Staphylococcus petrasii subsp. *pragensis* subsp. nov., occurring in human clinical material

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Seven coagulase-negative, oxidase-negative and novobiocin-susceptible staphylococci assigned tentatively as *Staphylococcus petrasii* were investigated in this study in order to elucidate their taxonomic position. All strains were initially shown to form a genetically homogeneous group separated from remaining species of the genus *Staphylococcus* by using a repetitive sequence-based PCR fingerprinting with the $(GTG)_5$ primer. Phylogenetic analysis based on 16S rRNA gene, *hsp60*, *rpoB*, *dnaJ*, *gap* and *tuf* sequences showed that the group is closely related to *Staphylococcus petrasii* but separated from the three hitherto known subspecies, *S. petrasii* subsp. *petrasii*, *S. petrasii* subsp. *croceilyticus* and *S. petrasii* subsp. *jettensis*. Further investigation using automated ribotyping, MALDI-TOF mass spectrometry, fatty acid methyl ester analysis, DNA–DNA hybridization and extensive biotyping confirmed that the analysed group represents a novel subspecies within *S. petrasii*, for which the name *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. is proposed. The type strain is NRL/St 12/356^T (=CCM 8529^T=LMG 28327^T).

Staphylococcus petrasii is a recently described coagulase-negative, oxidase-negative and novobiocin-susceptible species. At the time of description of *S. petrasii*, two subspecies, namely *Staphylococcus petrasii* subsp. *petrasii* and *Staphylococcus petrasii* subsp. *croceilyticus* were recognized (Pantůček *et al.*, 2013). Recently, another coagulase-negative species *Staphylococcus jettensis* (De Bel *et al.*, 2013) was revealed to be a member of the species *S. petrasii* and was reclassified as *Staphylococcus petrasii* subsp. *jettensis* (De Bel *et al.*, 2014).

The present taxonomic study deals with seven strains (Table 1) assigned tentatively to the species *S. petrasii*, but revealing characteristics differentiating them from the three hitherto known subspecies of *S. petrasii*. Six isolates (NRL/St 12/356^T, NRL/St 93/321, NRL/St 94/190, NRL/St 13/623, NRL/St 14/114 and NRL/St 14/221) were obtained from human clinical material in different clinical laboratories in the Czech Republic and were referred to the National Reference Laboratory for Staphylococci (National Institute of Public Health, Prague) for identification. The remaining strain LMG 28328 was isolated in 2013 from a

Abbreviations: MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; NRL/St, National Reference Laboratory for Staphylococci (Prague, Czech Republic).

The GenBank/EMBL/DDBJ accession numbers for *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. strains NRL/St 12/356^T, NRL/St 93/321, NRL/St 94/190, NRL/St 13/623, NRL/St 14/114, NRL/St 14/221 and LMG 28328 are KM873669–KM873675 (16S rRNA gene); KM873676–KM873682 (*hsp60*); KM873683–KM873689 (*rpoB*); KM873690–KM873696 (*dnaJ*); KM873697–KM873703 (*gap*); and KM873704–KM873710 (*tuf*).

Two supplementary tables and two supplementary figures are available with the online version of this paper.

Table 1. Origin of strains of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov.

Strain designation	Specimen	Diagnosis	Sex (age)	Locality	Date
NRL/St 12/356^T (=CCM 8529^T=LMG 28327^T)	Ejaculate	Prostatitis	M (58y)	Prague, CZ	May 2012
NRL/St 93/321 (=CCM 8530)	Wound pus	Hand wound infection	F (22y)	Prague, CZ	April 1993
NRL/St 94/190	Appendix pus	Appendicitis	M (52y)	Prague, CZ	February 1994
NRL/St 13/623	Blood	Pancreatitis	M (55y)	Ostrava, CZ	October 2013
NRL/St 14/114	Blood	Sepsis	M (45y)	Pilsen, CZ	February 2014
NRL/St 14/221	Wound pus	Phlegmona	M (56y)	Strakonice, CZ	March 2014
LMG 28328 (=CCM 8531)	Lambic beer brewery air	NA	NA	Brussels, Belgium	May 2013

NA, Not applicable; F, female; M, male; CZ, Czech Republic.

Lambic beer brewery air sample in Brussels (Belgium). The type and reference strains included in individual experiments and representing all three hitherto described subspecies of *S. petrasii* as well as representatives of the other related species of the genus *Staphylococcus* were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno) and/or from the BCCM/LMG Bacteria Collection (Ghent University, Belgium).

Initial genotypic screening of the isolates using the repetitive sequence-based PCR (rep-PCR) fingerprinting with the $(GTG)_5$ primer was performed as described previously (Švec *et al.*, 2010). The seven isolates revealed visually similar fingerprints (75 % similarity), grouped in a single cluster and separated from the remaining entries included in the in-house fingerprint database of the Czech Collection of Microorganisms covering representatives of all recognized species of the genus *Staphylococcus*. The dendrogram (Fig. S1, available in the online Supplementary Material) shows differentiation of the analysed strains from all three hitherto described subspecies of *S. petrasii* and the phylogenetically closest species.

The 16S rRNA gene (Pantůček *et al.*, 2005), *hsp60* (Kwok & Chow, 2003), *rpoB* (Mellmann *et al.*, 2006), *dnaJ* (Shah *et al.*, 2007), *gap* (Yugueros *et al.*, 2000), and *tuf* (Bergeron *et al.*, 2011) amplification from crude boiled cell extracts was performed as described previously. Sequencing was performed in the Eurofins MWG Operon sequencing facility (Ebersberg, Germany) and the obtained sequences were compared with those of other taxa of the genus *Staphylococcus* retrieved from the GenBank/EMBL/DDJB database. Determination of the closest relatives of the seven isolates was performed by using the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were reconstructed according to the neighbour-joining and maximum-likelihood methods to confirm the tree topology, implementing the Kimura-2 substitution model and using bootstrap values based on 1000 replications, with the MEGA software version 6 (Tamura *et al.*, 2013).

The topology of the neighbour-joining tree reconstructed with 16S rRNA gene sequences (Fig. 1) was similar to that of the maximum-likelihood tree (not shown) and showed that the isolates belonged to the species *S. petrasii*. However, the isolates clustered separately from the three subspecies *S. petrasii* subsp. *petrasii* (99.5 % 16S rRNA gene sequence similarity), *S. petrasii* subsp. *croceilyticus* (99.8 %), and *S. petrasii* subsp. *jettensis* (99.4 %). Because the 16S rRNA gene analysis has limited discriminatory power for the identification of some staphylococcal species, the phylogenetic position of the novel isolates was also assessed using concatenated multi-locus sequence data of five routinely used housekeeping genes, *hsp60*, *rpoB*, *dnaJ*, *gap* and *tuf*, as well as the corresponding amino acid sequences. The portions of analysed gene sequences corresponded to the following gene coordinates of *Staphylococcus aureus* subsp. *aureus* NCTC 8325 (GenBank accession no. NC_007795): 260–822 for *hsp60*, 1443–2263 for *rpoB*, 29–886 for *dnaJ*, 46–926 for *gap*, and 378–1019 for *tuf*. Neighbour-joining and maximum-likelihood phylogenetic trees for the five housekeeping genes including the closest relatives had a very similar topology, which corresponded with that of the 16S rRNA gene sequence tree. Both nucleotide and amino acid sequence-based phylogenetic analyses showed that the seven isolates represented a well-delineated group within the *S. petrasii* cluster, clearly separate from the three established subspecies (Fig. 2).

The capacity of matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to distinguish among subspecies of *S. petrasii* was examined. For this purpose, a main reference spectrum of the strains *S. petrasii* subsp. *petrasii* CCM 8418^T, *S. petrasii* subsp. *croceilyticus* CCM 8421^T, *S. petrasii* subsp. *jettensis* LMG 26879^T and NRL/St 12/356^T was added to the commercial database (version 3.3.1.0) of a Microflex instrument (Bruker) after cultivation of these strains on tryptic soy agar (Lab M) with 5 % horse blood (E&O Laboratories). Subsequently, ten clinical isolates were examined: three isolates of *S. petrasii* subsp. *petrasii* (Pantůček *et al.*, 2013), two isolates each of *S. petrasii* subsp. *croceilyticus* (Pantůček *et al.*, 2013) and *S. petrasii* subsp. *jettensis* (De Bel *et al.*, 2013), and three human clinical isolates of the present study. Nine of these strains yielded good species level identification results

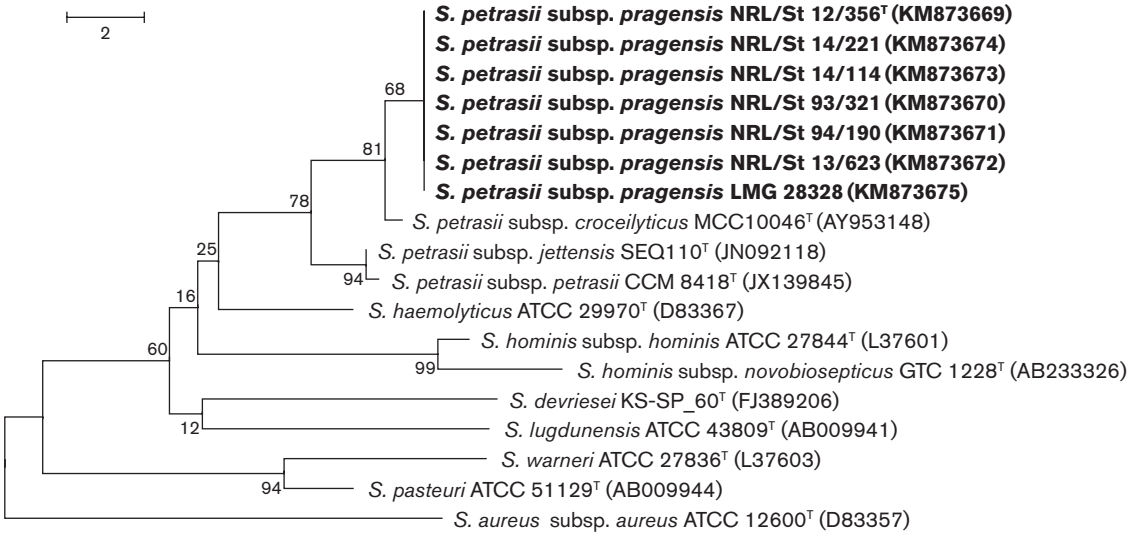

Fig. 1. Unrooted neighbour-joining tree based on 16S rRNA gene sequence comparison, showing the phylogenetic relationships of isolates of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. and reference type strains of related staphylococcal taxa. *Staphylococcus aureus* subsp. *aureus* ATCC 12600^T sequence was used as an outgroup. Bootstrap probability values (percentages of 1000 tree replications) are indicated at branch points. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. There were 1422 positions in the final dataset.

(log score >2.0) and the top score corresponded with the correct subspecies. In addition, a main log difference of 0.180 with the second subspecies match was obtained.

Automated ribotyping with the *Eco*RI restriction enzyme was performed using the RiboPrinter Microbial Characterization System (DuPont Qualicon) in accordance with the manufacturer's instructions. Numerical analysis and dendrogram construction was done using the BioNumerics 7.1 software (Applied Maths). The ribotype patterns were exported into the BioNumerics database using the load samples import script provided by the manufacturer. Numerical analysis of the obtained ribotypes clustered all seven isolates into a single cluster separated from the remaining phylogenetically closely related species of the genus *Staphylococcus* (Fig. S2). The similarity values between individual ribotypes from the studied strains ranged from 64.2 % to 95.9 %. The closest ribotype pattern was revealed by *Staphylococcus devriesei* CCM 7896^T (55.8 % similarity).

A Hewlett Packard 6890N gas chromatograph equipped with a flame-ionization detector and the Microbial Identification System (MIDI) was used to analyse the cellular fatty acid components. For this, bacterial cells were harvested from tryptic soy agar plates (BD) incubated for 48 h at 28 °C, and were subsequently analysed according to the manufacturer's instructions. The predominant fatty acid was anteiso-$C_{15:0}$, whilst iso-$C_{15:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$ and $C_{18:0}$ were present in variable and moderate amounts. These results are in line with previous reported patterns for coagulase-negative staphylococci (Freney *et al.*, 1999; Kotilainen *et al.* 1991).

High molecular mass genomic DNA for DNA–DNA hybridization experiments and mol% G+C content analysis was obtained using the protocol described by Gevers *et al.* (2001). The DNA G+C content of strain NRL/St 12/356^T was determined according to the HPLC method described by Mesbah & Whitman (1989) and was 33.0 mol%.

DNA–DNA hybridization between strain NRL/St 12/356^T and *S. petrasii* subsp. *petrasii* CCM 8418^T, *S. petrasii* subsp. *croceilyticus* CCM 8421^T, *S. petrasii* subsp. *jettensis* CCM 8494^T and the type strain of the phylogenetically closest species, *Staphylococcus haemolyticus* CCM 2737^T, was done using the microplate method described by Ezaki *et al.* (1989) according to the protocols described previously (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998). The hybridization temperature calculated from the G+C content of the analysed strains was 34 °C. The DNA–DNA relatedness values were calculated as means based on at least three independent hybridizations. Reciprocal reactions were performed and also considered as independent experiments. The DNA–DNA hybridization values obtained between strain NRL/St 12/356^T and the type strains of the subspecies of *S. petrasii* ranged from 88±6 % to 75±0 %, which confirmed that they are members of the same species (Table S1). The DNA–DNA hybridization value between NRL/St 12/356^T and *S. haemolyticus* CCM 2737^T was

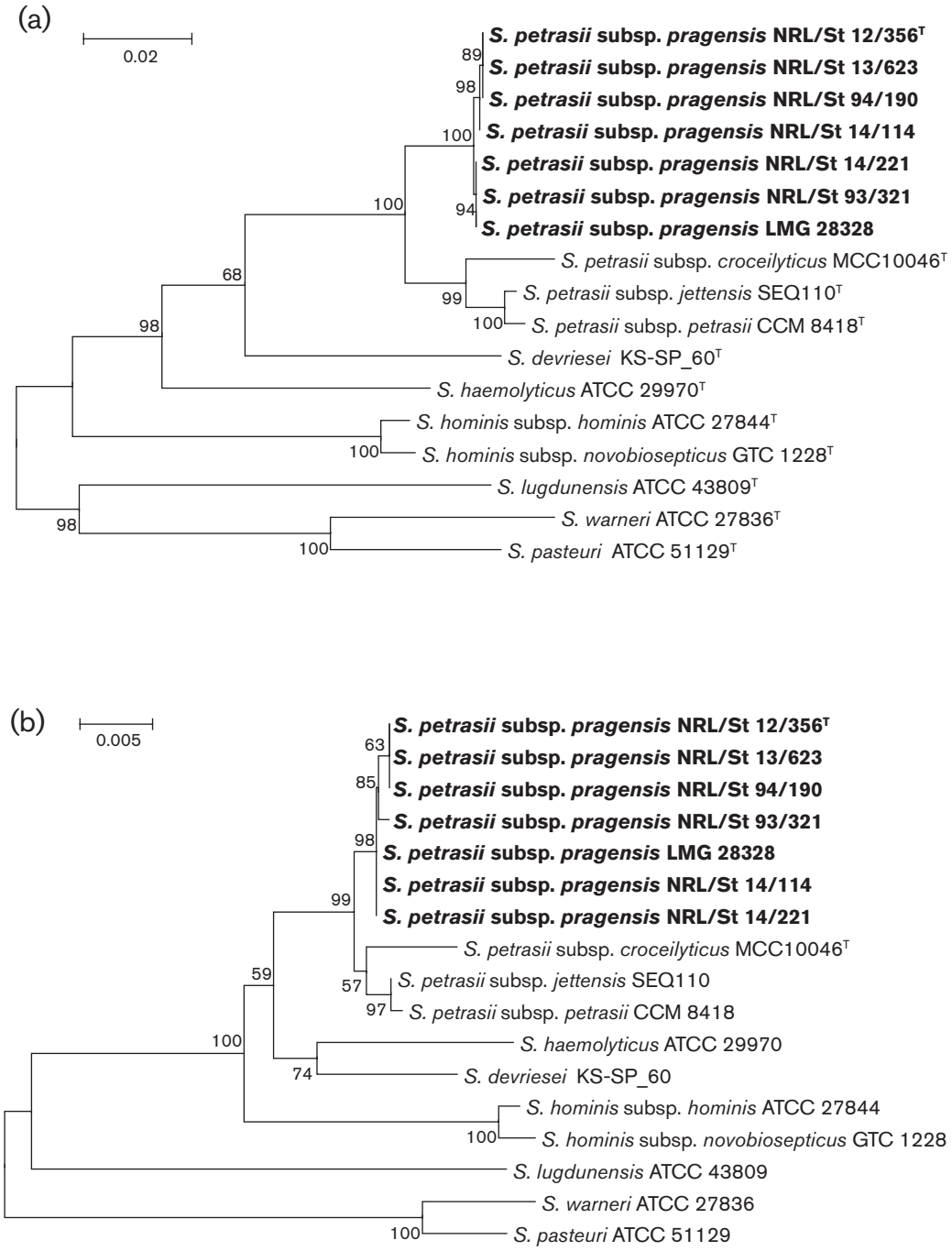

Fig. 2. a) Maximum-likelihood tree based on Kimura's two-parameter model reconstructed using concatenated partial gene sequences of five housekeeping genes (*hsp60*, *rpoB*, *dnaJ*, *gap* and *tuf*), showing the relationships of isolates of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. and staphylococcal reference type strains. The tree with the highest log-likelihood is shown and the percentage of trees from 1000 replicates in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were 3656 positions in the final dataset. b) Neighbour-joining tree reconstructed using concatenated partial sequences of five proteins Hsp60, RpoB, DnaJ, Gap and EF Tu, showing the relationships of isolates of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. and staphylococcal reference type strains. The bootstrap consensus tree inferred from 1000 replicates is shown and bootstrap probability values are indicated at branch points. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. There were 1243 amino acid positions in the final dataset.

51±6 %, which is clearly below the threshold value of 70 % generally accepted for bacterial species delineation.

Extensive phenotypic characterization using API 50CH, API Staph, ID 32 Staph, API ZYM (bioMérieux) and STAPHYtest 24 (Erba Lachema) commercial kits, phenotypic fingerprinting using the Biolog system with the Gram-positive identification test panel GP2 MicroPlate (Biolog), and conventional biochemical, physiological and growth tests relevant for the genus *Staphylococcus* were done as described by Pantůček *et al.* (2013). The obtained results are listed in the subspecies description. Extensive testing of clumping factor production using slide coagulase test and different commercial kits showed test-dependent results for *S. petrasii* subsp. *petrasii*. All strains revealed positive agglutination reactions when tested with Pastorex Staph plus test (Bio-Rad) and Staph Plus (DiaMondiaL), but negative results were obtained using the slide coagulase test and Staphylo La Seiken (Denka Seiken), Staphytect plus (Oxoid) and Staphylase test (Oxoid) kits. Representatives of *S. petrasii* subsp. *croceilyticus* and *S. petrasii* subsp. *jettensis*, as well as all seven studied isolates, revealed negative agglutination reactions using all aforementioned methods. Table 2 shows phenotypic characteristics differentiating the novel taxon represented by strain NRL/St 12/356^T from the phylogenetically closely related and phenotypically similar species. Table 3 shows the tests enabling differentiation of individual subspecies of *S. petrasii*. Susceptibility to antibiotics was tested by the disc diffusion method (Oxoid discs) on Mueller–Hinton agar (Oxoid, AST media) and interpreted according to the EUCAST v.4.0 (EUCAST 2014). Antimicrobial susceptibility profiles of the strains under study are given in the subspecies description.

In summary, data from the present study demonstrate that although the seven isolates represented by strain NRL/St 12/356^T belong to the species *S. petrasii*, they represent a taxon that can be distinguished both genotypically and phenotypically from established subspecies of *S. petrasii*. We therefore propose to classify these isolates as the novel subspecies *Staphylococcus petrasii* subsp. *pragensis*, with strain NRL/St 12/356^T (=CCM 8529^T=LMG 28327^T) as the type strain.

Description of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov.

Staphylococcus petrasii subsp. *pragensis* [prag.en′sis. N. L. masc. adj. *pragensis* of *Praga*, the Latin name for Prague (capital of the Czech Republic), pertaining to Prague where the type strain and two other strains were isolated].

Cells are Gram-stain-positive, non-spore-forming, non-motile, spherical cocci, occurring predominantly in pairs and clusters. Colonies on nutrient agar are circular with whole margins, flat, smooth, shiny, whitish, 1–3 mm in diameter and aerobic. Weak haemolytic activity on sheep-blood agar, production of δ-haemolysin revealed in a synergistic test with a β-haemolysin-producing strain. Grows at 45 °C; weak growth is observed at 15 °C. Growth in the presence of 12 % NaCl is strain-dependent. No growth occurs in thioglycolate medium. Positive results in tests for catalase, pyrrolidonyl arylamidase, arginine arylamidase, Voges–Proskauer reaction (acetoin) and hydrolysis of DNA. Negative results in tests for coagulase, clumping factor, hyaluronidase, thermonuclease, oxidase, urease and ornithine decarboxylase. Susceptible to novobiocin, polymyxin B and furazolidone, but resistant to bacitracine. Susceptible to lysostaphin and resistant to lysozyme. Hydrolysis of aesculin, Tween 80 and

Table 2. Key tests for phenotypic differentiation of *Staphylococcus petrasii* subsp. *pragensis* subsp. nov. from phylogenetically related and phenotypically similar species of the genus *Staphylococcus*

Species: 1, *S. petrasii* subsp. *pragensis* subsp. nov. (data from this study); 2, *S. petrasii* (including subspecies *petrasii*, *croceilyticus* and *jettensis*) (Pantůček *et al.*, 2013; De Bel *et al.*, 2013, 2014); 3, *S. haemolyticus* (Schleifer & Bell, 2009); 4, *S. warneri* (Schleifer & Bell, 2009); 5, *S. hominis* subsp. *hominis* (Schleifer & Bell, 2009); 6, *S. pasteuri* (Schleifer & Bell, 2009); 7, *S. devriesei* (Supré *et al.*, 2010); 8, *S. hominis* subsp. *novobiosepticus* (Schleifer & Bell, 2009); 9, *S. lugdunensis* (Schleifer & Bell, 2009). +, 90 % or more strains positive; −, 90 % or more strains negative; d, 11–89 % strains positive; (+), delayed reaction; w, weak reaction; td, test dependent reaction; in the case of *S. petrasii* subsp. *pragensis* subsp. nov. the test results are: +, 100 % strains positive; −, 100 % strains negative.

Characteristic	1	2	3	4	5	6	7	8	9
Clumping factor	−	td*	−	−	−	−	−	−	+
Novobiocin resistance (5 μg)	−	−	−	−	−	−	−	+	−
Thioglycolate (growth)	−	−	(+)	+	−w	+	+	−w	+
Pyrrolidonyl arylamidase	+	+	+	−	−	−	−	−	+
Urease	−	d	−	+	+	+	d	+	d
Haemolysis	w	d	(+)	d	−w	+	+	−	w

*Data obtained in this study; strains of *S. petrasii* subsp. *petrasii* reveal positive agglutination reaction when tested with Pastorex Staph plus test (Bio-Rad) and Staph Plus (DiaMondiaL), while negative when tested using slide coagulase test and Staphylo La Seiken (Denka Seiken), Staphytect plus (Oxoid) and Staphylase test (Oxoid) kits.

Table 3. Phenotypic differentiation of subspecies of *Staphylococcus petrasii*. Subspecies: 1, *S. petrasii* subsp. *pragensis* subsp. nov.; 2, *S. petrasii* subsp. petrasii; 3, *S. petrasii* subsp. croceilyticus; 4, *S. petrasii* subsp. *jettensis*. +, Positive; −, negative; w, weak reaction; d, strain dependent.

Characteristic	1	2	3	4
Acid production from:				
D-Arabinose	−	−	+	−
L-Fucose	−	−	+	−
Mannose	−	+	−	−
Arginine dihydrolase	d	+	+	+
DNase	+	w	−	−
β-Glucuronidase	d	−	+	d
Urease	−	+	+	−
Utilization of D-mannitol	−	d	−	+

gelatin is negative. Esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present, but not lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, α-mannosidase or α-fucosidase activities. Acid is produced from glycerol, D-glucose, maltose and sucrose, but not from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl β-D-xyloside, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, cellobiose, melibiose, inulin, raffinose, starch, glycogen, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, and 2-ketogluconate. Test results were strain-dependent for acid production from ribose (3 strains positive among 7 strains), galactose (2), fructose (3), mannitol (1), salicin (1), lactose (1), trehalose (6), melezitose (4), turanose (5), and 5-ketogluconate (2); activity of alkaline phosphatase (5), leucine arylamidase (5), valine arylamidase (2), cystine arylamidase (1), β-glucuronidase (1), α-glucosidase (5), β-glucosidase (2), *N*-acetyl-β-glucosaminidase (1), arginine dihydrolase (3) and nitrate reduction (1). Carbon source utilization results via respiration determined in the Biolog GP2 MicroPlate test panel is shown in Table S2. Tested strains were susceptible to cefoxitin, ciprofloxacin, clindamycin, erythromycin, trimethoprim/sulfamethoxazole, fusidic acid, gentamicin, rifampicin, tigecycline, mupirocin, tetracycline, chloramphenicol, linezolid, benzylpenicilin, vancomycin and teicoplanin with the exception of strain NRL/St 12/356^T (resistant to erythromycin and tetracycline) and strain NRL/St 13/623 (resistant to benzylpenicilin).

The type strain NRL/St 12/356^T (=CCM 8529^T=LMG 28327^T) was retrieved from an ejaculate specimen from a 58-year-old male suffering with prostatitis in Prague (Czech Republic) in May 2012. The DNA G+C content of the type strain is 33.0 mol% (HPLC). Most characteristics of the type strain are in agreement with the general subspecies description. In addition, strain NRL/St 12/356^T displays the following characteristics: acid production from trehalose, melezitose and turanose, but not from ribose, galactose, fructose, mannitol, salicin, lactose or 5-ketogluconate; exhibits α-glucosidase activity, but not alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase or arginine dihydrolase activity; negative for nitrate reduction and growth in 12 % NaCl.

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