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## Identification of clinically relevant viridans group streptococci by phenotypic and genotypic analysis

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1 **Title:** Identification of clinically relevant viridans group streptococci by phenotypic and  
2 genotypic analysis.

3

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13

14 **Running title:** Viridans groups streptococci species identification.

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17

## ABSTRACT

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**Purpose:** Two phenotypic and three molecular methods were assessed for their ability to identify viridans group streptococci (VGS) to the species level.

**Methods:** A panel of 23 clinical isolates, comprising strains isolated from infective endocarditis, blood cultures, pleural and peritoneal fluid, and 19 type/reference strains were analyzed. Identification was performed using two conventional phenotypic methods, API<sup>®</sup> rapid ID 32 Strep and VITEK<sup>®</sup> 2 system; and genotypic analysis of the nucleotide sequence of the housekeeping gene *sodA*, restriction patterns generated by restriction fragment length polymorphism (RFLP) of the 16S rRNA gene and multilocus sequence analysis (MLSA) of 7 housekeeping genes.

**Results:** The API<sup>®</sup> rapid ID 32 Strep accurately speciated 79% of the strains assessed, while the VITEK<sup>®</sup> 2 generated a successful identification for 55%, presenting limitations particularly with regard to species belonging to the mitis group. RFLP of the 16S rRNA gene correctly speciated 24% of the strains, having failed to allocate a species for 36% of the isolates examined. In contrast, sequence analysis of the *sodA* gene provided a correct identification for 95% of the strains assessed, while identification using the MLSA technique was unsuccessful due to practical limitations.

**Conclusions:** The results generated herein indicate that no single methodology can be used to provide an accurate identification to the species level of all VGS although, nucleotide sequence analysis of the *sodA* gene proved to be useful in providing reliable speciation.

**Keywords:** *Streptococcus*, MLSA, API, VITEK, RFLP, *sodA*

## INTRODUCTION

43

44

45         Viridans group streptococci (VGS) encompass four phylogenetic clusters: the  
46         mitis, mutans, salivarius and anginosus groups, which are part of the natural human  
47         flora, being isolated mainly from the oral cavity, gastrointestinal and genitourinary tract  
48         [1]. The pathogenic potential of the species comprising the VGS is variable, with the  
49         natural competence of these organisms giving rise to a genetic exchange with  
50         *Streptococcus pneumoniae*, a species with recognized virulence traits [2]. It is believed  
51         that this interspecies gene transfer is responsible for the emerging tolerance or resistance  
52         to  $\beta$ -lactam antimicrobial agents amongst VGS, particularly *S. mitis* [3]. VGS can  
53         behave as opportunistic pathogens causing diseases that range from mild infections to  
54         life threatening conditions. *S. mutans* for instance, has a strong association with the  
55         development of dental caries [4], while *S. sanguinis*, *S. oralis* and *S. mitis* are  
56         responsible for 40-60% of native-valve infective endocarditis [5, 6]. Members of the  
57         anginosus group are causative agents of pyogenic abscesses in a number of body sites  
58         [7]. Other opportunistic infections caused by VGS include septicaemia in neutropenic  
59         patients [8], and occasionally cases of meningitis have been reported [9].

60         Currently, no recognised “gold standard” exists that is able to identify many of  
61         the species satisfactorily, which has led to an inadequate reporting of streptococcal  
62         infections, and potentially has serious implications for diagnosis and treatment [11-13].  
63         Accurate identification of VGS to the species level is therefore important to characterise  
64         the pathogenic potential of individual species and monitor trends in antimicrobial  
65         susceptibility and emerging infections, such as septicaemia in immunocompromised  
66         individuals [14].

67           Although presenting recognized limitations, phenotypic tests such as the API®  
68 rapid ID 32 Strep and the VITEK® 2 system remain the techniques most frequently used  
69 in clinical laboratories, achieving a speciation on the basis of the organisms biochemical  
70 properties [15, 16]. The demand for simple, effective and reliable methods for species  
71 identification both in the clinical and research laboratories has resulted in an increasing  
72 utilization and development of molecular based techniques [17]. Nucleotide analysis of  
73 the *sodA* gene, encoding a manganese-dependent superoxide dismutase enzyme (Mn-  
74 SOD), has been proposed as an accurate identification tool [18, 19]. Other genotypic  
75 tests previously described include analysis of restriction patterns of the total  
76 chromosome (pulsed-field gel electrophoresis) [20, 21] or within specific genes [22].  
77 RFLP of ribosomal RNA encoding genes has been used as an identification method for  
78 several bacterial and yeast species including *Leuconostoc*, *Malassezia* and  
79 *Streptococcus* [23-25]. More recently, a MLSA technique has been developed for  
80 identification of VGS based on the nucleotide sequence analysis of seven housekeeping  
81 genes [26]. Using an electronic database comprising the nucleotide sequences acquired  
82 from 420 VGS strains, this methodology reportedly allows unknown strains to be  
83 assigned to viridans species via the internet [26].

84           Here we report the applicability of three molecular based techniques, as well as,  
85 two commercially available methods, widely used in routine clinical laboratories, in the  
86 identification of VGS to the species level.

87

88

## MATERIALS AND METHODS

89

90 **Bacterial strains and culture conditions.** A collection of 23 clinical isolates,  
91 comprising 14 streptococci recovered from patients diagnosed as Duke definite for

92 infective endocarditis, 7 strains isolated from blood cultures, 1 pleural fluid isolate and  
93 1 peritoneal fluid (Glasgow Caledonian University culture collection, Glasgow Dental  
94 School, Barts and The London NHS Trust) were examined (Table 1). For assessment of  
95 the accuracy of the identification provided by each method 10 type and 9 reference  
96 strains were included (Table 1). The strains designated ATCC and NCTC were obtained  
97 from the American Type Culture Collection (Manassas, USA) and National Collection  
98 of Type Cultures (London, UK), respectively.

99 All strains were grown at 37°C in an atmosphere enriched with 5% CO<sub>2</sub> on  
100 Columbia base agar (Oxoid, Hampshire, UK) supplemented with 5% (v/v) defibrinated  
101 horse blood (Oxoid) and cultivated in brain heart infusion broth (BHI) (Oxoid).

102

103 **Phenotypic identification.** The API<sup>®</sup> rapid ID 32 Strep and VITEK<sup>®</sup> 2 system kits  
104 were purchased from bioMérieux, UK. Phenotypic analysis by both methods was  
105 performed according to the manufacturer's instructions. The API results were obtained  
106 as a numerical profile after visual reading and the identification was achieved by  
107 manually entering the code into the identification software (apiweb – API<sup>®</sup> rapid ID 32  
108 Strep V3.0, bioMérieux, France). Identification through the VITEK<sup>®</sup> 2 system was  
109 provided by the associated computer software (V1.02), through comparison of the  
110 biochemical profile of the strains tested against the biochemical profiles of the strains  
111 present in the software database.

112

113 **Genotypic identification**

114 **DNA manipulations.** Streptococcal DNA was extracted using the DNeasy<sup>®</sup> Tissue kit  
115 (Qiagen, West Sussex, UK) and quantified using a ND-1000 spectrophotometer  
116 (NanoDrop Technologies Inc, Wilmington, USA).

117

118 **Polymerase chain reaction (PCR) analysis.** DNA amplifications were performed  
119 using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City,  
120 USA). All oligonucleotide primer sequences are depicted in table 2. The PCR reactions  
121 consisted of 1× NH<sub>4</sub> amplification buffer, 1.5mM MgCl<sub>2</sub>, 1mM dNTP mix, 1μM of  
122 each primer, 10ng of genomic DNA and 0.025U/μl *Taq* polymerase (Bioline, London,  
123 UK) in a final volume of 50μl.

124 Amplification of the *sodA* gene was performed on all strains using an annealing  
125 temperature of 51.4°C.

126 *S. oralis* NCTC 11427<sup>T</sup> and *S. oralis* 12601 were selected to assess the MLSA  
127 technique for VGS species identification. Genes *map*, *pfl* and *tuf* were amplified with  
128 55°C annealing temperature, while primers for genes *ppaC*, *pyK* and *rpoB* were  
129 annealed at 50°C, as previously published [26]. PCR amplifications were performed  
130 with 30 cycles of denaturation (94°C, 30 seconds), annealing (X°C, 30 seconds) and  
131 extension (72°C, 30 seconds), followed by a final extension step (72°C, 5 minutes) [26].  
132 All generated amplicons were resolved by electrophoresis using a 1.0% (w/v)  
133 SeaPlaque<sup>®</sup> agarose gel (Cambrex, Bio Science Rockland, Inc., USA), excised from the  
134 gel and purified using the QIAquick<sup>®</sup> gel extraction kit (Qiagen).

135 The amplification of the 16S rRNA gene was performed with 35 cycles of  
136 denaturation (94°C for 1 minute), annealing (60°C for 1 minute) and extension (72°C for  
137 1.5 minutes), followed by a final extension step at 72°C for 10 minutes [22]. The  
138 resulting products were resolved by electrophoresis using a 1% (w/v) agarose gel  
139 (Invitrogen, Paisley, UK), after which the amplicons were recovered and purified from  
140 the PCR reaction mixture using QIAquick<sup>®</sup> PCR Purification Kit (Qiagen).

141

142 **Sequencing analysis.** Nucleotide sequence analysis of the *sodA* gene was performed  
143 based on the nucleotide sequence analysis of a single DNA strand of all VGS examined.  
144 Evaluation of MLSA was performed by sequencing both DNA strands of the amplified  
145 fragments of the *sodA*, *map*, *pfl*, *tuf*, *ppaC*, *pyK* and *rpoB* genes of *S. oralis* NCTC  
146 11427<sup>T</sup> and *S. oralis* 12601 (infective endocarditis isolate). Sequencing was performed  
147 using 15ng of each amplicon, 3.5µM primer and 50°C annealing temperature or 64µM  
148 primer and 45°C annealing temperature by The Sequencing Service (College of Life  
149 Sciences, University of Dundee, UK) using Applied Biosystems Big-Dye Ver 3.1  
150 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.  
151 Species identification was achieved through nucleotide comparison against published  
152 sequences available in the microbial genomes database using the Basic Local Alignment  
153 Search Tool (BLAST) available online (<http://www.ncbi.nlm.nih.gov/BLAST/>).

154

155 **RFLP of the 16S rRNA gene.** RFLP of the amplified 16S rRNA genes was carried out  
156 as described in previous studies [22], with modifications regarding the restriction  
157 enzyme digestion conditions. Double restriction enzyme digestions were performed  
158 with *Hae*III and *Hpa*II (New England Biolabs, Hitchin, UK) according to the  
159 manufacturer's instructions and the resulting restriction fragments were resolved by  
160 electrophoresis using a 2% (w/v) agarose gel (Invitrogen). A library of the restriction  
161 patterns was produced for the reference strains and identification of the clinical isolates  
162 was achieved using Dice similarity coefficient. A dendrogram was then constructed  
163 showing the clusters generated by the Unweighted pair group method with arithmetic  
164 mean (UPGMA, BioNumerics software, Applied Maths NV, Belgium).

165

166

## RESULTS

167

168 **Species identification through API<sup>®</sup> rapid ID 32 Strep system.** The API<sup>®</sup> rapid ID 32  
169 Strep system provided an accurate identification to the species level for 33 strains  
170 (79%), without the need to perform additional tests. Eight strains (19%) were given a  
171 correct identification to the group level, these included 7 strains from the mitis group,  
172 which presented a number of unusual reactions, and type strain *S. sobrinus* ATCC  
173 33478<sup>T</sup> was given an inconclusive identification, stating *sobrinus/S. downei*. Since the  
174 biochemical range of this method is unable to differentiate between these two species  
175 further tests were recommended.

176

177 **Species identification through the Vitek<sup>®</sup> 2 system.** The results generated by the  
178 Vitek<sup>®</sup> 2 accurately speciated 23 strains (55%) while, 9 isolates of the mitis group were  
179 allocated to the correct group (21%). The result of all *S. oralis* and *S. mitis* was  
180 presented as *S. mitis/S. oralis* with a high level of confidence, since this methodology  
181 does not differentiate between these species. Assignment of *S. sanguinis* reference and  
182 type strains to *S. mitis/S. oralis* occurred in two instances (*S. sanguinis* NCTC 7863 and  
183 *S. sanguinis* ATCC10556<sup>T</sup>), possibly due to a negative reaction in both arginine tests,  
184 which is characteristic of *S. mitis* and *S. oralis* strains [27]. *S. parasanguinis* ATCC  
185 15912<sup>T</sup> was speciated as *S. gordonii*, presenting a single obvious atypical reaction  
186 (methyl- $\beta$ -D-glucopyranoside positive). No identification was generated for 8 strains  
187 (19%) represented in the software database, including isolates from the mitis, salivarius  
188 and anginosus group, with 2 (5%) isolates having been misidentified.

189

190 **Species identification through sequence analysis of the *sodA* gene.** The PCR  
191 amplification of an internal fragment of the *sodA* gene was successful for all strains,

192 resulting in a single amplicon with the expected size of approximately 440bp. Sequence  
193 analysis of the *sodA* gene resulted in the correct speciation of 40 strains (95%), with *S.*  
194 *sobrinus* ATCC 33478<sup>T</sup> having been incorrectly assigned to *S. thermophilus* with 89%  
195 nucleotide similarity and failure to generate sequence data occurred for one isolate,  
196 despite repeated attempts.

197

198 **Species identification through RFLP of the 16S rRNA gene.** Amplification of the  
199 16S rRNA gene of all VGS strains generated a single amplicon with the expected size  
200 of approximately 1500bp. With the similarity threshold set at 90%, analysis of the  
201 RFLP revealed 11 clusters incorporating 27 strains (64%), 24% of which were correctly  
202 speciated (10/42) and 21% of isolates (9/42) were allocated to the correct VGS group  
203 while 19% (8/42) were misidentified (fig. 1). The remaining 15 strains (36%) examined,  
204 including species of the mutans, mitis and anginosus group, were excluded from the  
205 generated clusters despite the presence of other members of the same species and/or  
206 VGS group (fig. 1).

207

208 **Species identification through MLSA.** The PCR amplification of the 7 housekeeping  
209 genes was successful for both strains examined, generating single amplicons of the  
210 expected sizes. Using the forward primers the nucleotide sequence generated for 3  
211 genes (3/7) of the type strain *S. oralis* NCTC 11427<sup>T</sup> and 2 genes (2/7) of the clinical  
212 isolate *S. oralis* 12601 did not reach the length required by the MLSA software and both  
213 strains failed to yield sequence results using the *ppaC*-F primer. When analyzing the  
214 complementary DNA strand using the reverse primers, the *ppaC* gene of *S. oralis*  
215 NCTC 11427<sup>T</sup> and *S. oralis* 12601 remained below the number of base pairs necessary  
216 to proceed with the analysis. In addition, no nucleotide sequence was obtained for the

217 *pfl* and *pyk* genes of the type strain and *map*, *pyk*, *rpoB* and *sodA* genes of the clinical  
218 isolate. When modifications were introduced to the published protocol, an improvement  
219 was seen for 2 of the genes of interest however, this was not beneficial overall as it  
220 caused no improvement on either strand of the *ppaC* gene and decreased the number of  
221 base pairs acquired for the *sodA* gene. Since the required read length of the 7  
222 housekeeping genes was not obtained for either of the strains examined, no analysis  
223 could be performed.

224

225 **Identification concordance between the methods assessed.** Comparison of the  
226 identification allocated to each strain by the techniques assessed, revealed a species  
227 level agreement between the two phenotypic methods for 18 strains (43%). In contrast,  
228 the results generated by the genotypic methods were concordant for 10 isolates (24%).  
229 The species allocated by the VITEK<sup>®</sup> 2 and sequence analysis of the *sodA* gene were  
230 similar for 21 strains (50%) however, when the identifications produced by the VITEK<sup>®</sup>  
231 2 were compared with those of the RFLP of the 16S rRNA gene, only 6 strains (14%)  
232 were allocated to the same species. The API<sup>®</sup> rapid ID 32 Strep and the RFLP of the  
233 16S rRNA gene were concordant for 8 strains (19%) while, the highest levels of  
234 agreement were obtained when comparing the API<sup>®</sup> rapid ID 32 Strep and sequence  
235 analysis of the *sodA* gene, with 32 isolates (76%) being similarly speciated.

236

237

## DISCUSSION

238

239 The results presented in this study reflect the complex taxonomy of the VGS  
240 which has lead to difficulties in defining a “gold standard” technique that would provide  
241 accurate identification to the species level. The API<sup>®</sup> rapid ID 32 Strep system remains

242 one of the most frequently used methodologies in the clinical laboratory for streptococci  
243 identification to the species level [15]. Previous reports demonstrated the low sensitivity  
244 of this technique for speciation of members of the anginosus and mitis group [6, 28-30],  
245 which is in agreement with the results found in the present study as all the discrepant  
246 speciations by this method occurred with members of the mitis group. Further  
247 limitations of this system, and others of this nature, are associated with the subjectivity  
248 inherent with the interpretation of the colorimetric reactions which can interfere with the  
249 identification obtained. In addition, the availability of an up-to-date database  
250 incorporating the taxonomic re-evaluations of this diverse group of bacteria is crucial in  
251 acquiring accurate results [31]. Despite being a simple and non time consuming method,  
252 the API<sup>®</sup> rapid ID 32 Strep only provides reliable identification to the group level.

253         The VITEK<sup>®</sup> 2 system is an automated machine that is capable of identifying  
254 numerous bacteria, including VGS, based on kinetic analysis of the fluorescence,  
255 turbidity and colorimetric readings of 43 tests measuring enzymatic activities, carbon  
256 source and antibiotic resistance [32]. Difficulties in the speciation of VGS members  
257 using the VITEK<sup>®</sup> 2 have been reported [17, 33] and the results generated by this  
258 technique in the present assessment reinforce these findings in that only 55% of the  
259 strains examined were accurately speciated. In addition, the supplementary tests  
260 required for two of the most clinically relevant VGS species, *S. mitis* and *S. oralis*,  
261 proves to be a major limitation also extending the time required to obtain identification.  
262 In agreement with previous reports, the results provided by the VITEK<sup>®</sup> 2 suggest this  
263 methodology may be better suited for identification to the species level of organisms  
264 other than VGS, such as  $\beta$ -haemolytic streptococci [14].

265         The sequence analysis of an internal fragment of the *sodA* gene resulted in an  
266 accurate speciation of 95% of all strains assessed, revealing the high reliability of this

267 method for VGS speciation. Traditionally, the sequencing of this housekeeping gene has  
268 been carried out by cloning the amplified fragment into a vector prior to sequencing [18,  
269 19, 34]. In this study, elimination of this step made this technique less time-consuming  
270 and more cost-effective, without significant consequence to its efficiency. GenBank  
271 includes a comprehensive and freely available microbial genomic database which,  
272 despite not guaranteeing the accuracy and quality of the data deposited, is an up-to-date  
273 database which incorporates the *sodA* nucleotide sequences of VGS reference/type  
274 strains and clinical isolates [18, 19, 34]. These factors have contributed to the high  
275 levels of reliable identification generated by this methodology.

276         The sequence analysis of the 16S rRNA gene as been widely applied for  
277 speciation of streptococci having greatly contributed to a better understanding of the  
278 classification of such a complex bacterial group [35]. The high level of nucleotide  
279 similarities between closely related species, particularly *S. oralis*, *S. mitis* and *S.*  
280 *pneumoniae* soon became evident, questioning the discriminatory capability of this  
281 methodology [18]. Restriction enzyme digestion of the 16S rRNA gene has been  
282 suggested as an alternative method to gene sequence analysis for streptococcal  
283 speciation, potentially offering more precise results [22]. The use of a single restriction  
284 enzyme (*HpaII*) for discrimination of mutans group species was previously described  
285 [22] however, in that study, not all strains presented distinctive patterns requiring a  
286 second restriction endonuclease (*HaeIII*) to allow differentiation. Given the number and  
287 diversity of strains being assessed in the present evaluation, a simultaneous double  
288 restriction reaction was applied. Traditionally, species identification was performed by  
289 visual comparison of the restriction patterns resolved by agarose gel electrophoresis  
290 however, computer analysis is less subjective and a more practical approach for high  
291 throughput of strains [25]. Analysis of the constructed dendrogram demonstrated the

292 limited capacity of RFLP of the 16S rRNA gene to speciate VGS (24%) and therefore,  
293 low concordance with the other methodologies evaluated. This unsatisfactory results  
294 may have been influenced by the low resolution, or inability to visually detect, small  
295 restriction fragments. Other limitations of this technique have also been reported. These  
296 included the inability to differentiate between *S. parasanguinis* and *S. mitis*, despite the  
297 use of three separate restriction endonuclease combinations, due to the sequence  
298 homology of the 16S rRNA gene shared amongst strains within a VGS group [25].  
299 Based on the findings generated using the panel of strains assessed, RFLP of the 16S  
300 rRNA genes was considered unsuitable for streptococcal identification to the species  
301 level.

302         Unlike the MLST technique successfully applied in the genetic differentiation  
303 between serotypes of *S. aureus* and *S. pneumoniae*, MLSA has recently been described  
304 for VGS species identification based on the sequence data acquired for 7 housekeeping  
305 genes [26]. The results obtained in the present study suggest that MLSA of VGS  
306 species, at present is not a technique sufficiently robust to allow inter-lab variability and  
307 cannot be used routinely.

308         In conclusion, no individual method assessed was able to unequivocally assign  
309 the correct species to all VGS strains. The difficulties in achieving accurate speciation  
310 were particularly noticeable in members of the mitis group, which correlates with the  
311 genetic homology shared among the species included in this VGS group. Comparison of  
312 the data provided indicated that nucleotide sequence analysis of the *sodA* gene is a  
313 useful and reliable technique for VGS species identification.

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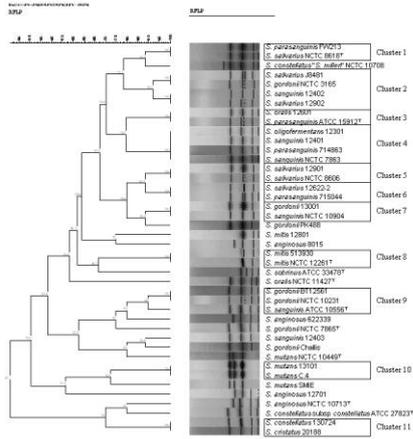
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434 **Table 1** – VGS species assessed in this study and reference strains included for  
 435 evaluation of the methods accuracy.

<b>Phylogenetic group</b>	<b>Species</b>	<b>Number of strains</b>	<b>Reference strain included</b>
<b>Mutans</b>	<i>S. mutans</i>	4	<i>S. mutans</i> NCTC 10449 <sup>T</sup>
	<i>S. sobrinus</i>	1	<i>S. sobrinus</i> ATCC 33478 <sup>T</sup>
<b>Salivarius</b>	<i>S. salivarius</i>	6	<i>S. salivarius</i> NCTC 8618 <sup>T</sup>
<b>Anginosus</b>	<i>S. anginosus</i>	4	<i>S. anginosus</i> NCTC 10713 <sup>T</sup>
	<i>S. constellatus</i>	3	<i>S. constellatus</i> subsp <i>constellatus</i> ATCC 27823 <sup>T</sup>
<b>Mitis</b>	<i>S. mitis</i>	3	<i>S. mitis</i> NCTC 12261 <sup>T</sup>
	<i>S. sanguinis</i>	6	<i>S. sanguinis</i> ATCC 10556 <sup>T</sup>
	<i>S. parasanguinis</i>	4	<i>S. parasanguinis</i> ATCC 15912 <sup>T</sup>
	<i>S. gordonii</i>	7	<i>S. gordonii</i> NCTC 7865 <sup>T</sup>
	<i>S. oralis</i>	2	<i>S. oralis</i> NCTC 11427 <sup>T</sup>
	<i>S. cristatus</i>	1	None included
	<i>S. oligofermentans</i>	1	None available

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438 **Table 2** – PCR primers to amplify *sodA*, 16S rRNA, *map*, *pfl*, *tuf*, *ppaC*, *pyK* and *rpoB*  
 439 genes.

Primers	Sequence (5' to 3')	Gene amplified	Expected	Origin
			product size (bp)	
sodA-F	<u>T</u> <u>R</u> <u>C</u> <u>A</u> <u>Y</u> <u>C</u> <u>A</u> <u>T</u> <u>G</u> <u>A</u> <u>Y</u> <u>A</u> <u>A</u> <u>R</u> <u>C</u> <u>A</u> <u>C</u> <u>C</u> <u>A</u> <u>T</u> *	<i>sodA</i>	436	[18]
sodA-R	<u>A</u> <u>R</u> <u>R</u> <u>T</u> <u>A</u> <u>R</u> <u>T</u> <u>A</u> <u>M</u> <u>G</u> <u>C</u> <u>R</u> <u>T</u> <u>G</u> <u>Y</u> <u>T</u> <u>C</u> <u>C</u> <u>C</u> <u>A</u> <u>R</u> <u>A</u> <u>C</u> <u>R</u> <u>T</u> <u>C</u> *			
8UA	AGAGTTTGATCCTGGCTCAG	16S	1521	[22]
1492R	TACGGGTACCTTGTTACGACTT	rRNA		
map-F	<u>G</u> <u>C</u> <u>W</u> <u>G</u> <u>A</u> <u>C</u> <u>T</u> <u>C</u> <u>W</u> <u>T</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>G</u> <u>G</u> <u>C</u> <u>W</u> <u>T</u> <u>A</u> <u>T</u> <u>G</u> <u>C</u>	<i>map</i>	459	
map-R	<u>T</u> <u>T</u> <u>A</u> <u>R</u> <u>T</u> <u>A</u> <u>A</u> <u>G</u> <u>T</u> <u>T</u> <u>C</u> <u>Y</u> <u>T</u> <u>T</u> <u>C</u> <u>T</u> <u>T</u> <u>C</u> <u>D</u> <u>C</u> <u>C</u> <u>T</u> <u>T</u> <u>G</u>			
pfl-F	AACGTTGCTTACTCTAAACAAACTGG	<i>pfl</i>	458	
pfl-R	<u>A</u> <u>C</u> <u>T</u> <u>T</u> <u>C</u> <u>R</u> <u>T</u> <u>G</u> <u>G</u> <u>A</u> <u>A</u> <u>G</u> <u>A</u> <u>C</u> <u>A</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>W</u> <u>G</u> <u>T</u> <u>C</u> *			
tuf-F	GTTGAAATGGAAATCCGTGACC	<i>tuf</i>	531	[26]
tuf-R	GTTGAAGAATGGAGTGTGACG			
ppaC-F	<u>G</u> <u>A</u> <u>C</u> <u>C</u> <u>A</u> <u>Y</u> <u>A</u> <u>A</u> <u>T</u> <u>G</u> <u>A</u> <u>A</u> <u>T</u> <u>T</u> <u>Y</u> <u>C</u> <u>A</u> <u>R</u> <u>C</u> <u>A</u> <u>A</u> <u>T</u> <u>C</u> *	<i>ppaC</i>	683	
ppaC-R	<u>T</u> <u>G</u> <u>A</u> <u>G</u> <u>G</u> <u>N</u> <u>A</u> <u>C</u> <u>M</u> <u>A</u> <u>C</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>S</u> <u>T</u> <u>T</u> <u>A</u> <u>C</u> <u>G</u> *			
pyk-F	<u>G</u> <u>C</u> <u>G</u> <u>G</u> <u>T</u> <u>W</u> <u>G</u> <u>A</u> <u>A</u> <u>W</u> <u>T</u> <u>C</u> <u>C</u> <u>G</u> <u>T</u> <u>G</u> <u>G</u> <u>T</u> <u>G</u> *	<i>pyK</i>	557	
pyk-R	<u>G</u> <u>C</u> <u>A</u> <u>A</u> <u>G</u> <u>W</u> <u>G</u> <u>C</u> <u>T</u> <u>G</u> <u>G</u> <u>G</u> <u>A</u> <u>A</u> <u>G</u> <u>G</u> <u>A</u> <u>A</u> <u>T</u> *			
rpoB-F	<u>A</u> <u>A</u> <u>R</u> <u>Y</u> <u>T</u> <u>I</u> <u>G</u> <u>G</u> <u>M</u> <u>C</u> <u>C</u> <u>T</u> <u>G</u> <u>A</u> <u>A</u> <u>G</u> <u>A</u> <u>A</u> <u>A</u> <u>T</u> *	<i>rpoB</i>	740	
rpoB-R	<u>T</u> <u>G</u> <u>I</u> <u>A</u> <u>R</u> <u>T</u> <u>T</u> <u>T</u> <u>R</u> <u>T</u> <u>C</u> <u>A</u> <u>T</u> <u>C</u> <u>A</u> <u>A</u> <u>C</u> <u>C</u> <u>A</u> <u>T</u> <u>G</u> <u>T</u> <u>G</u> *			

440 \* The underlined degenerate base sites correspond to positions within the gene that may  
 441 differ between species.  
 442  
 443