹	784-805(10365-11009)	forward	SGGATTAGATACCCSDGTAGTC	GGGATTAGATACCCGGGTAGTC : 23 GGGATTAGATACCCGAGTAGTC : 1 GGGATTAGATACCCGTGTAGTC : 0 GGGATTAGATACCCCGGTAGTC : 973 GGGATTAGATACCCAGTAGTC : 47 GGGATTAGATACCCCTGTAGTC : 172	90.4%
		reverse	GACTACHSGGGTATCTAATCCS	CGGATTAGATACCCGGGTAGTC : 9000 CGGATTAGATACCCGAGTAGTC : 353 CGGATTAGATACCCGTGTAGTC : 51 CGGATTAGATACCCCGGTAGTC : 4 CGGATTAGATACCCAGTAGTC : 0 CGGATTAGATACCCCTGTAGTC : 1	
864-893(11582-13404) ²	871-890(12677-13180)	forward	TAARGGAATTGGCGGGGRGRG	TAAGGGAATTGGCGGGGGGG : 0 TAAGGGAATTGGCGGGGGAG : 115 TAAGGGAATTGGCGGGAGGG : 0 TAAGGGAATTGGCGGGAGAG : 20	84.8%
		reverse	CYCYCCCGCCAATTCCYTТА	TAAAGGAATTGGCGGGGGGG : 165 TAAAGGAATTGGCGGGGGAG : 9530 TAAAGGAATTGGCGGGAGGG : 0 TAAAGGAATTGGCGGGAGAG : 137	

Supplementary Table S2. Degenerate and haplotype primers for 16S rRNA genes of Archaea. Notes: ^aThe numbering is based on the *Escherichia coli* (NC_000913.3) system of nomenclature. ^bThe numbering is based on the alignment. ¹Region identified by PMPrimer and in a previous study. ²Region only identified by PMPrimer.

Conserved region	Primer region	Direction	Degenerate primer	Haplotype primer:count	Coverage
160-182 ^a (163-185) ^b	163-181(166-184)	forward	ATCGCYAAGGARATCGAVC	ATCGCTAAGGAGATCGAGC : 1 ATCGCTAAGGAGATCGACC : 0 ATCGCTAAGGAGATCGAAC : 0 ATCGCTAAGGAAATCGAGC : 2 ATCGCTAAGGAAATCGACC : 0 ATCGCTAAGGAAATCGAAC : 0	99.3%
		reverse	GBTCGATYTCCTTRGCGAT	ATCGCCAAGGAGATCGAGC : 550 ATCGCCAAGGAGATCGACC : 3 ATCGCCAAGGAGATCGAAC : 17 ATCGCCAAGGAAATCGAGC : 1 ATCGCCAAGGAAATCGACC : 0 ATCGCCAAGGAAATCGAAC : 0	99.3%
193-209(196-212)	194-209(197-212)	forward	ACGARAAGATYGGYGC	ACGAGAAGATTGGTGC : 0 ACGAGAAGATTGGCGC : 4 ACGAGAAGATCGGTGC : 16 ACGAGAAGATCGGCGC : 554	100.0%
		reverse	GCRCCRATCTTYTCGT	ACGAAAAGATTGGTGC : 0 ACGAAAAGATTGGCGC : 0 ACGAAAAGATCGGTGC : 0 ACGAAAAGATCGGCGC : 4	100.0%
514-531(517-534)	514-531(517-534)	forward	GTCATCACBGTYGARGAG	GTCATCACGGTTGAGGAG : 0 GTCATCACGGTTGAAGAG : 0 GTCATCACGGTCGAGGAG : 7 GTCATCACGGTCGAAGAG : 0	97.9%
		reverse	CTCYTCRACVGTGATGAC	GTCATCACTGTTGAGGAG : 0 GTCATCACTGTTGAAGAG : 0 GTCATCACTGTCGAGGAG : 4 GTCATCACTGTCGAAGAG : 2	97.9%

				GTCATCACCGTTGAGGAG : 3	
				GTCATCACCGTTGAAGAG : 0	
				GTCATCACCGTCGAGGAG : 542	
				GTCATCACCGTCGAAGAG : 8	
727-755(730-758)	736-754(739-757)	forward	CTGATCATCGCYGARGAYG	CTGATCATCGCTGAGGATG : 0	95.5%
				CTGATCATCGCTGAGGACG : 3	
				CTGATCATCGCTGAAGATG : 0	
				CTGATCATCGCTGAAGACG : 14	
	reverse		CRTCYTCTRGCGATGATCAG	CTGATCATCGCCGAGGATG : 10	95.5%
				CTGATCATCGCCGAGGACG : 520	
				CTGATCATCGCCGAAGATG : 0	
				CTGATCATCGCCGAAGACG : 5	
1048-1079(1051-1082)	1056-1075(1059-1078)	forward	GAACAGCGAYWSCGACTACG	GAACAGCGATAGCGACTACG : 0	92.4%
				GAACAGCGATACCGACTACG : 0	
				GAACAGCGATTGCCGACTACG : 0	
				GAACAGCGATTCCGACTACG : 9	
	reverse		CGTAGTCGSWRTCCTGTTC	GAACAGCGACAGCGACTACG : 10	92.4%
				GAACAGCGACACCGACTACG : 0	
				GAACAGCGACTGCCGACTACG : 0	
				GAACAGCGACTCCGACTACG : 515	
1081-1097(1084-1100)	1081-1097(1084-1100)	forward	GAGAAGYTSCAGGARCG	GAGAAGTTGCAGGAGCG : 6	99.1%
				GAGAAGTTGCAGGAACG : 0	
				GAGAAGTTCCAGGAGCG : 0	
				GAGAAGTTCCAGGAACG : 0	
	reverse		CGYTCCTGSARCTTCTC	GAGAAGCTGCAGGAGCG : 560	99.1%
				GAGAAGCTGCAGGAACG : 4	
				GAGAAGCTCCAGGAGCG : 3	
				GAGAAGCTCCAGGAACG : 0	
1099-1115(1102-1118)	1099-1113(1102-1116)	forward	YTGGCCAARCTSGCC	TTGGCCAAGCTGGCC : 5	96.4%

		reverse	GGCSAGYTTGGCCAR	TTGGCCAAGCTCGCC : 0	96.4%
				TTGGCCAAACTGGCC : 0	
				TTGGCCAAACTCGCC : 0	
				CTGGCCAAGCTGGCC : 543	
				CTGGCCAAGCTCGCC : 4	
				CTGGCCAAACTGGCC : 5	
1159-1187(1162-1190)	1170-1187(1173-1190)	forward	CAAGCAYCGYATCGARGA	CTGGCCAAACTCGCC : 0	98.1%
				CAAGCATCGTATCGAGGA : 0	
				CAAGCATCGTATCGAAGA : 0	
				CAAGCATCGCATCGAGGA : 8	
				CAAGCATCGCATCGAAGA : 0	
				CAAGCATCGCATCGAGGA : 0	
		reverse	TCYTCGATRCGRTGCTTG	CAAGCACCGTATCGAGGA : 17	98.1%
				CAAGCACCGTATCGAAGA : 0	
				CAAGCACCGCATCGAGGA : 519	
				CAAGCACCGCATCGAAGA : 23	
				GAGGGCATTGTTGCC : 0	
				GAGGGCATTGTCGCC : 3	
1219-1235(1222-1238)	1219-1233(1222-1236)	forward	GARGGCATYGTYGCC	GAGGGCATCGTTGCC : 4	98.3%
				GAGGGCATCGTCGCC : 549	
				GAAGGCATTGTTGCC : 0	
				GAAGGCATTGTCGCC : 0	
				GAAGGCATCGTTGCC : 0	
				GAAGGCATCGTCGCC : 12	
		reverse	GGCRACRATGCCYTC	GAAGGCATTGTTGCC : 0	98.3%
				GAAGGCATTGTCGCC : 0	
				GAAGGCATCGTTGCC : 0	
				GAAGGCATCGTCGCC : 12	

Supplementary Table S3. Degenerate and haplotype primers for *hsp65* (*groEL2*) genes of Mycobacteriaceae. Notes: ^aThe numbering is based on the *hsp65* gene of *Mycobacterium Tuberculosis* (NC_000962.3). ^bThe numbering is based on the alignment.

Conserved region	Primer region	Direction	Degenerate primer	Haplotype primer:count	coverage
58-89 ^a (58-89) ^{b1}	58-78(58-78)	forward	CACGTTGACCAYGGTAAACD	CACGTTGACCATGGTAAAACG : 12	100.0%
				CACGTTGACCATGGTAAAACA : 116	
				CACGTTGACCATGGTAAAAC : 183	
		reverse	HGTTTTACCRTGGTCAACGTG	CACGTTGACCACGGTAAAACG : 0	100.0%
				CACGTTGACCACGGTAAAACA : 0	
				CACGTTGACCACGGTAAAAC : 4	
160-182(163-185) ¹	160-180(163-183)	forward	CCWGAAGAAAAAGARCGTGGT	CCAGAAGAAAAAGAGCGTGGT : 2	100.0%
				CCAGAAGAAAAAGAACGTGGT : 312	100.0%
		reverse	ACCACGYTCTTTTCTTCWGG	CCTGAAGAAAAAGAGCGTGGT : 0	
				CCTGAAGAAAAAGAACGTGGT : 1	
		forward	CGTCACTAYGCDACGTWGAC	CGTCACTATGCGCACGTAGAC : 0	98.4%
				CGTCACTATGCGCACGTTGAC : 48	
223-245(226-248) ²	223-243(226-246)			CGTCACTATGCACACGTAGAC : 0	
				CGTCACTATGCACACGTTGAC : 51	
				CGTCACTATGCTCACGTAGAC : 14	
				CGTCACTATGCTCACGTTGAC : 150	
		reverse	GTCWACGTGHGCRTAGTGACG	CGTCACTACGCGCACGTAGAC : 0	98.4%
				CGTCACTACGCGCACGTTGAC : 0	
				CGTCACTACGCACACGTAGAC : 0	
				CGTCACTACGCACACGTTGAC : 0	
				CGTCACTACGCTCACGTAGAC : 0	
				CGTCACTACGCTCACGTTGAC : 47	
		forward	GCTGACTAYGTKAAAAAYATGA	GCTGACTATGTGAAAAATATGA : 0	99.4%
				GCTGACTATGTGAAAAACATGA : 12	
256-278(259-281) ²	256-277(259-280)			GCTGACTATGTAAAAATATGA : 8	
		reverse	TCATRTTTTTMACRTAGTCAGC	GCTGACTATGTAAAAACATGA : 235	99.4%
				GCTGACTACGTGAAAAATATGA : 0	
				GCTGACTACGTGAAAAACATGA : 0	

				GCTGACTACGTTAAAAATATGA : 0	
				GCTGACTACGTTAAAAACATGA : 58	
292-314(298-320) ²	296-314(302-320)	forward	TGGAYGGHGSTATCTTAGT	TGGATGGAGGTATCTTAGT : 0	98.7%
				TGGATGGAGCTATCTTAGT : 2	
				TGGATGGCGGTATCTTAGT : 5	
				TGGATGGCGCTATCTTAGT : 0	
				TGGATGGTGGTATCTTAGT : 0	
	reverse			TGGATGGTGCTATCTTAGT : 0	
			ACTAAGATASDCCRTCCA	TGGACGGAGGTATCTTAGT : 0	98.7%
				TGGACGGAGCTATCTTAGT : 67	
				TGGACGGCGGTATCTTAGT : 172	
				TGGACGGCGCTATCTTAGT : 8	
			TGGACGGTGGTATCTTAGT : 57		
			TGGACGGTGCTATCTTAGT : 0		
334-353(340-359) ¹	334-353(340-359)	forward	CCAATGCCWCAAACKCGTGA	CCAATGCCACAAACGCGTGA : 4	100.0%
				CCAATGCCACAAACTCGTGA : 275	
	reverse		TCACGMGTTTGWGGCATTGG	CCAATGCCTCAAACGCGTGA : 0	100.0%
				CCAATGCCTCAAACTCGTGA : 36	
364-383(370-389) ²	367-383(373-389)	forward	TCWCGTAAYGTWGGTGT	TCACGTAATGTAGGTGT : 0	99.7%
				TCACGTAATGTTGGTGT : 5	
				TCACGTAACGTAGGTGT : 22	
				TCACGTAACGTTGGTGT : 284	
	reverse		ACACCWACRTTACGWGA	TCTCGTAATGTAGGTGT : 0	99.7%
				TCTCGTAATGTTGGTGT : 0	
				TCTCGTAACGTAGGTGT : 0	
				TCTCGTAACGTTGGTGT : 3	
424-461(430-467) ¹	439-461(445-467)	forward	YTAGAATTAGTWGAAATGGAAGT	TTAGAATTAGTAGAAATGGAAGT : 166	100.0%
				TTAGAATTAGTTGAAATGGAAGT : 146	
	reverse		ACTTCCATTTWACTAATTCTAR	CTAGAATTAGTAGAAATGGAAGT : 0	100.0%

				CTAGAATTAGTTGAAATGGAAGT : 3					
463-482(469-488) ¹	463-481(469-487)	forward	CGTGACTTATTAWSYGAAT	CGTGACTTATTAAGTGAAT : 1	93.7%				
				CGTGACTTATTAAGCGAAT : 150					
				CGTGACTTATTAAGTGAAT : 3					
				CGTGACTTATTAACCGAAT : 0					
		reverse	ATTCRSWTAATAAGTCACG	CGTGACTTATTATGTGAAT : 0	93.7%				
				CGTGACTTATTATGCCAAT : 0					
				CGTGACTTATTATCTGAAT : 141					
				CGTGACTTATTATCCGAAT : 0					
601-617(607-623) ²	601-617(607-623)	forward	CCAACWCCAGAMCGTGA	CCAACACCAGAACGTGA : 58	100.0%				
				CCAACACCAGACCGTGA : 1					
		reverse	TCACGKTCTGGWGTGG	CCAACTCCAGAACGTGA : 227	100.0%				
				CCAACTCCAGACCGTGA : 29					
				628-662(634-668) ²	634-653(640-659)	forward	ATGATGCCWGTWGARGACGT	ATGATGCCAGTAGAGGACGT : 7	99.4%
								ATGATGCCAGTAGAAGACGT : 0	
		ATGATGCCAGTTGAGGACGT : 259							
		ATGATGCCAGTTGAAGACGT : 3							
reverse	ACGTCYTCWACWGGCATCAT	ATGATGCCTGTAGAGGACGT : 0	99.4%						
		ATGATGCCTGTAGAAGACGT : 0							
		ATGATGCCTGTTGAGGACGT : 44							
		ATGATGCCTGTTGAAGACGT : 0							
691-710(697-716) ¹	692-709(698-715)	forward	GTGTTGAACGTGGDCAAR	GTGTTGAACGTGGGCAAG : 0	100.0%				
				GTGTTGAACGTGGGCAAA : 1					
				GTGTTGAACGTGGACAAG : 1					
		reverse	YTTGHCCACGTTCAACAC	GTGTTGAACGTGGACAAA : 5	100.0%				
				GTGTTGAACGTGGTCAAG : 0					
				GTGTTGAACGTGGTCAAA : 308					
772-791(781-800) ²	772-789(781-798)	forward	GGTGTWGARATGTTCCGT	GGTGTAGAGATGTTCCGT : 0	100.0%				
		reverse	ACGGAACATYTCWACACC	GGTGTAGAAATGTTCCGT : 238	100.0%				

				GGTGTTGAGATGTTCCGT : 4		
				GGTGTTGAAATGTTCCGT : 73		
826-842(835-851) ¹	826-842(835-851)	forward	GGTGCDYTATTACGWGG	GGTGCGTTATTACGAGG : 0	98.7%	
				GGTGCGTTATTACGTGG : 40		
				GGTGCGCTATTACGAGG : 0		
				GGTGCGCTATTACGTGG : 0		
				GGTGCATTATTACGAGG : 1		
	reverse	CCWCGTAATARHGCACC			GGTGCATTATTACGTGG : 151	
					GGTGCACTATTACGAGG : 0	98.7%
					GGTGCACTATTACGTGG : 0	
					GGTGCTTTATTACGAGG : 0	
					GGTGCTTTATTACGTGG : 122	
				GGTGCTCTATTACGAGG : 0		
				GGTGCTCTATTACGTGG : 1		
955-972(964-981) ²	955-972(964-981)	forward	CGTCAYACDCCWTTCTTC	CGTCATACGCCATTCTTC : 19	99.4%	
				CGTCATACGCCTTTCTTC : 0		
				CGTCATACACCATTCTTC : 63		
				CGTCATACACCTTTCTTC : 0		
				CGTCATACTCCATTCTTC : 100		
	reverse	GAAGAAWGGHGTGTGACG			CGTCATACTCCTTTCTTC : 0	
					CGTCACACGCCATTCTTC : 7	99.4%
					CGTCACACGCCTTTCTTC : 0	
					CGTCACACACCATTCTTC : 2	
					CGTCACACACCTTTCTTC : 0	
				CGTCACACTCCATTCTTC : 120		
				CGTCACACTCCTTTCTTC : 2		
991-1016(1000-1025) ²	997-1016(1006-1025)	forward	TTYCGTACTACWGAYGTAAC	TTTCGTACTACAGATGTAAC : 0	99.7%	
				TTTCGTACTACAGACGTAAC : 0		
	reverse	GTTACRTCWGTAGTACGRAA			TTTCGTACTACTGATGTAAC : 0	99.7%
					TTTCGTACTACTGACGTAAC : 1	

				TTCCGTACTACAGATGTAAC : 0	
				TTCCGTACTACAGACGTAAC : 12	
				TTCCGTACTACTGATGTAAC : 16	
				TTCCGTACTACTGACGTAAC : 285	
1030-1052(1039-1061) ²	1033-1052(1042-1061)	forward	CCAGAAGGHAYWGAAATGGT	CCAGAAGGCACTGAAATGGT : 2	99.0%
				CCAGAAGGTACTGAAATGGT : 252	
				CCAGAAGGAACTGAAATGGT : 2	
				CCAGAAGGCATTGAAATGGT : 0	
				CCAGAAGGTATTGAAATGGT : 4	
	reverse			CCAGAAGGAATTGAAATGGT : 0	
			ACCATTTCWRTDCCTTCTGG	CCAGAAGGCACAGAAATGGT : 0	99.0%
				CCAGAAGGTACAGAAATGGT : 52	
				CCAGAAGGAACAGAAATGGT : 0	
				CCAGAAGGCATAGAAATGGT : 0	
			CCAGAAGGTATAGAAATGGT : 0		
			CCAGAAGGAATAGAAATGGT : 0		
1138-1166(1147-1175) ²	1142-1162(1151-1171)	forward	GACGTACWGTWGGWTCAGGCG	GACGTACTGTTGGTTCAGGCG : 0	96.2%
				GACGTACAGTTGGTTCAGGCG : 0	
				GACGTACTGTAGGTTTCAGGCG : 7	
				GACGTACAGTAGGTTTCAGGCG : 0	
				GACGTACTGTTGGATCAGGCG : 96	96.2%
	reverse		CGCCTGAWCCWACWGTACGTC	GACGTACAGTTGGATCAGGCG : 15	
				GACGTACTGTAGGATCAGGCG : 185	
				GACGTACAGTAGGATCAGGCG : 0	

Supplementary Table S4. Degenerate and haplotype primers for *tuf* genes of Staphylococci. Notes: ^aThe numbering is based on the the *tuf* gene of *Staphylococcus aureus* (NC_007795.1). ^bThe numbering is based on the alignment. ¹Region identified by PMPrimer and in a previous study. ²Region only identified by PMPrimer.

Amplicon	Forward primer	Reverse primer	Length	Coverage	Taxon-specificity		
					Genus	Species	Subspecies
300-bp fragment length							
334-353(340-359) to 601-617(607-623)	CCAATGCCWCAAACKCGTGA	TCACGKTCTGGWGTGG	284	100%	100%	98.11%	98.11%
58-89(58-89) to 334-353(340-359)	CACGTTGACCAYGGTAAACD	TCACGMGTTTGWGGCATTGG	296	100%	100%	90.57%	90.57%
600-bp fragment length							
58-89(58-89) to 424-461(430-467)	CACGTTGACCAYGGTAAACD	TCACGWACTTCCATTTCWACT	404	100%	100%	96.23%	96.23%
160-182(163-185) to 601-617(607-623)	GAAGAAAAAGARCGTGGT	TCACGKTCTGGWGTGG	458	100%	100%	98.11%	98.11%
334-353(340-359) to 772-791(781-800)	CCAATGCCWCAAACKCGTGA	ACGGAACATYTCWACACC	458	100%	100%	98.11%	98.11%
58-89(58-89) to 601-617(607-623)	CACGTTGACCAYGGTAAACD	TCACGKTCTGGWGTGG	560	100%	100%	98.11%	98.11%
>600-bp fragment length							
160-182(163-185) to 772-791(781-800)	GAAGAAAAAGARCGTGGT	ACGGAACATYTCWACACC	632	100%	100%	98.11%	98.11%
58-89(58-89) to 772-791(781-800)	CACGTTGACCAYGGTAAACD	ACGGAACATYTCWACACC	734	100%	100%	98.11%	98.11%

Supplementary Table S5. Amplicons suitable for different fragment lengths in *tuf* genes of Staphylococci.