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Title

Species identification of staphylococci by amplification and sequencing of the *tuf* gene compared to the *gap* gene and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Running title: *tuf* sequence-based identification of Staphylococci

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KEY-WORDS: *Staphylococcus* identification, *tuf* sequencing, *gap* sequencing, phylogeny, MALDI-TOF-MS

Abstract:

Staphylococcal species notably, coagulase-negative staphylococci are frequently misidentified using phenotypic methods.

The partial nucleotide sequences of the *tuf* and *gap* genes were determined in 47 reference strains to assess their suitability, practicability and discriminatory power as target molecules for staphylococcal identification. The partial *tuf* gene sequence was selected and further assessed with a collection of 186 strains including 35 species and sub-species. Then, to evaluate the efficacy of this genotyping method versus the technology of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS), the 186 strains were identified using MALDI-TOF-MS (Axima® Shimadzu) coupled to the SARAMIS® database (AnagnosTec). The French National Reference Center for staphylococci identification method was used as a reference.

One hundred eighty-for strains (98.9%) were correctly identified by *tuf* gene sequencing. Only one strain was misidentified, and one was unidentified. MALDI-TOF-MS identified properly 138 isolates (74.2%). Four strains were misidentified, 39 were unidentified, 5 were identified at the group (*hominis*/*warneri*) level and 1 strain was identified at the genus level.

These results confirm the value of MALDI-TOF-MS identification for common species in clinical laboratory practice and the value of the partial *tuf*-gene sequence for the identification of all staphylococcal species as required in a reference laboratory.

Introduction

According to current knowledge, including the newly described species published in 2009-2010, the *Staphylococcus* genus groups together 45 species and 21 subspecies [1, 17, 35, 40, 48]. Staphylococcal species are widely distributed in various environments: the skin and mucous membranes of humans and animals as well as soil, sand, and water. Some staphylococcal species are used as starter cultures for sausage manufacturing in the food industry (*Staphylococcus xylosus* and *S. carnosus*) [7], whereas others are mainly associated with animal diseases such as *S. pseudintermedius* in dogs. Of the 45 species and 21 subspecies, only half have been cultured from human specimens. *S. aureus* is the most clinically relevant staphylococcal species, but coagulase-negative staphylococci (CoNS) are increasingly recognized as etiologic agents of clinical manifestations in humans. CoNS have been identified as a major cause of hospital-acquired infections that typically affect immunocompromised patients with implanted medical devices [52]. Treatment is difficult because many CoNS species carry multiple antibiotic resistances, notably methicillin resistance in approximately 55-75% of nosocomial isolates, as well as glycopeptide resistance, which was initially described in CoNS strains [5, 36]. Identification to the species level is necessary to provide a better understanding of pathogenic potential of various CoNS and could help therapeutic clinical decision [18]. Furthermore, the accurate identification to the species level in reference laboratories is important to establish the role of each staphylococcal species as an infectious agent and to conduct epidemiologic investigations.

Several manual and automated phenotypic identification systems are available, such as the ID32 STAPH® strip (bioMérieux), the VITEK 2 GP® identification card (bioMérieux) and the PID 61 Phoenix system (Becton Dickinson), but none of these systems are able to accurately identify all staphylococcal species [8, 22, 28]. These methods have been designed mainly for the most frequently encountered species in human clinical samples and are not able to identify rare species and atypical strains such as metabolic variants of common species. More recently, peptide spectra obtained by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) have been used to identify CoNS; this technique has a good performance overall for species encountered in clinical practice [6, 10, 11, 45, 47] . Sample preparation and analysis techniques are simple and can be performed

within minutes. In addition to phenotypic methods, several PCR-sequencing-based methods have been developed for the identification of *Staphylococcus spp.*: the 16S rRNA [4, 15], *hsp*60 [13, 26], *sod*A [37], *rpo*B [9, 31], *fem*A [51], *tuf* [18, 30] and *gap* [27, 53, 54] genes have been used as targets. Many studies have demonstrated that genotyping methods are superior to phenotypic methods [18, 28]. However, the sequences of some genes are not sufficiently discriminative to differentiate closely related *Staphylococcus* species, and the databases only include a limited number of species. Previous studies suggest that the *tuf* and *gap* genes constitute the most discriminative targets to differentiate closely related *Staphylococcus* species [12]. The *tuf* gene, which encodes the elongation factor (EF-Tu), is involved in peptide chain formation and is a part of the core genome [44]. PCR-based assays targeting the *tuf* gene have been developed for different bacterial genera such as *Enterococcus* [21] *Mycobacterium* [33] and *Staphylococcus* [30]. In the latter case, Martineau *et al*. used hybridization probes (and not DNA sequencing) to differentiate 27 species. The *gap* gene encodes a 42-kDa transferrin-binding protein (Tpn) located within the bacterial cell wall that possesses a glycolytic function, converting D-glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate [34]. Partial sequencing of the gap gene has been proposed as an alternative molecular tool for the taxonomic analysis of *Staphylococcus* species [12].

We constructed a *tuf* and *gap* gene sequence database of 47 staphylococcal-type strains and evaluated the performance of this database as a molecular identification tool using a 186-strain collection from the French National Reference Center for staphylococci (CNRSta). Finally, to ascertain the rank of this PCR-sequencing approach among the panel of newly developed techniques, the same collection of strains was also tested using MALDI-TOF-MS technology.

Materials and Methods

Bacterial strains. Type strains representing 47 *Staphylococcus* species and subspecies (Table 1) were used in this study. In addition, 186 strains collected by the CNRSta (Lyon, France) from 1980 to 2008, of both human and animal origin and representing 35 staphylococcal species and subspecies, were included. They were distributed as follows: *S. arlettae* (n = 4), *S. aureus* (n = 9), *S. auricularis* (n = 5), *S. capitis* subsp. *capitis* (n = 5), *S. capitis* subsp. *urealyticus* (n = 6), *S. caprae* (n = 6), *S. carnosus* (n = 3), *S. chromogenes* (n = 4), *S. cohnii* subsp. *cohnii* (n = 5), *S. cohnii* subsp. *urealyticus* (n = 5), *S. epidermidis* (n = 7), *S. equorum* (n = 3), *S. felis* (n = 4), *S. gallinarum* (n = 4), *S. haemolyticus* (n = 8), *S. hominis* subsp. *hominis* (n = 6), *S. hominis* subsp. *novobiosepticus* (n = 4), *S. hyicus* (n = 1), *intermedius* group with *S. delphini* (n = 4), *S. intermedius* (n = 16) *S. pseudintermedius* (n = 4), *S. lentus* (n = 2), *S. lugdunensis* (n = 6), *S. pasteuri* (n = 5), *S. pettenkoferi* (n = 1), *S. piscifermentans* (n = 3), *S. saprophyticus* (n = 7), *S. schleiferi* subsp. *coagulans* (n = 5), *S. schleiferi* subsp. *schleiferi* (n = 8), *S. sciuri* subsp. *carnaticus* (n = 2), *S. sciuri* subsp. *sciuri* (n = 3), *S. sciuri* subsp. *rodentium* (n = 3), *S. simiae* (n = 6), *S. simulans* (n = 6), *S. succinus* (n = 2), *S. warneri* (n = 7), *S. xylosus* (n = 7).

CNR identification. Identification of the above 186 isolates was performed using phenotypic (biochemical characteristics) and genotypic methods. The genus *Staphylococcus* was defined as a Gram-positive cocci with a positive catalase reaction, O/129 compound resistance, bacitracin resistance and nitrofurantoin susceptibility. Coagulase activity on rabbit plasma, heat-stable DNase and the agglutination test (clumping factor, protein A) were used to distinguish *S. aureus* and coagulase-negative Staphylococci (CoNS). In the case of negative coagulase activity or discordant tests, species identification was performed using the ID32 STAPH strip (bioMerieux, Marcy l'Etoile, France). In the case of incorrect identification (unacceptable probability, low confidence factor or no identification), additional tests suggested by the bioMérieux identification system were performed, such as novobiocin susceptibility, oxidase reactions, or the deferoxamine test.

When phenotypic tests were not sufficient for the identification of staphylococcal species, molecular methods were used. Sixty-nine strains required supplementary tests for identification, among which were species not included in the ID32 STAPH database (2006). Fifteen strains were identified by amplification of the 16S-23S

intergenic spacer regions and the restriction enzyme analysis technique as described by Mendoza *et al.* [32]. In addition, other molecular methods were used, such as PCR sequencing of the partial *sod*A gene [37], ribotyping [39] and DNA-DNA hybridization [43]. A PCR based on the amplification of a *S. pasteuri*-specific random amplified polymorphism DNA (RAPD) fragment was performed to identify *S. pasteuri* species [50]. The same technique (amplification of a specific fragment generated by RAPD) was used to identify *S. capitis* ([50] and unpublished). An *agr*-PCR, described by Jarraud *et al.,* permitted the identification of atypical *S. aureus* strains (*i.e.,* lactose negative, mannitol negative, catalase negative or coagulase negative) [20].

Excluding *S. cohnii* subsp. *cohnii* and *S. cohnii* subsp. *urealyticus,* which are discriminated by the ID32 STAPH strip, subspecies were determined using phenotypic or genotypic tests according to the original description of each subspecies: (i) colony pigmentation for *S. capitis* subsp. *capitis* (negative) and *S. capitis* subsp. *urealyticus* (positive) [2], (ii) coagulase activity on rabbit plasma for *S. schleiferi* subsp. *schleiferi* (negative) and *S. schleiferi* subsp. *coagulans* (positive) [19], (iii) novobiocin susceptibility for *S. hominis* subsp. *hominis* (negative) and *S. hominis* subsp. *novobiosepticus* (positive) [24], (iv) nitrate reduction for *S. saprophyticus* subsp. *saprophyticus* (negative) and *S. saprophyticus* subsp. *bovis* (positive) [16] and (v) ribotyping methods for the three subspecies of *S. sciuri* [23, 29].

Bacterial growth and DNA isolation. Chromosomal DNA from all staphylococcal strains were obtained from overnight cultures grown on horse blood trypticase soy agar plates (bioMérieux, Marcy l'Etoile, France) at 37°C. Colonies were suspended in 10 mM/L Tris-HCl buffer, pH 7.0. After centrifugation at 3,450 x *g* for 2 min, the bacterial pellet was resuspended in 100 µL of Tris buffer (10 mM) containing 10 µL of lysostaphin (1 mg/ml) (Sigma, Saint Quentin Fallavier, France), and the mixture was incubated at 37°C for 30 min. DNA purification was completed on the QIAcube apparatus (QIAGEN, Courtaboeuf, France).

Bacterial identification by *tuf* and *gap* sequencing. Based on multiple sequence alignments, a region of the *tuf* gene that is highly conserved among staphylococci was chosen to design the PCR primers. A 660-bp *tuf* DNA fragment was amplified

using the primers stat1 (TTA TCA CGT AAC GTT GGT G) and stat2 (CAT TTC WGT ACC TTC TGG). The PCR program consisted of an initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 40 sec., and a final extension step for 10 min at 72°C. Gap1-for and Gap2-rev were used to amplify a 931-bp fragment of the *gap* gene as previously described [54]. PCR products were electrophoresed in a 0.8% agarose gel and visualized with SYBR® Safe DNA gel stain (Invitrogen) under UV light to confirm the correct size of the amplified product. Amplicons were sequenced using Genoscreen (Institut Pasteur, Lille, France). Both strands were sequenced with stat1 and stat2 oligonucleotides, or with Gap1-for and Gap2-rev for the reference strains. The coding strand was sequenced only for the other 186 strains.

Phylogenetic analysis. Multiple sequence alignments were performed using the ClustalW Program. Phylogenetic trees were generated with the neighbor-joining algorithm [41] applied to synonymous distances (Ks) using the SeaView program [14]. The degree of data support for the tree topology was quantified using the bootstrap method with 500 replications. The *tuf* and *gap* sequences of *Bacillus subtilis* were obtained from GenBank (accession no. NC_000964) and used as the outgroup in the phylogenetic analysis. The identification of the 186 strains of the CNR collection was based on their phylogenetic position and their similarities to the reference strain sequences.

Nucleotide sequence accession numbers. The GenBank accession numbers of the staphylococcal *tuf* and *gap* sequences determined in this study are listed in Table 1.

MALDI-TOF-MS

Staphylococcal strains were sub-cultivated 3 times on Columbia sheep blood agar plates (bioMérieux, Marcy l'Etoile, France) before MALDI-TOF-MS testing. One colony was directly deposited on a MALDI-TOF-MS target plate, and each strain was spotted 4 times. The preparation was overlaid with 1 µl of matrix solution (saturated α-cyano-4-hydroxycinnamic acid). The matrix-sample was crystallized by air-drying at room temperature. Samples were then processed in the MALDI-TOF mass

spectrometer Axima Assurance® (Shimadzu, Champs sur Marne, France) using an accelerating voltage of 20 kV in linear mode. The spectra were analyzed in the mass-to-charge ratio (m/z) range of 2 000-20 000. Five hundred laser shots were recorded for each spectrum. Quality controls (*i.e.,* duplicate spots of the *Escherichia coli* CCUG 10979 strain) were performed for each target plate. To identify the strains, the spectra obtained for each isolate were compared to the SARAMIS database for January 2009 (AnagnosTec, Potsdam, Germany). This database includes more than 2 600 SuperSpectra™, which can be used for automatic microorganism identification, over 35 000 single spectra, and notably 38 *Staphylococcus* species and subspecies. The results of the matching process are expressed as percentages. Values greater than 80% provide reliable identification based on a SuperSpectra™. Values between 30 and 80% allow provide identification based on a single spectrum. No identification has been achieved for a score below 30%, as specified by the manufacturer. The SirWeb-MALDI-TOF software (I2A, Perols, France) was used for all experiments to generate the analysis and to export the results to the laboratory informatics system.

Results

***tuf* and *gap* amplification and sequencing.** The utility of amplification–sequencing of the *tuf* and *gap* genes for the identification of staphylococcal species was first determined by analyzing 47 reference strains representing 21 staphylococcal species and 23 subspecies. A partial *tuf* gene sequence (660 bp) was amplified using the primers designed for this study (stat1 and stat2), sequenced and compared. An amplification signal was obtained for all strains tested, and a complete reference database of partial *tuf* gene sequences from the type strains was created for this study. The obtained data were deposited in the GenBank database (accession numbers are presented in Table 1). Similarly, amplification of the partial *gap* gene (931 bp) was performed for the 47 reference strains using the primers described by Yugueros *et al.* [54]. An implemented GenBank database was generated by depositing the 20 missing *gap* sequences that were not deposited by Ghebremedhin [12]. Overall, three species (*S. fleurettii*, *S. vitulinus* and *S. felis*) could not be amplified using the *gap*-specific primers.

***Staphylococcus* phylogeny derived from *tuf* and *gap* sequences**. Multiple alignments of the partial *tuf* and *gap* DNA sequences were carried out using the ClustalX® software, and phylogenetic trees were constructed by the neighbor-joining method. Bootstrap support values are indicated at the tree nodes (Figs. 1 and 2). The global topology of the *tuf* tree is in agreement with that constructed by *gap* gene analysis. The two trees revealed three common major clusters (bootstrap values > 90): (i) the *"sciuri* group" (bootstrap value of 97 with *tuf*, 100 with *gap*) including the 3 subspecies of *S. sciuri*, *S. lentus*, *S. vitulinus* and *S. fleurettii,* (ii) the *"intermedius* group" (bootstrap value of 93 with *tuf*, 98 with *gap*) comprising *S. intermedius*, *S. delphini* and *S. pseudintermedius,* and (iii) the *"simulans* group" (bootstrap value of 91 with *tuf*, 100 with *gap*) including *S. simulans*, *S. piscifermentans*, and *S. carnosus*. *S. epidermidis* and *S. saccharolyticus* formed another major cluster in the *gap* tree with a bootstrap value of 95, whereas these two species were not related in the *tuf* tree. In agreement with other methods, the two trees clustered *S. schleiferi*, *S. hyicus*, *S. chromogenes, S. muscae,* and *S. lutrae* with the *S. intermedius* group (bootstrap value of 36 with *tuf*, 67 with *gap*), *S. haemolyticus* with *S. lugdunensis* and *S. hominis* and *S. warneri* with *S. pasteuri*, and finally *S. aureus* with *S. simiae*. In both trees, the "*saprophyticus* group" included *S. saprophyticus*, *S. cohnii*, and *S.*

xylosus, with the addition of *S. succinus* and *S. gallinarum* in the *tuf* tree (Figs. 1 and 2). These analyses revealed that the two gene sequences allowed the discrimination of all *Staphylococcus* species, because subspecies of the same species were always clustered together with the exclusion of any other *Staphylococcus* species. Bootstrap values were typically higher for *gap*. Thus, the *gap* gene had a greater discriminatory power than *tuf* for the differentiation of *Staphylococcus* species. However, *tuf* demonstrated greater practicability; a 660-bp amplicon of *tuf* was sufficient for the analysis, *versus* 900 bp for the *gap* gene. In addition, *tuf* provided a more universal analysis, because it resulted in the amplification of all species, in contrast with *gap* (Table 1). Therefore, the *tuf* gene was selected for further analysis.

Species identification of CNRSta laboratory collection strains by *tuf* sequencing. The *tuf* gene-based identification matched at the species level 184/186 strains obtained from CNRSta (98.9%) (Table 2). Note that for the *S. intermedius* group, identification was considered correct when the *tuf* sequence assigned the identification to the group and not necessarily to the three recently defined species constituting this group: *S. delphini*, *S. pseudintermedius* and *S. intermedius* [42]. For the remaining two strains, one was identified as S*. schleiferi* by CNRSta and as *S. warneri* by *tuf* sequencing, with the latter identification confirmed by *gap* sequencing. The second strain identified as *S. warneri* by CNRSta and confirmed to be *S. warneri* by *gap* sequencing could not be identified by *tuf* sequencing for reasons unknown. Similarly to other molecular methods, *tuf* did not discriminate *Staphylococcus* subspecies, except for *S. cohnii* subsp. *cohnii* and *S. cohnii* subsp. *urealyticus.*

Comparison of MALDI-TOF-MS and *tuf* sequencing for species identification. Forty-four of the 47 reference strains together with the 186 strains from the CNRSta collection were analyzed using the MALDI-TOF technology. The two anaerobic strains (*S. aureus* subsp. *anaerobius* and *S. saccharolyticus*) and *S. fleurettii* were not tested. Five species, *S. kloosii*, *S. muscae*, *S. piscifermentans*, *S. simiae* and two subspecies of *S. succinus* not included in the SARAMIS® database, provided an incorrect (*S. simiae* identified as *S. aureus*) or no identification (Table 3). Concerning the species or subspecies included in the SARAMIS® database, seven reference strains were not identified (*S. auricularis*, *S. caprae*, *S. hyicus*, *S. intermedius*, *S. pasteuri*, *S. pettenkoferi* and *S. schleiferi* subsp. *schleiferi*), whereas for some of

these species, a correct identification was obtained for several isolates from the CNRSta collection (Table 3). For instance, the reference strain of *S. caprae* was not identified, whereas 5 out of 6 isolates from the CNRSta collection were properly identified by MALDI-TOF-MS. The reference strain of *S. warneri* and 5 out of 7 *S. warneri* isolates of the CNRSta collection were identified only at the group *hominis*/*warneri* level. Overall, 138 out of 186 strains (74.2%) from the CNRSta collection were identified at the species level by MALDI-TOF-MS, and one *S. warneri* strain was identified at the genus level. Four strains were misidentified, and 39 were unidentified. Five *S. warneri* strains were assigned to the group *hominis*/*warneri*. After exclusion of the CoNS species not included in the database (*i.e., S. simiae, S. kloosii, S. muscae, S. piscifermentans,* and *S. succinus)*, the final percentage of correct identifications in the CNRSta collection reached 81.5%.

Discussion

The use of nucleic acid targets provides an alternative technique for the accurate identification of *Staphylococcus* species. Because of the large amount of 16S rDNA sequence data available in public databases, this gene has been the favorite choice in many studies. However due to its lack of discriminatory power, the 16S rDNA sequence is not sufficient for the reliable identification of staphylococcal species [4]. Thus, several targets have been exploited to identify species belonging to the *Staphylococcus* genus. Among these, the *sod*A [37], *rpo*B [9, 31], *hsp*60 [25], *dna*J [46], *gap* [12, 27] and *tuf* gene [30] sequences have been found to be useful for staphylococcal species identification. Major interest in the use of *tuf* results from the small required size of the amplicon (660 bp) together with the ability to use non-degenerate oligonucleotide primers; these two conditions have not been achieved simultaneously by most other targets. The *tuf* gene has thus emerged as a reliable molecular tool for the accurate identification of *Staphylococcus* species [12, 18, 49]. However, published studies have been limited to the most common staphylococcal species encountered in human diseases [30]. In the present study, we extended the sequence analysis of the *tuf* gene to a total of 47 species and subspecies. Thus, the present study is the most extensive *tuf*-gene sequence-based study to date on staphylococcal species and sub-species.

Considering the phylogeny derived from the *tuf* gene, the global topology of the *tuf* tree, notably the presence of three major clusters, is in agreement with trees constructed based on the analysis of the other genes listed above [9, 12, 26, 30, 37, 46, 49]. The strains belonging to the *"sciuri* group" form an identical cluster in all phylogenetic trees derived from 16S rDNA, *rpo*B, *sod*A, *hsp*60, *dna*J, *gap* and *tuf* gene sequences. These strains are all novobiocin resistant and oxidase positive. Similarly, the "*intermedius* group" clusters with *S. schleiferi*, *S. hyicus*, *S. chromogenes, S. muscae* and *S. lutrae* by phylogenetic analysis of the 16S rDNA, *rpo*B, *hsp*60, *dna*J, *gap* and *tuf* genes. In contrast, the phylogeny obtained using *sod*A is slightly discordant with those phylogenies, because *S. schleiferi, S. hyicus*, *S. muscae*, and *S. chromogenes* do not cluster with the "*intermedius* group" using *sod*A [37]. The third major cluster, the "simulans group," is conserved with *sod*A, *rpo*B, *hsp*60, *dna*J, *gap* and *tuf* gene analysis but not with 16S rDNA. In addition to these major clusters, the "*saprophyticus* group" appears to be partially conserved in the *tuf* phylogeny (*S. saprophyticus*, *S. cohnii*, *S. xylosus*, *S. gallinarum*) with a low

bootstrap value, in contrast with other gene-derived phylogenies including *S. arlettae*, *S. kloosi*, and *S. equorum* in the "*saprophyticus* group" [9, 25, 37] [12, 30, 46, 49]. The other groups with low bootstrap values in the *tuf* phylogeny appeared to be poorly conserved in the phylogenies derived from other genes.

The present study resulted in the creation of an almost complete reference database of partial *tuf* gene sequences from type strains. Indeed, the completeness of the database is essential for reliable identification. Prior to the present work, numerous species were either not identified or misidentified when relying on the *tuf* GenBank database; for instance, *S. carnosus* could be misidentified as *S. simulans*, *S. gallinarum* as *S. saprophyticus*, *S. lentus* as *S. sciuri*, and *S. piscifermentans* as *S. simulans*. However, both the percentage of similarity (below 97%) and the topology of the *tuf*-based phylogenetic tree should demonstrate the lack of robustness of such results.

Considering sub-species identification, *tuf* and *gap* sequencing did not allow discrimination at the subspecies level except for *S. cohnii* subsp. *cohnii* and *S. cohnii* subsp. *urealyticus,* as demonstrated for other genes [12, 37]. Thus, molecular methods are clearly not suitable for identification at the subspecies level, a restriction with almost no consequences in clinical practice.

Because mass spectrometry is becoming increasingly popular for bacterial identification, we wondered whether it would outcompete *tuf* sequencing in the identification of the 47 species and subspecies of staphylococci. It is noteworthy that no studies have yet explored such a diversity of staphylococcal species. When comparing the *tuf*-based identification with the MALDI-TOF-MS technology, we concluded for an overall superiority of the molecular method even though the MALDI-TOF-MS based method is faster and more cost effective than the molecular method. As expected the MALDI-TOF-MS with 74.2% of correct identification out-competed the ID32 STAPH that identified 62.9% of isolates. When excluding species not included in the databases these percentages were 81.5% *versus* 75% respectively. The slight inferiority of the MALDI-TOF-MS versus the *tuf*-based method was rather unexpected given the number of enthusiastic reports on the performance of this technology for species identification [6, 10, 11, 45, 47]. Dupont *et al.* analyzed 230 isolates of CoNS representing 20 species. They obtained correct identifications for 93.2% of the isolates using MALDI-TOF-MS, and this percentage reached 97.4% with exclusion of the species not included in their database [11]. Similarly, Dubois *et*

al. used the MALDI-TOF-MS Biotyper® to identify a collection of 156 strains representing 22 different species and obtained concordant identifications for 99.3% of the species [10]. There are several reasons to explain these apparent discrepancies. First, the SARAMIS database is said to comprise 38 species and subspecies; however, only 15 species or subspecies have a SuperSpectra®. It appears that a reliable identification can only be obtained in the latter cases. A similar limitation has been pointed out by Seng *et al.* for the Biotyper database [45]. It is important to note that this drawback has limited consequences in routine clinical practice, because the most frequent species encountered in humans are well represented in both the SARAMIS® and Biotyper® databases. Hence, correct identification scores as high as 99.3% can be reported for bloodstream isolates in certain studies using MALDI-TOF-MS [47]. Expanding the database to include more species and more strains tested per species would improve the performance of this promising method. Second, the strain collection tested in the present study (the CNRSta collection) not only contains numerous species that are exceptional in clinical practice (but not necessarily never encountered) but also includes isolates of rare species, which were difficult to identify using classical methods and were thus referred to us as a reference laboratory. A third possible reason for the slight inferiority of the MALDI-TOF-MS approach in the present study was that our strain collection contained isolates that had been stored at -20°C for durations ranging from months to several years. This storage period may have altered the phenotypic expression of proteins and thus decreased the performance of the MALDI-TOF-MS approach, which essentially depends on the expression of ribosomal proteins, without affecting the efficiency of the DNA sequencing approach. In conclusion, the *tuf*-based approach appears to be particularly suited for a reference laboratory in which typical and atypical strains of all staphylococcal species are encountered, whereas at present, MALDI-TOF remains more appropriate for routine microbiology practices in clinical laboratories.

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software.

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Table 1: Sources and gene accession numbers of the bacterial reference strains used in this study

Strain	Source	*tuf* gene accession number	*gap* gene accession number
S. aureus subsp. *anaerobius*	ATCC 35844	HM352930	HM352968
S. aureus subsp. *aureus*	CCM 885	HM352919	HM352967
S. arlettae	DSM 20672	HM352954	DQ321674[a]
S. auricularis	ATCC 33753	HM352956	DQ321675[a]
S. capitis subsp. *capitis*	CCM 2734	HM352920	DQ321676[a]
S. capitis subsp. *urealyticus*	ATCC 49326	HM352921	HM352966
S. caprae	CCM 3573	HM352928	DQ321677[a]
S. carnosus subsp. *carnosus*	DSM 20501	HM352953	DQ321678[a]
S. chromogenes	CCM 3387	HM352952	DQ321680[a]
S. cohnii subsp. *cohnii*	CCM 2736	HM352938	DQ321681[a]
S. cohnii subsp. *urealyticus*	ATCC 49330	HM352939	HM352971
S. delphini	DSM 20771	HM352940	DQ321682[a]
S. epidermidis	CCM 2124	HM352922	DQ321683[a]
S. equorum subsp. *equorum*	DSM 20674	HM352959	DQ321684[a]
S. equorum subsp. *linens*	DSM 15097	HM352965	HM352977
S. felis	ATCC 49168	HM352941	Failed amplification
S. fleurettii	CIP 106114	HM352961	Failed amplification
S. gallinarum	CCM 3572	HM352942	DQ321686[a]
S. haemolyticus	CCM2737	HM352923	DQ321687[a]
S. hominis subsp. *hominis*	DSM 20328	HM352924	DQ321688[a]
S. hominis subsp. *novobiosepticus*	ATCC 700236	HM352925	HM352973
S. hyicus	CCM 2368	HM352943	DQ321689[a]
S. intermedius	CCM 5739	HM352933	DQ321690[a]
S. kloosii	DSM 20676	HM352951	DQ321691[a]
S. lentus	ATCC 29070	HM352944	DQ321692[a]
S. lugdunensis	ATCC 43809	HM352926	DQ321693[a]
S. lutrae	DSM 10244	HM352945	HM352978
S. muscae	CCM 4175	HM352957	DQ321694[a]
S. pasteuri	ATCC 51129	HM352929	HM352972
S. pettenkoferi	CIP 107711	HM352963	HM352976
S. piscifermentans	JCM 6057	HM352955	HM352979
S. pseudintermedius	LMG 22219	HM352962	HM352982
S. saccharolyticus	DSM 20359	HM352932	HM352969
S. saprophyticus subsp. *bovis*	CCM 4410	HM352934	HM352975
S. saprophyticus subsp. *saprophyticus*	CCM 883	HM352935	DQ321695[a]
S. schleiferi subsp. *schleiferi*	ATCC 43808	HM352936	DQ321696[a]
S. schleiferi subsp. *coagulans*	JCM 7470	HM352937	HM352980
S. sciuri subsp. *carnaticus*	ATCC 700058	HM352946	HM352983
S. sciuri subsp. *rodentium*	ATCC 700061	HM352948	HM352984
S. sciuri subsp. *sciuri*	ATCC 29062	HM352947	HM352985
S. simiae	CCM 7213	HM352931	HM352970
S. simulans	ATCC 27848	HM352949	DQ321698[a]
S. succinus subsp. *casei*	DSM 15096	HM352964	HM352981
S. succinus subsp. *succinus*	ATCC 700337	HM352958	HM352974
S. vitulinus	ATCC 51145	HM352960	Failed amplification
S. warneri	CCM 2730	HM352927	DQ321699[a]
S. xylosus	ATCC 29971	HM352950	DQ321700[a]

ATCC: American Type Culture Collection; CCM: Czech Collection of Microorganisms; DSM = DSMZ: German Collection of Microorganisms and Cell Cultures; JCM: Japan Collection of Microorganisms; LMG = BCCM/LMG = Belgian Coordinated Collections of Microorganisms; CIP: Institute Pasteur Collection

[a] Sequences deposited by Ghebremedhin *et al.* [12]

Fig. 1 Phylogenetic tree of the reference strains based on *tuf* sequences, computed by the neighbor-joining method applied to synonymous distances (Ks). Bootstrap support percentages ≥ 90% are indicated. The tree was rooted using *Bacillus subtilis*.

Fig. 2 Phylogenetic tree of reference strains based on *gap* sequences, computed by the neighbor-joining method applied to synonymous distances (Ks). Bootstrap support percentages ≥ 90% are indicated. The tree is rooted at its center.

Table 2: *Staphylococcus* species and subspecies identified by *tuf* sequencing *versus* CNRSta

Species and subspecies (number of strains)	CNRSta identification		*tuf* identification at the species level *versus* CNRSta identification
	ID32 STAPH (number of correct IDs)	Complementary tests (number of tests performed)	
S. arlettae (n = 4)	1	ITS-PCR (2), *sod*A (1)	4/4
S. aureus (n = 9)	7	Accuprobe (1) *agr* PCR (1)	9/9
S. auricularis (n = 5)	5		5/5
S. capitis subsp. *capitis* (n = 5)	4	Specific PCR (1)	5/5
S. capitis subsp. *urealyticus* (n = 6)	2	Specific PCR (4)	6/6
S. caprae (n = 6)	4	ITS-PCR (1) DNA-DNA hybridization (1)	6/6
S. carnosus (n = 3)	0	ITS-PCR (1) specific probes (2) [38]	3/3
S. chromogenes (n = 4)	2	Pigmentation (2)	4/4
S. cohnii subsp. *cohnii* (n = 5)	5		5/5
S. cohnii subsp. *urealyticus* (n = 5)	5		5/5
S. delphini (n = 5)	0	DNA-DNA hybridization (1) sequencing (4) [3]	5/5[a]
S. epidermidis (n = 7)	6	ITS-PCR (1)	7/7
S. equorum (n = 3)	1	DNA-DNA hybridization (2)	3/3
S. felis (n = 4)	0	DNA-DNA hybridization (4)	4/4
S. gallinarum (n = 4)	4		4/4
S. haemolyticus (n = 8)	7	ITS-PCR (1)	8/8
S. hominis subsp. *hominis* (n = 6)	5	β-glucuronidase (1)	6/6
S. hominis subsp. *novobiosepticus* (n = 4)	0	ITS-PCR (4)	4/4
S. hyicus (n = 1)	1		1/1
S. intermedius group (n = 15)	13	ITS-PCR (2)	15/15
S. lentus (n = 2)	2		2/2
S. lugdunensis (n = 6)	6		6/6
S. pasteuri (n = 5)	0	Specific PCR (5) [50]	5/5
S. pettenkoferi (n = 1)	0	DNA-DNA hybridization (1)	1/1
S. piscifermentans (n = 3)	0	ITS-PCR (1) DNA-DNA hybridization (2)	3/3
S. pseudintermedius (n = 4)	0	Sequencing (4) [3]	4/4[a]
S. saprophyticus subsp. *saprophyticus* (n = 5)	5		5/5
S. saprophyticus subsp. *bovis* (n = 2)	0	DNA-DNA hybridization (2)	2/2
S. schleiferi subsp. *schleiferi* (n = 9)	8		8/9
S. schleiferi subsp. *coagulans* (n = 5)	0	ITS-PCR (4), DNA-DNA hybridization (1)	5/5
S. scirui subsp. *carnaticus* (n = 2)	2		2/2
S. sciuri subsp. *sciuri* (n = 3)	3		3/3
S. sciuri subsp. *rodentium* (n = 3)	3		3/3
S. simiae (n = 6)	0	DNA-DNA hybridization (6)	6/6
S. simulans (n = 6)	6		6/6
S. succinus (n = 2)	0	*sod*A PCR (2)	2/2
S. warneri (n = 7)	7		6/7
S. xylosus (n = 7)	7		7/7
	117 (62.9%)	69 (37.1%)	184/186 **(98.9%)**

[a] Assigned to group *intermedius* by *tuf* sequencing

Table 3. MALDI-TOF-MS identification based on quadruplicate runs of each strain

	MALDI identification	
Species	Reference strains	*CNRSta collection strains*
S. arlettae	1/1	4/4
S. aureus	1/1	9/9
S. auricularis [a]	0/1	0/5
S. capitis subsp. *capitis*	1/1	3/5
S. capitis subsp. *urealyticus*	1/1	3/6
S. caprae	0/1	5/6
S. carnosus	1/1	2/3
S. chromogenes	1/1	3/4
S. cohnii subsp. *cohnii*	1/1	5/5
S. cohnii subsp. *urealyticus*	1/1	1/5
S. delphini	1/1	3/4
S. epidermidis	1/1	7/7
S. equorum subsp. *equorum*	1/1	3/3
S. equorum subsp. *linens*	1/1	-
S. felis	1/1	2/4
S. gallinarum	1/1	4/4
S. haemolyticus	1/1	8/8
S. hominis subsp. *hominis*	1/1	6/6
S. hominis subsp. *novobiosepticus*	1/1	4/4
S. hyicus	0/1	1/1
S. intermedius	0/1	-
S. intermedius group		21/24
S. kloosii [a]	0/1	-
S. lentus	1/1	1/2
S. lugdunensis	1/1	6/6
S. lutrae	1/1	-
S. muscae [a]	0/1	-
S. pasteuri	0/1	3/5
S. pettenkoferi	0/1	0/1
S. piscifermentans [a]	0/1	0/3
S. pseudintermedius	1/1	0/7
S. saprophyticus subsp. *bovis*	1/1	2/5
S. saprophyticus subsp. *saprophyticus*	1/1	5/5
S. schleiferi subsp. *coagulans*	1/1	4/7
S. schleiferi subsp. *schleiferi*	0/1	4/5
S. sciuri subsp. *carnaticus*	1/1	3/3
S. sciuri subsp. *rodentium*	1/1	3/3
S. sciuri subsp. *sciuri*	1/1	2/3
S. simiae [a]	0/1	4/6 [b]
S. simulans	1/1	6/6
S. succinus subsp. *casei* [a]	0/1	-
S. succinus subsp. *succcinus* [a]	0/1	0/2
S. vitulinus	1/1	-
S. warneri	1/1[c]	5/7[c]
S. xylosus	1/1	4/7

	31/44 (**70,5%**)	138/186 (**74,2%**)

[a]Absent in the SARAMIS database

[b]False identification: *S. aureus* instead of *S. simiae*

[c]Identified as a member of the *hominis*/*warneri* group

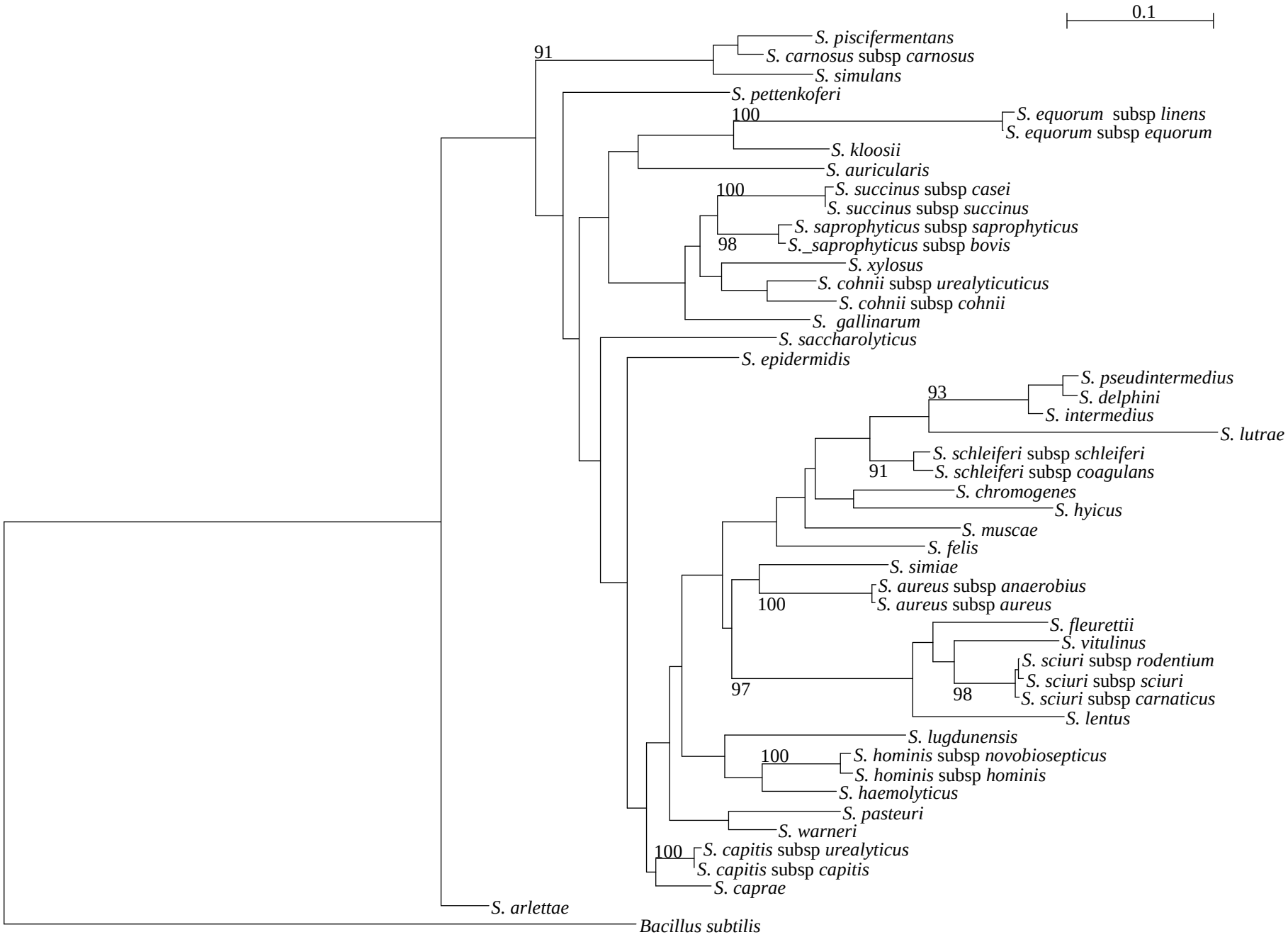

Fig. 1. Phylogenetic tree of the reference strains based on *tuf* sequences, computed by the neighbor-joining method applied to synonymous distances (Ks). Bootstrap support percentages ≥ 90% are indicated. The tree was rooted using *Bacillus subtilis*.

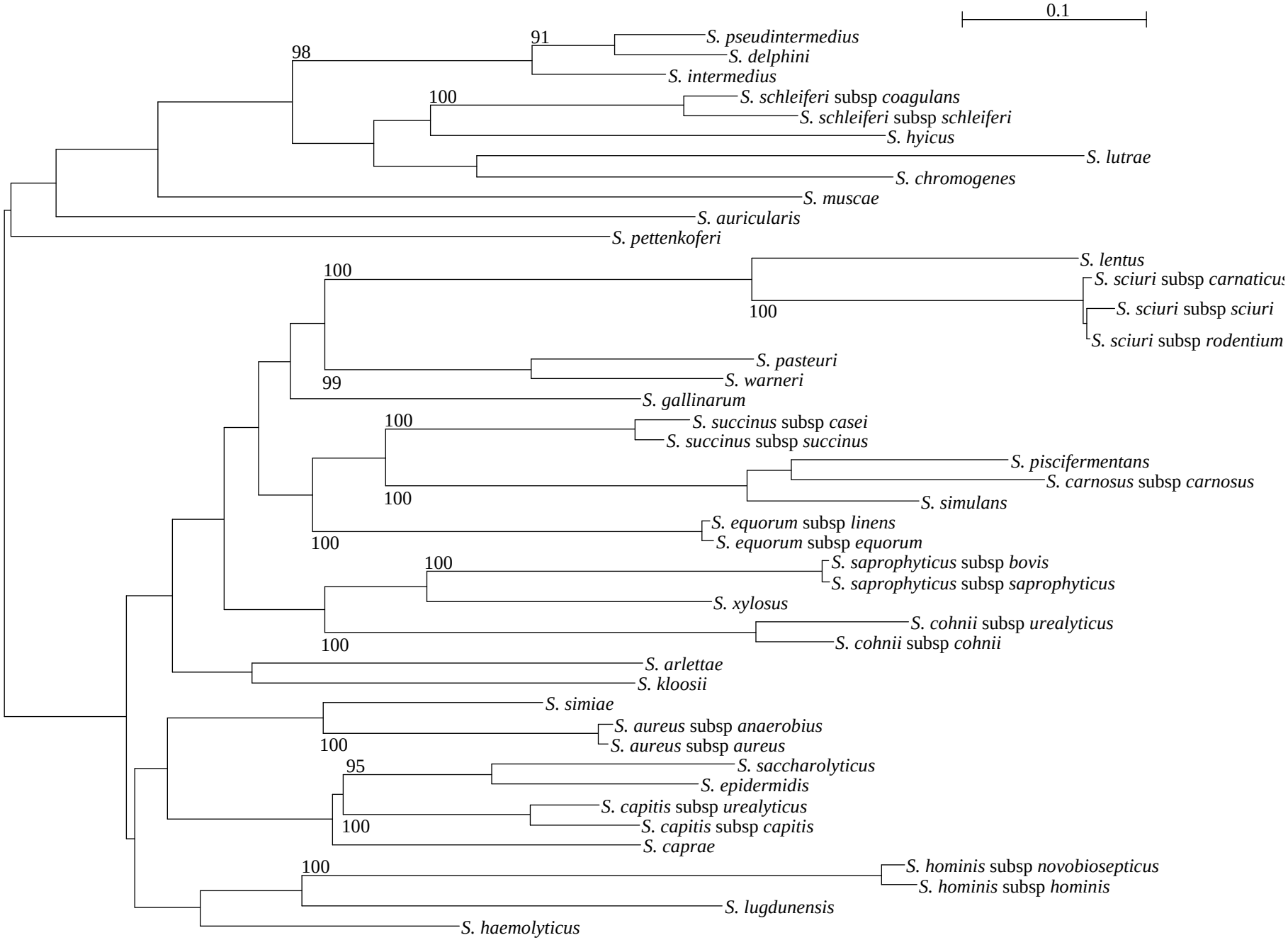

Fig. 2. Phylogenetic tree of reference strains based on *gap* sequences, computed by the neighbor-joining method applied to synonymous distances (Ks). Bootstrap support percentages ≥ 90% are indicated. The tree is rooted at its center.