High intraspecies heterogeneity within *Staphylococcus sciuri* and rejection of its classification into *S. sciuri* subsp. *sciuri*, *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium*

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A polyphasic taxonomic approach was applied to strains of the species *Staphylococcus sciuri* in order to clarify the taxonomic legitimacy of the delineation of *S. sciuri* into *S. sciuri* subsp. *sciuri*, *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium*. A group of 81 *S. sciuri* isolates obtained from human (*n*=62) and veterinary (*n*=17) clinical materials and foods (*n*=2) and ten reference and type strains obtained from the Czech Collection of Microorganisms were characterized by extensive biotyping using conventional tests and commercial identification kits (ID 32 Staph, STAPHYTest, Biolog Microbial ID System), matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry, automated ribotyping with EcoR1 restriction enzyme, 16S–23S rRNA gene intergenic transcribed spacer PCR fingerprinting and repetitive-sequence-based PCR fingerprinting with the (GTG)n primer. Selected strains representing different ribotypes were further characterized using sequencing of the ρB-subunit of RNA polymerase (*rpoB*) gene. Individual techniques revealed high heterogeneity within the analysed *S. sciuri* strains but differentiation of the investigated strains into groups corresponding to the aforementioned *S. sciuri* subspecies and supported by these techniques was not clearly revealed. Based on obtained results and data retrieved from literature we propose rejecting the separation of *S. sciuri* species into *S. sciuri* subsp. *sciuri*, *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium* and we suggest reclassification these subspecies as *S. sciuri* with the type strain W.E. Kloos SC 116T (=ATCC 29062T=BCRC 12922T=CCUG 15598T=CNCTC 5683T=DSM 20345T=JCM 2425T=NCTC 12103T).

*Staphylococcus sciuri* is a coagulase-negative, oxidase-positive and novobiocin-resistant species occurring mainly in animal sources, however, it may be isolated also from human clinical materials, foods and/or from the environment. It is a natural reservoir of the mecA gene encoding methicillin resistance (Wu et al., 1998; Rolo et al., 2014) and is considered to be a source of its horizontal transfer into other staphylococcal species (Antignac & Tomasz, 2009; Mašičková et al., 2013). The species *S. sciuri* was described by Kloos et al. (1976) who originally recognised two subspecies, namely *S. sciuri* subsp. *sciuri* and *S. sciuri* subsp. *lentus*. Subsequently, Schleifer et al. (1983) reclassified these subspecies as two separate species *S. sciuri* and *Staphylococcus lentus* on the basis of DNA–DNA hybridization experiments. The current delineation of *S. sciuri* into *S. sciuri* subsp. *sciuri*, *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium* was proposed by Kloos et al. (1997) who differentiated these three subspecies on the basis of a few

**Abbreviations:** ITS-PCR, intergenic transcribed spacer PCR; rep-PCR, repetitive-sequence-based PCR.

The GenBank/EMBL/DDBJ accession numbers for partial RNA-polymerase gene (*rpoB*) sequences of 38 analysed *S. sciuri* strains are KU975566–KU975603.

Five supplementary figures and three supplementary tables are available with the online Supplementary Material.
phenotypic characteristics and results of ribotyping with EcoRI restriction endonuclease.

This study was set up to assess the taxonomic legitimacy of the delineation of S. sciuri into S. sciuri subsp. sciuri, S. sciuri subsp. carnaticus and S. sciuri subsp. rodentium. An extensive literature search showed that only a few publications describing identification of any of the S. sciuri subspecies have been published (Dakic et al., 2005; Marsou et al., 1999; Stepanovic et al., 2005). However, the authors of the vast majority of studies dealing with this bacterial group identified these staphylococci only to the species level (Nagase et al., 2002; Rolo et al., 2014; Shittu et al., 2004; Stepanovic et al., 2003) indicating that reliable differentiation of subspecies of S. sciuri has been a difficult and controversial task since their description.

A group of 91 S. sciuri strains was investigated in this study. Ten reference and type strains representing all three subspecies of S. sciuri and further reference strains were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic, http://www.sci.muni.cz/ccm/) (Table S1, available in the online Supplementary Material). An additional 81 S. sciuri isolates originating from human (n=62) and veterinary (n=17) clinical materials and two strains from foods were obtained from the National Reference Laboratory for Staphylococci (NRL/St), National Institute of Public Health, Prague, Czech Republic (Table S2). These strains were isolated at different laboratories in the Czech Republic and were referred to NRL/St for identification. Individual strains were retrieved from different individuals and/or sources (different clinical cases, dates and sites) to prevent analysis of duplicate isolates of a single strain. They were initially classified as S. sciuri on the basis of biochemical tests relevant for the genus Staphylococcus and matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) results.

Initial phenotypic characterization using ID 32 Staph (bio-Mérieux) and STAPHYTest 24 (Erba Lachema) commercial kits and conventional biochemical, physiological and growth tests relevant for the genus Staphylococcus were performed as described by Pantuček et al. (2013). The results obtained corresponded with those described for S. sciuri but they did not allow us to reliably differentiate individual subspecies of S. sciuri when compared with biochemical test results available in the literature. The test results previously reported to be discriminatory, e.g. acid production from D-xylene, D-melezitose or D-cellobiose are variable in at least one of the subspecies of S. sciuri (Kloos et al., 1997; Stepanovic et al., 2005; Schleifer & Bell, 2009; Becker & Von Eiff, 2011) which complicates evaluation of the identification results. Moreover, results for melezitose acidification described originally by Kloos et al. (1997) were found to be different in a later study (Marsou et al., 1999).

To achieve extensive biochemical identification the Biolog system with the Gram-positive identification panel the GP2 MicroPlate was utilized. The Biolog system was used to identify 57 strains as S. sciuri and two strains as S. sciuri subsp. rodentium; four strains were identified only to the genus Staphylococcus. Strain P563 was erroneously assigned to S. lentus and the remaining 28 strains were not identified at all. In general, the Biolog system, using an extended set of substrate-assimilation tests, revealed high heterogeneity within the phenotypic fingerprints of analysed S. sciuri strains (Table S3), as demonstrated by the numerical analysis of obtained results (Fig. S1), but no clustering indicating separation of the strains among S. sciuri subspecies was apparent.

MALDI-TOF MS was used as a rapid screening method for the identification of isolates received in the NRL/St. All strains were analyzed using a Microflex LT system (Bruker Daltonics) and obtained mass spectra were processed using the Flex Analysis (version 3.3) and identified within the BioTyper database version 3.2.1.0. as described previously (Pantuček et al., 2013). Out of 81 strains, the MALDI-TOF MS method assigned 23 strains to S. sciuri subsp. sciuri with log scores ranging from 2.0 to 2.135 and a further 54 strains were assigned to S. sciuri subsp. sciuri with log scores ranging from 1.701 to 1.999. Three strains (P538, P541 and P543) were identified as unreliable S. sciuri subsp. sciuri with log scores of 1.613, 1.641 and 1.613, respectively, and the last strain (P592) was assigned as unreliable S. sciuri subsp. carnaticus with a log score of 1.609.

Ribotyping with EcoRI restriction endonuclease has been presented as a key differentiating method in the publication describing the three subspecies of S. sciuri (Kloos et al., 1997). In our study, automated ribotyping with EcoRI restriction enzyme was performed using the RiboPrinter Microbial Characterization System (DuPont Qualicon) in accordance with the manufacturer’s instructions. Numerical analysis and dendrogram reconstruction was performed using the BioNumerics 7.1 software (Applied Maths). The ribotype patterns were imported into the BioNumerics software using the load samples import script provided by the manufacturer. The dendrogram demonstrating EcoRI ribotypes variability within all analysed strains of S. sciuri is shown in Fig. 1. The cluster analysis grouped most of the investigated strains into a few clusters, revealing distinctive fingerprint patterns; only a few strains revealed more distantly separated fingerprints. However, no clear distribution of the type, reference and the isolates corresponding to the three recognised subspecies was apparent. Ribotyping represents a group of techniques which may methodologically differ in individual laboratories (e.g. different probes, hybridization temperatures, etc.), thus yielding different results. For example, Chesneau et al. (2000) found EcoRI ribotype profiles revealed by the type strains of S. sciuri subsp. sciuri and S. sciuri subsp. rodentium to be identical but the fingerprints revealed by the RiboPrinter system were different for these two strains in our study. Due to the low reproducibility of different ribotyping methods we also visually closely scrutinized the S. sciuri subspecies ribotype patterns published by Kloos et al. (1997). In our opinion the fingerprints obtained by these authors do not clearly
Fig. 1. Dendrogram based on cluster analysis of EcoRI ribotype patterns obtained using the RiboPrinter system from strains of S. sciuri. The dendrogram was calculated with Pearson's correlation coefficients with the UPGMA clustering method (r, expressed as percentage similarity values). Origin of P strains is highlighted by the text font style. The 62 isolates originating from human clinical materials are written in plain type the 17 strains from veterinary clinical materials are underlined and two strains isolated from food are double underlined.
support differentiation of the three subspecies as advocated in the subspecies description paper. Kloos et al. (1997) differentiated individual subspecies on the basis of the presence and/or intensity of a few bands. *S. sciuri* subsp. *rodentium* was distinguished by a typical band at about 8.5 kbp and another band at about 4.8 kbp which was found in most of the isolates of *S. sciuri* subsp. *rodentium*. *S. sciuri* subsp. *carnaticus* was differentiated by a dark band at about 12 kbp, however this band was found in less than half of the strains analysed and the visual examination of the fingerprints published by Kloos et al. (1997) also showed, that such a dark band may be seen also in strains of *S. sciuri* subsp. *sciuri*. The subspecies *S. sciuri* subsp. *sciuri* was distinguished by a high-intensity band at about 4.2 kbp and by the presence of two separable bands at about 4.2 and 4.3–4.5 kbp. However, the high-intensity band at about 4.2 kbp may be seen also in ribotype patterns revealed by two remaining subspecies and the two separable bands were present only in about half of the strains of *S. sciuri* subsp. *sciuri*. As indicated by the results discussed above, only *S. sciuri* subsp. *rodentium* revealed a unique band at about 8.5 kbp, although its size also slightly varied in individual fingerprints. Thus, based on the visual examination of fingerprints published by Kloos et al. (1997) we concluded that these data were not sufficient for a reliable differentiation of the three subspecies of *S. sciuri*. It is necessary to point out here that the numerical analysis of our ribotyping results separated a group of a few strains including the *S. sciuri* subsp. *rodentium* type strain CCM 4657\(^T\) in a sub-cluster (Fig. 1) and this group was also differentiated by *rpoB* gene sequencing (see below). However, separation of this group was not further confirmed by other molecular and, most importantly, phenotypic methods applied in this study.

Analysis of the 16S–23S rRNA gene intergenic transcribed spacer (ITS-PCR) represents another method described in the literature as a valuable technique for identification of staphylococci including the differentiation of *S. sciuri* subspecies (Couto et al., 2001; Dakić et al., 2005). ITS-PCR fingerprinting with primers G1 (5′-GAA GTC GTA ACA AGG-3′) and L1 (5′-CAA GGC ATC CAC CGT-3′) was performed using a method described by Jensen et al. (1993) with a few modifications. Briefly, the PCR was performed in a final volume of 25 µl containing 12.5 µl FastStart PCR Master (Roche), 2.5 µl (=1 µM) each of G1 and L1 primer, 2 µl of a cell lysate obtained by the alkaline extraction procedure described previously (Švec et al., 2008) and 5.5 µl sterile deionized water. The DNA fragments obtained were separated in 2 % (w/v) agarose gels in 0.5× TBE buffer for 16 h at 1.4 V cm\(^{-1}\). Fingerprint profiles were digitised and processed using the BioNumerics software (Applied Maths). In correspondence with repetitive-sequence-based PCR (rep-PCR) fingerprinting and automated ribotyping the ITS-PCR fingerprints obtained did not differentiate the analysed strains into subspecies-specific clusters (Fig. S2). Analogously to ribotyping also, ITS-PCR methods developed and performed by different laboratories gave different results. Our technique was not able to differentiate the type strain of *S. sciuri* subsp. *rodentium* from the type strains of phylogenetically closely related *S. vitulinus* and *S. feurettii* (Fig. S3). In a similar way Marsou et al. (1999, 2001) did not differentiate *S. sciuri* subsp. *sciuri* from *S. vitulinus* using ITS-PCR fingerprinting. Such diverse results indicate that the applicability of ITS-PCR assays for a reproducible and reliable taxonomic characterization of staphylococcal (sub)species is questionable.

Genotypic screening of the isolates using the rep-PCR fingerprinting with the (GTG)\(_5\) primer was performed as described previously (Švec et al., 2010). This method demonstrated a good discriminatory power for the differentiation of the majority of staphylococcal (sub)species in our previous study based on investigation of representatives of individual (sub)species but it failed to generate (sub)species-specific fingerprints from a few species of the genus *Staphylococcus* (*S. caprae*, *S. equorum*, *S. pseudintermedius*, *S. saprophyticus* and *S. xylosus*) including *S. sciuri* (Švec et al., 2010). Data obtained with an extended *S. sciuri* group in this study confirmed our previously published observations and revealed high heterogeneity within the analysed strains of *S. sciuri*. Cluster analysis of the fingerprints obtained distributed individual strains into multiple clusters without any relation to possible subspecies-specific subclusters (Fig. S4).

Our literature searches revealed that numerous studies evaluating the discriminatory power of different DNA-based fingerprinting methods and/or studies implementing these methods for the identification of staphylococci do not cover all three subspecies (e.g. Carretto et al., 2005; Ghebremedhin et al., 2008) or include only the type strains of subspecies of *S. sciuri*. For example Couto et al. (2001) differentiated the type strains of subspecies of *S. sciuri* using ITS-PCR and Dakić et al. (2005) identified clinical isolates of *S. sciuri* on the basis of visual comparison of obtained ITS-PCR fingerprints with those revealed by type strains of subspecies of *S. sciuri*. Similarly, Marsou et al. (1999) used ribotype patterns from the type strains as reference entries for identification of clinical isolates of *S. sciuri*. These results obtained with single representatives of (sub)species do not allow reliable assessment of intra(sub)species variability revealed by individual fingerprinting techniques and thus cannot reliably evaluate applicability of these methods for differentiation of subspecies of *S. sciuri*.

Molecular identification techniques based on gene sequences have, in general, limited value for the differentiation of staphylococcal subspecies. For example, Poyart et al. (2001) was not able to differentiate reliably type strains of *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *sciuri* using *sodA* gene sequencing. In contrast, results of previous studies indicated that the type and collection cultures of subspecies of *S. sciuri* revealed slight differences when *hsp60*, *rpoB*, *dnaJ*, gap and *tuf* genes were compared (Bergeron et al., 2011; Pantucěk et al., 2013) but, according to our best knowledge, any comprehensive study evaluating reliably differentiation
of subspecies of S. sciuri using gene sequences and/or investigating intra(sub)species gene sequence variability have not been published. For these reasons, we selected 38 strains representing individual ribotype pattern subclusters revealed within our studied group and characterized their rpoB gene sequences. The rpoB gene was selected due to its good discriminative power within staphylococcal (sub)species (Drancourt & Raoult, 2002; Mellmann et al., 2006; Pantuček et al., 2013). The rpoB gene sequencing and analysis of obtained sequences was performed as described by Švec et al. (2015). The rpoB gene sequences obtained showed pairwise similarity values ranging from 96.8 to 100 %. Numerical analysis using the maximum-likelihood treeing method revealed apparent heterogeneity among the tested representative strains and revealed two major clusters with 99 % bootstrap support and numerous subclusters that received moderate bootstrap support (Fig. S5). Detection of these two major rpoB clusters implies existence of two lineages, however these groups could not be differentiated phenotypically. Ribotype, rep-PCR as well as ITS-PCR patterns revealed in these strains were distributed almost randomly among different rpoB gene subclusters and did not display any subspecies-specific distribution, indicating an inherent genotypic variability.

All genotypic and phenotypic results obtained in this study as well as the data retrieved from the literature demonstrated that the separation of S. sciuri into the three currently recognised subspecies is doubtful and it is not taxonomically fully justified. The techniques applied revealed high heterogeneity between the investigated isolates of S. sciuri, however, no clear differentiation of the investigated strains into groups corresponding to the three subspecies of S. sciuri was apparent. Based on all the aforementioned data we propose rejecting the separation of S. sciuri into S. sciuri subsp. sciuri, S. sciuri subsp. carnaticus and S. sciuri subsp. rodentium and we suggest reclassification these subspecies as S. sciuri with the type strain W.E. Kloos SC 1161 (=ATCC 29062=BCRC 12927=CCM 3473=CCUG 15598=CNCTC 5683=DSM 20345=JCM 2425=NCTC 12103).


In addition to the species description given by Kloos et al. (1976, 1997) the following characteristics were revealed by testing of 91 S. sciuri strains included in this study. The test was scored as positive when ≥90 % strains revealed positive reaction and as negative when ≥90 % strains revealed negative test results. Biochemical characteristics obtained by ID32 STAPH (bioMérieux) and STAPHYtest 16 (ErbaLachema) test kits are positive for ascinulin hydrolysis, nitrate reduction and acid production from D-glucose, D-fructose, D-mannose, D-maltose, D-trehalose, D-mannitol, D-saccharose and D-cellobiose; negative for production of urease, arginine dihydrolase, ornithine decarboxylase, aco- toin (Voges-Proskauer test), arginine arylamidase, pyrrolidonyl arylamidase, β-galactosidase and acid production from D-raffinose; and variable for production of alkaline phosphatase, β-glucuronidase and acid production from xylose, D-lactose, D-turanose, L-arabinose, D-ribose and N-acetyl-glucosamine. The strain-dependent characteristics obtained by ID32 STAPH and STAPHYtest 16 are as follows: positive production of alkaline phosphatase and β-glucuronidase and acid production from lactose, D-turanose and L-arabinose; negative acid production from xylose, D-ribose and N-acetyl-glucosamine.

Utilization of carbon sources via respiration tested by the Biolog system with the Gram-positive identification panel GP2 MicroPlate is positive for dextrin, D-fructose, α-D-glucose, maltose, maltotriose, D-mannitol, D-mannose, palatinose, D-psicose, D-ribose, salicin, D-sorbitol, sucrose, D-trehalose, turanose, D-xylose, pyruvic acid methyl ester, propionic acid, L-alanine, L-glutamic acid, L-serine, glycerol, thymidine, uridine and D-L-α-glycerol-phosphate and negative for α- and β-cyclodextrin, inulin, mannan, L-fucose, D-galacturonic acid, myo-inositol, α-D-lactose, lactulose, α- and β-methyl-D-galactoside, α-methyl-D-mannoside, sedoheptulose, stachyose, xylitol, α- and β-hydroxybutyric acid, p-hydroxyphenylactic acid, lactamide, D-malic acid, L-pyrogulatic acid, putrescine, adenosine-5’-monophosphate and α-D-glucose-1-phosphate. Variable test results obtained with the Biolog system are shown in Table S3.

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References


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