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TAXONOMIC DESCRIPTION

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Staphylococcus borealis sp. nov., isolated from human skin and blood

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Abstract

When analysing a large cohort of *Staphylococcus haemolyticus*, using whole-genome sequencing, five human isolates (four from the skin and one from a blood culture) with aberrant phenotypic and genotypic traits were identified. They were phenotypically similar with yellow colonies, nearly identical 16S rRNA gene sequences and initially speciated as *S. haemolyticus* based on 16S rRNA gene sequence and MALDI-TOF MS. However, compared to *S. haemolyticus*, these five strains demonstrate: (i) considerable phylogenetic distance with an average nucleotide identity <95% and inferred DNA–DNA hybridization <70%; (ii) a pigmented phenotype; (iii) urease production; and (iv) different fatty acid composition. Based on the phenotypic and genotypic results, we conclude that these strains represent a novel species, for which the name *Staphylococcus borealis* sp. nov. is proposed. The novel species belong to the genus *Staphylococcus* and is coagulase- and oxidase-negative and catalase-positive. The type strain, 51-48^T, is deposited in the Culture Collection University of Gothenburg (CCUG 73747^T) and in the Spanish Type Culture Collection (CECT 30011^T).

INTRODUCTION

Members of the genus *Staphylococcus*, currently consisting of 54 species and 22 subspecies with validly published names (based on the List of Prokaryotic Names with Standing in Nomenclature, https://lpsn.dsmz.de), are most often found on the skin and mucus membranes of mammals and birds [1]. Staphylococci, and particularly the coagulase-positive *Staphylococcus aureus*, are a major cause of clinical disease in both humans and animals [2–4]. The coagulase-negative staphylococci (CoNS) colonize different niches of the human skin [5] and are part of the commensal human host microbiota. However, over the last decades some CoNS species such as *Staphylococcus epidermidis*, *Staphylococcus hominis* and *Staphylococcus haemolyticus* have emerged as important opportunistic pathogens primarily causing disease in patients with foreign body implants or impaired immunity [2].

As part of a previous study analysing a large cohort of *S. haemolyticus* [6], we detected five bacterial strains with aberrant phenotypic and genotypic traits. All five strains originated from the same geographic location, Tromsø, in North Norway. Four strains were isolated from skin swabs from the groin and armpit of healthy volunteers [5], and one strain was isolated from blood culture in 1997 at the University Hospital of North Norway [7]. The five strains were all initially identified as *S. haemolyticus* based on 16S rRNA gene sequencing and matrix-assisted laser desorption ionization time-of-flight

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Keywords: Coagulase-negative staphylococci; *Staphylococcus borealis*; *Staphylococcus haemolyticus*; *Staphylococcus*; urease; whole-genome sequencing.

Abbreviations: ANI, average nucleotide identity; CFA-FAME, cell fatty acid–fatty acid methyl ester; CoNS, coagulase-negative staphylococci; dDDH, digital DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator; HSP, high-scoring segment pairs; ML, maximum-likelihood; MSA, multi sequence alignment; OGRI, overall genome related index; UBCG, Up-to-date Bacterial Core Gene.

The 16S rRNA gene sequence of *Staphylococcus borealis* 51-48^T is available under the accession number MT586030. The genome sequence data from *Staphylococcus borealis* 51-48^T is available under these accessions: BioSample number SAMN15197055 and assembly accession number GCA_013345165.1. The genome sequence data from *Staphylococcus borealis* strains 57-14, 57-74, 58-22 and 58-52 are available under BioSample numbers SAMN15197056, SAMN15197057, SAMN15197058 and SAMN15197059, and assembly accession numbers GCA_013345185.1, GCA_013345175.1, GCA_013345195.1 and GCA_013345205.1, respectively.

One supplementary figure and four supplementary tables are available with the online version of this article.

Table 1. Overview of genomic information for all eight *Staphylococcus borealis* strains and the *Staphylococcus haemolyticus* type strain

Isolate ID	Genome size	Contigs	CDS	N50	G+C content (mol%)	Coverage	Accession
51-48^T(=CCUG 73747^T=CECT 30011^T)	2797948 bp	5	2529	2689815	33.75	292.2×	GCA_013345165.1
57-14 (=CCUG 73748=CECT 30010)	2626230 bp	36	2403	645817	33.66	337.7×	GCA_013345185.1
57-74 (=CCUG 73749)	2615713 bp	41	2398	390616	33.66	463.8×	GCA_013345175.1
58-22 (=CCUG 73750)	2666192 bp	40	2475	391465	33.69	319.4×	GCA_013345195.1
58-52 (=CCUG 73751)	2664706 bp	30	2420	805534	33.54	387.0×	GCA_013345205.1
Staphylococcus sp. 170179	2629435 bp	48	2324	212499	33.58	334.0×	GCA_009735325.1
SNUC119	2521961 bp	166	2288	42538	33.80	50×	GCA_003580835.1
SNUC1342	2522218 bp	99	2290	119419	33.80	93×	GCA_003042555.1
S. haemolyticus NCTC 11042^T	2569468 bp	4	2323	2515409	32.90	100×	GCA_900458595.1

mass spectrometry (MALDI-TOF MS) using a Microflex LT instrument (Bruker Daltonics), Flex Control software and the MALDI Biotyper 3.1 software (Bruker Daltonics). The genome of the blood culture strain was published as *S. haemolyticus* under the accession number GCA_001224225.1 in 2015 [7].

We decided to perform further analyses of these five strains due to the differences observed in both genotypic and phenotypic features compared to other *S. haemolyticus* strains. Based on results from established phenotypic and genotypic methods for species identification [8, 9], we propose that these five strains belong to a new staphylococcal species hereafter designated *Staphylococcus borealis* sp. nov.

WHOLE-GENOME SEQUENCING (WGS) AND PHYLOGENETIC ANALYSIS

Genomic DNA from the blood culture strain was isolated according to Chachaty and Saulnier [10] with the addition of RNase A (10 mg ml^{-1}; Qiagen) for Illumina sequencing, and the MasterPure Gram Positive DNA Purification Kit (Lucigen) for isolating genomic DNA for PacBio sequencing. Genomic DNA from the four commensal isolates was isolated by using the Wizard Genomic DNA kit (Promega). WGS was performed using the Illumina Genome Analyzer II (for the blood culture strain) and Illumina MiSeq (for the four commensal strains), as described previously [6, 7]. Strain 51-48^T was additionally sequenced with a PacBio RS II instrument (Pacific Biosciences) at the Norwegian Sequencing Centre (NSC), Oslo, Norway. Assembly of Illumina reads was done using Shovill version 0.8.0 (https://github.com/tseemann/shovill). For the PacBio long reads, consensus sequences were generated and assembled with HGAP version 3 (Pacific Biosciences, SMRT Analysis Software version 2.3.0) [11]. The assembly was polished with Pilon version 1.23 [12], using the Illumina sequences (BioSample, SAMEA1035138; SRA, ERS066311) generated previously by Cavanagh *et al.* [7]. Mapping of Illumina sequences and the PacBio assembly were done using BWA-MEM (version 0.715-r1140) [13]. The resultant draft genome sequences were deposited in GenBank under the BioProject PRJNA638539.

As these strains were initially identified as *S. haemolyticus* based on both 16S rRNA gene sequence similarity and MALDI-TOF, we performed a core-genome phylogeny analysis on all available *S. haemolyticus* genomes deposited in the National Center for Biotechnology Information (NCBI) at the time to see whether there were additional isolates related to *S. borealis*. Two draft genomes isolated from cattle in Canada (SNUC119, assembly accession nos. GCA_003580835.1 and SNUC 1342; assembly accession no. GCA_003042555.1) [14] clustering with the five proposed *S. borealis* strains were identified. Additionally, a recent publication presented the draft genome of a novel *Staphylococcus* species isolated from human skin in Denmark (*Staphylococcus* sp. strain 170179, accession no. GCA_009735325.1); the authors reported that the closest related genome was the clinical *S. borealis* strain (51-48^T, GCA_001224225.1) [15]. These three additional draft genomes were included in all the comparative genomic analyses. All eight genomes were annotated with Prokka (version 1.13) [16] for downstream analysis.

The genome size range was 2521961–2797948 bp, with 2288–2529 coding sequences (CDSs). The G+C content of the novel species ranged from 33.54 to 33.80 mol% (Table 1), which is in the range of 33–40 mol% expected for species of the genus *Staphylococcus* [1]. The G+C content was 0.64–0.9 mol% higher than that of *S. haemolyticus* NCTC 11042^T.

The draft genome of strain 51-48^T was 2797948 bp long and had 292.2× depth of sequencing coverage. The draft genome had 33.75 mol% G+C content and contained a total of 2529 CDS, 22 rRNA (Among the 22 rRNA, one 5S rRNA and one 16S rRNA were partial sequences) genes (eight copies of each 5S rRNA, seven copies of 16S rRNA and seven copies of 23S rRNA), one tmRNA and 67 tRNAs.

The 16S rRNA gene sequences of *S. borealis* 51-48^T, 57-14, 57-74, 58-22 and 58-52 was determined by Sanger sequencing (forward primer; 5′-TACATGCAAGTCGAGCGAAC-3′ and

Table 2. Overview of results for species identity of the closest related *Staphylococcus* species and subspecies, compared to the proposed type strain of *Staphylococcus borealis* 51-48^T

The numbers in brackets are the threshold values for species delineation

Staphylococcal type strains	16S rRNA gene (98.7%)	ANIb (<95%)	ANIm (<95%)	Tetra (<0.989)	dDDH (<70%)
S. devriesei NCTC 13828^T	99.25	79.82	84.92	0.95612	23.8
S. petrasii subsp. *petrasii* CCM8418^T	99.39	80.10	85.49	0.96349	23.3
S. petrasii subsp *croceolyticus* CCM8421^T	99.39	80.35	85.72	0.96578	23.5
S. petrasii subsp. *jettensis* SEQ110^T	99.51	80.28	85.65	0.96301	23.6
S. petrasii subsp. *pragensis* NRL/St 12/356^T	99.46	80.55	85.74	0.96981	23.6
S. hominis subsp. *hominis* DSM 20328^T	99.25	78.54	85.11	0.95310	22.6
S. hominis subsp. *novobiosepticus* GTC 1228^T	98.83	78.67	85.38	0.95569	23.0
S. haemolyticus NCTC 11042^T	99.86	87.40	88.66	0.98571	34.2
SNUC 119	99.93	97.67	98.14	0.99807	82.1
SNUC 1342	100	97.65	98.18	0.99727	82.4
Staphylococcus sp. 170179	–	99.54	99.80	0.99903	98.0
S. borealis 57-14	100	99.58	99.82	0.99895	97.2
S. borealis 57-74	100	99.56	99.78	0.99879	97.4
S. borealis 58-22	99.93	99.56	99.83	0.99910	98.0
S. borealis 58-52	99.93	99.74	99.81	0.99905	98.1

reverse primer 5′-ACCTTCCGATACGGCTACCT-3′). The 16S rRNA sequence of SNUC119, SNUC1342 and 170179 was retrieved from the genome assemblies. The 16S rRNA gene sequences from the *S. borealis* strains were analysed using the EzBioCloud online tool 16S-based ID [17]. These results showed that the highest similarities were found to *S. haemolyticus* NCTC 11042^T (99.79% for strain SNUC119, 99.86% for strains 51-48^T, 57-14, 57-74 and SNUC1342; 99.93% for 58-22 and 58-52) and *Staphylococcus petrasii* subsp. *jettensis* (99.5% for isolates 51-48^T, 57-14, 57-74 and SNUC1342; 99.4% for 58-22, 58-52 and SNUC119) (Table 2). The full-length 16S rRNA gene was detected in all strains but SNUC119 (96.7% of full length). For the Danish strain 170179 the 16S rRNA gene was fragmented with some parts missing, and was omitted

Table 3. Percent identity between housekeeping genes of *S. borealis* 51-48^T and *S. haemolyticus* NCTC 11042^T and the intraspecies variations for each gene within each species

Intraspecies variation within *S. haemolyticus* was based on representatives from each phylogenetic group of a diverse collection of *S. haemolyticus* strains [6]. For the *S. borealis* strains of human origin (51-48, 57-14, 57–74, 58-22, 58-52 and 170179) all housekeeping genes with the exception of the 16S rRNA gene were identical. Within all housekeeping genes (except 16S rRNA), *S. borealis* specific conserved bases were observed (specific bases/SNPs found in all *S. borealis* strains and in no *S. haemolyticus* strains)

	Housekeeping genes (identity cut-off)								
	tuf **(98%) [22]**	***gap*** **(96%) [21]**	***sodA*** **(97%) [23]**	***rpoB*** **(93.6%) [24]**	***hsp60*** **(93%) [26]**	***dnaJ*** **(88.8%) [25]**	***recA*** **[27]**	***gyrB*** **[28]**	**16S (98.7%)**
S. haemolyticus NCTC 11042^T versus *S. borealis* 51-48^T	99.2%	99.4%	97.2%	96.1%	91.4%	93.1%	91.2%	94.7%	99.86%
S. borealis intraspecies variation	100%	99.7–100%	99.8–100%	99.3–100%	99.1–100%	99.4–100%	99–100%	99.5–100%	99.93–100%
Conserved SNPs only in *S. borealis*	4	9	12	113	117	61	70	83	0
S. haemolyticus intraspecies variation	99–100%	99.3–100%	98.2–100%	98.9–100%	98.3–100%	97.7–100%	97.0–100%	98.7–100%	99.67–100%

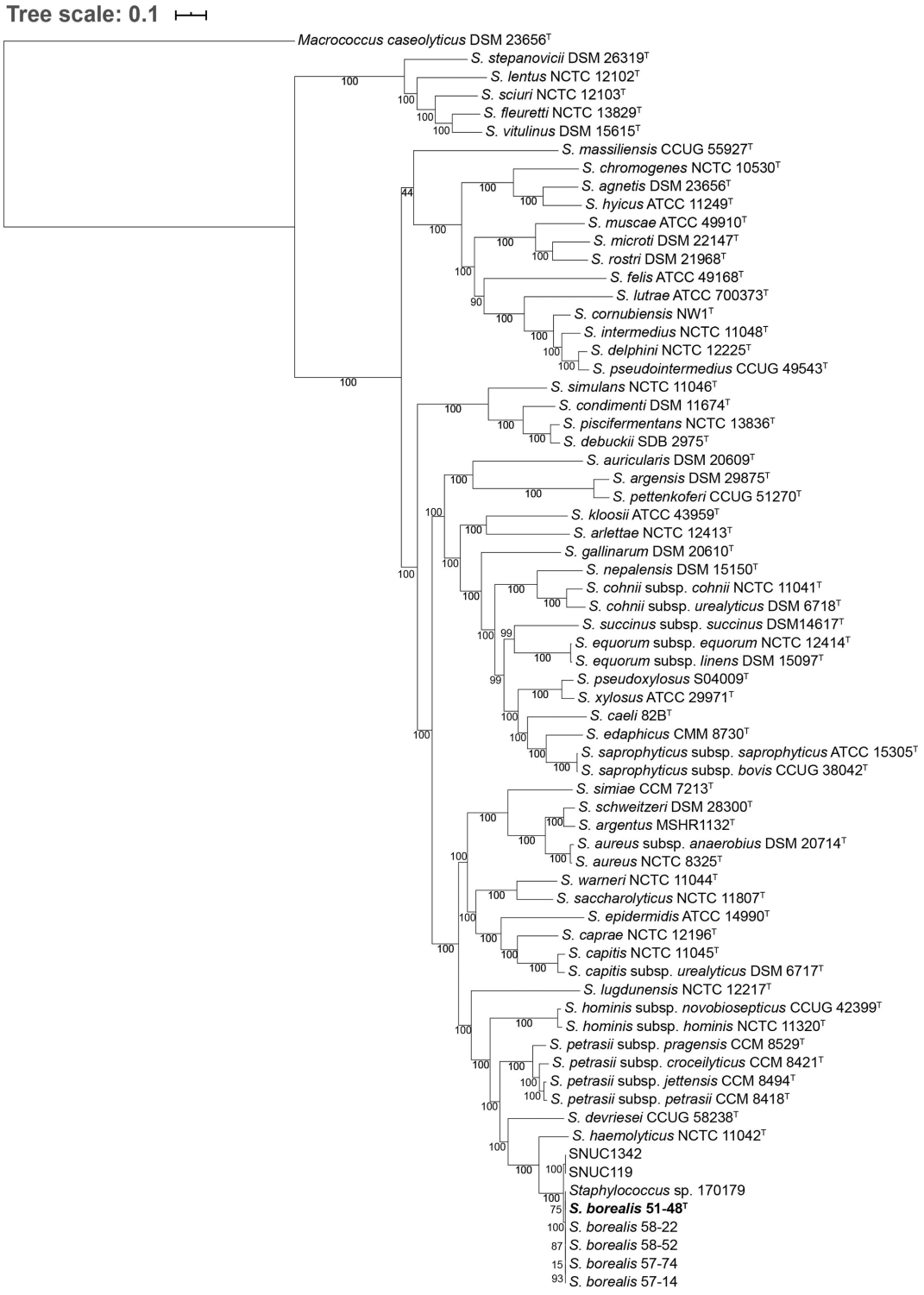

Fig. 1. Phylogenetic relationship of Staphylococcal type strains and the eight *S. borealis* strains based on core genes. The maximum-likelihood method was used and bootstrapping was set to 100 replicates, using the RAxML software. *Macrococcus caseolyticus* was used for rooting the tree.

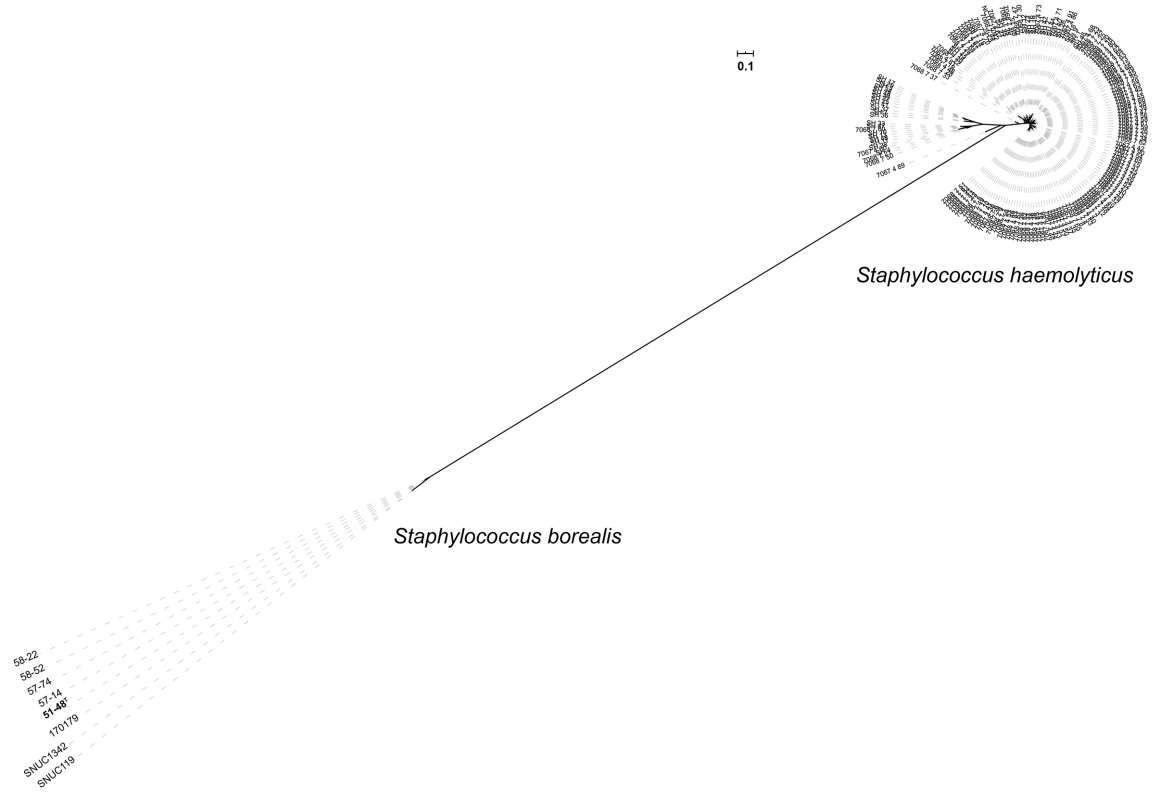

Fig. 2. SNP-based core-genome phylogenetic tree using the kSNP3 suite, of 169 *S. haemolyticus* strains and the eight *S. borealis* strains.

from the analysis. In total 16 staphylococcal species and subspecies showed 16S rRNA gene identity >98.7%.

The complete 16S rRNA gene sequences of all staphylococcal type strains were downloaded from the EzBioCloud database [17] and a multi sequence alignment (MSA) was created in MEGA7 [18] using the software MUSCLE (MUltiple Sequence Comparison by Log-Expectation) [19]. The maximum-likelihood (ML) method was used and bootstrapping was set to 500 replicates, using the RAxML software [20]. Fig. S1 (available in the online version of this article) shows the reconstructed phylogenetic tree generated from the MSA of the 16S rRNA gene sequence of the staphylococci type strains and accession numbers are listed in Table S1.

We also retrieved 16S rRNA gene sequences from a diverse collection of *S. haemolyticus* (five representative strains from each of six phylogenetic groups [6]) and compared them to *S. borealis*. We identified intraspecies variations in 16S rRNA gene among the diverse groups of *S. haemolyticus*, with some strains having identical 16S rRNA gene sequences to *S. borealis*, emphasizing that 16S rRNA cannot be used to distinguish between the two species.

As the 16S rRNA gene was unable to discriminate *S. haemolyticus* from *S. borealis* we investigated whether other single housekeeping genes could distinguish the two species. We analysed the sequence identity between *S. haemolyticus* NCTC 11042^T and *S. borealis* 51-48^T of the following housekeeping genes: *gap* [21], *tuf* [22], *sodA* [23], *rpoB* [24], *dnaJ* [25], *hsp60* [26], *recA* [27] and *gyrB* [28]. Additionally, we looked at intraspecies variations within the eight *S. borealis* strains and within a diverse group of 30 *S. haemolyticus* strains (the same strains as used in 16S rRNA comparison). Even though only the *hsp60* gene meets the cut-off criteria for different species, we believe that the genes *rpoB*, *dnaJ*, *hsp60*, *recA* and *gyrB* can be used to discriminate between *S. haemolyticus* and *S. borealis* as *S. borealis* contains many unique signature bases (found in all eight *S. borealis* strains and not identified in any of the tested *S. haemolyticus*). These results are summarized in Table 3.

We also performed multilocus sequence typing (MLST) using the *S. haemolyticus*-specific MLST-scheme [29]. All *S. borealis* strains were non-typeable following this scheme, and we observed variations ranging from 23 to 79 SNPs for each of the seven genes to the closest allele in the MLST database, including gaps and insertions, further supporting the identification of a new species.

Genome-based phylogeny plays a central role in taxonomy and phylogeny of bacteria and provides higher resolution than 16S rRNA/single gene phylogeny [30]. WGS comparisons were performed according to the recommended minimal standards for description of new staphylococcal species [8]. We used the up-to-date bacterial core gene set (UBCG) [30], which produced an alignment based on 92 single-copy core

Table 4. Antibiotic resistance genes

The resistance genes listed in the table can confer resistance to the following antimicrobials: *ANT4*, aminoglycoside; *ble*, bleomycin; *ermC*, erythromycin; *fusC*, fusidic acid; *mgrA*, global regulator (*β*-lactams and quinolones); *qacC*, quaternary ammonium compounds; *vga(A)*, streptogramin A lincosamides and/or pleuromutilins identified in the different *S. borealis* strains, based on the antibiotic databases CARD, MEGARes and NCBI. For each resistance gene the percentage identity with the genes identified in the *S. borealis* strain is presented. For numbers marked in bold the resistance phenotype was also confirmed

	ANT4	*ble*	*ermC*	*fusC*	*mgrA*	*tetK*	*qacC*	*vga(A)*
51-48^T			**100%**		93%			98.5%
57-14					93%			98.5%
57-74					93%			98.5%
58-22					93%			98.5%
58-52			**100%**	**100%**	93%		100%	98.5%
170179	100%	100%		100%	93%			98.5%
SNUC119					93%	100%		98.5%
SNUC1342					93%			98.5%

genes extracted from WGS of staphylococcal type strains available in GenBank (accession numbers listed in Table S1). From the concatenated gene sequences extracted by UBCG, a ML phylogenetic tree was inferred using RAxML (raxmlGUI2 beta) [20] using the GTRGAMMA model and 100 bootstrap replications [31]. The results from the phylogenomic tree confirmed that the eight *S. borealis* strains belong to a novel CoNS species forming their own well-supported branch (Fig. 1) most closely related to *S. haemolyticus*, *S. hominis*, *S. petrasii* and *Staphylococcus devriesei*.

We used the overall genome related index (OGRI) methods to calculate average nucleotide identity (ANI) and tetra-nucleotide analysis using the online tool JSpeciesWS [32]. The digitalDNA–DNA hybridization (dDDH) values were calculated using the Genome-to-Genome Distance Calculator

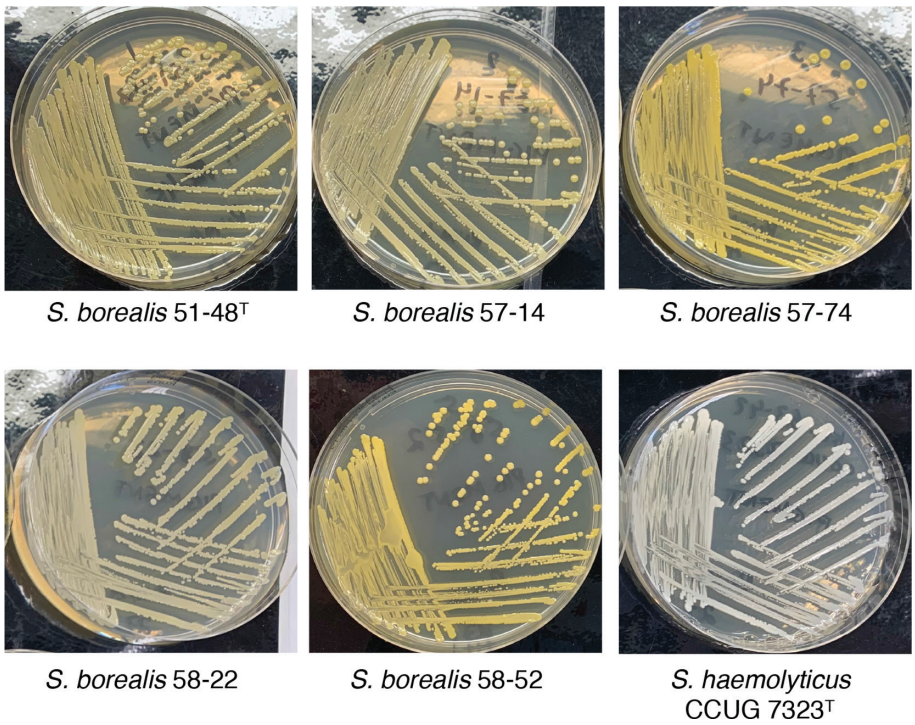

Fig. 3. Yellow pigmentation of the five *S. borealis* isolates from this study, in comparison to *S. haemolyticus* CCUG 7323^T shown on P-agar.

Table 5. Biochemical tests, based on APIStaph, API 32 Staph and API Coryne, of the five *Staphylococcus borealis* isolates and the type strain of *Staphylococcus haemolyticus*

		Staphylococcus borealis					*Staphylococcus haemolyticus*
Culture Collection University of Gothenburg (CCUG)		**73747^T**	**73748**	**73749**	**73750**	**73751**	**7323^T**
Local strain identification number		**51-48**	**57-14**	**57-74**	**58-22**	**58-52**	**63-42**
Test							
Glucose	GLU	+	+	+	+	+	+
Fructose	FRU	+	+	+	+	+	–
Arabinose	ARA	–	–	–	–	–	–
Ribose	RIB	+	+	+	+	+	+
Mannose	MNE	–	–	–	–	+	–
Xylose	XYL	–	–	–	–	–	–
Sucrose	SAC	+	+	+	+	+	+
Lactose	LAC	–	–	–	–	–	–
Turanose	TUR	–	+	+	–	–	–
Cellobiose	CEL	–	–	–	–	–	–
Maltose	MAL	+	+	+	+	+	+
Trehalose	TRE	+	+	+	+	+	+
Melibiose	MEL	–	–	–	–	–	–
Raffinose	RAF	–	–	–	–	–	–
Glycogen	GLYG	–	–	–	–	–	–
N-Acetyl-glucosamine	NAG	+	–	+	+	–	+
Methyl α-D-glucopiranoside	MDG	–	+	+	+	+	–
Mannitol	MAN	+	+	+	+	+	–
Xylitol	XLT	–	–	–	–	–	–
Nitrate	NIT	+	+	+	+	+	+
Acetoin production	VP	–	–	+	–	+	+
Novobiocin	NOVO	–	–	–	–	–	–
Gelatin	GEL	–	–	–	–	–	–
Aesculin	ESC	+	–	+	–	+	–
Catalase	CAT	+	+	+	+	+	+
Urease	URE	+	+	+	+	+	–
N-Acetyl-*β*-glucosaminidase	*β*NAG	–	–	–	–	–	–
α-Glucosidase	αGLU	–	–	–	–	–	–
β-Galactosidase	*β*GAL	–	–	–	–	–	–
β-Glucuronidase	*β*GUR	+	–	–	+	–	+
Alkaline phosphatase	PAL	+	–	–	+	–	+
Pyrazinamidase	PYZ	+	+	+	+	+	+
Arginine arylamidase	ArgA	–	–	–	–	–	–

Continued

Table 5. Continued

		Staphylococcus borealis					*Staphylococcus haemolyticus*
Culture Collection University of Gothenburg (CCUG)		**73747^T**	**73748**	**73749**	**73750**	**73751**	**7323^T**
Local strain identification number		**51-48**	**57-14**	**57-74**	**58-22**	**58-52**	**63-42**
Pyrrolidonyl arylamidase	PyrA	+	+	+	+	+	+
Ornithine decarboxilase	ODC	–	–	–	–	–	–
Arginine dihydrolase	ADH	+	+	+	+	+	+

(GGDC) version 2.1 [33]. The GGDC results were based on the recommended formula 2 (sum of all identities found in high-scoring segment pairs (HSPs), divided by the overall HSP length), which is independent of genome size. Both the ANI and dDDH values between the *S. borealis* strains and related staphylococci were much lower than those considered to represent the same species [9]. The results from all OGRI methods confirm that the eight *S. borealis* strains belong to a novel species, which is related to, but distinctly different from, *S. haemolyticus*. The OGRI values between the closest-related staphylococcal type strains and the *S. borealis* strains are summarized in Table 2. The OGRI values between the individual *S. borealis* strains confirmed that these were different strains and that they belonged to the same species (Tables S2 and S3).

Based on the WGS data we reconstructed a core-genome SNP-based ML tree using the KSNP3 package [34] including 169 *S. haemolyticus* strains, our five *S. borealis* strains and the three draft genomes similar to *S. borealis* found in NCBI. The resultant ML tree clearly demonstrates that the five *S. borealis* strains and the strains included from the NCBI form a distinct cluster separated from *S. haemolyticus* (Fig. 2).

Based on the OGRI analysis we clearly see a significant difference in the genomes between *S. borealis* and the closest-related staphylococcal species. To what extent this represents *S. borealis*-specific genes or SNP variability within genes of similar functions, the OGRI tools does not answer. Thus, in order to identify genes specific for *S. borealis*, a genome comparison between the eight *S. borealis* strains and 169 whole-genome sequences of *S. haemolyticus* strains [6] was performed. We used the pan-genome tool Roary version 3.11.2 [35] at default settings but changed the parameter for minimum percentage identity for BLASTp to 70% in order to identify genes significantly different between the two species. We then extracted the genes found in all eight *S. borealis* which were found in 1% or less of the 169 *S. haemolyticus* strains. We identified 74 *S. borealis* specific genes (Table S4), and among these genes we found a urease operon (*ureABCEFGD*). Performing pan-genome analysis with the default minimum percentage for BLAST at 95% produced a *S. borealis* pan-genome of 3267 genes, of which 1480 were *S. borealis* specific and not shared with any *S. haemolyticus* strains. The common core genome between the two species comprised 861 genes, which totals 34.6% of the average *S. borealis* gene content.

All eight *S. borealis* genomes contained capsule-like genes, similar to both the *S. haemolyticus* described capsule (*capA-capG*, and *capK-capM*) and to *S. aureus* capsule genes (*cap5H-cap5J* and *cap5/8L-cap5/8P*). The presence of capsule-like genes was initially identified by performing a local BLAST of the *S. borealis* genomes against the virulence factor database (VFDB) [36], and was subsequently manually inspected in all eight strains. The six human-associated strains contained the same capsule-like operon, while the two Canadian animal associated strains had a slightly different type. Whether these are functional genes, and their role in this species remains to be investigated.

Antibiotic resistance genes were identified using the following databases: the Comprehensive Antibiotic Resistance Database (CARD) [37], NCBI AMRFinderPlus [38] and MEGARes [39]. The identified antibiotic resistance genes are summarized in Table 4. All eight strains contained a *vga(A)* gene variant (98.48% identity). *Vga(A)* variants confer different levels of resistance to streptogramin A, lincosamides and/or pleuromutilins [40–42]. All eight strains also harboured *mgrA*, a global regulator shown to play a role in regulation of virulence factors and contributing to decreased susceptibility to antibiotics like quinolones and β-lactams [43].

PHENOTYPIC TESTS AND METABOLIC PROFILING

The phenotype and metabolic profiling were only performed on the five *S. borealis* strains identified in our own collection. Coagulase activity was determined using the Staphaurex Plus Latex Agglutination Test (Thermo Scientific). DNAse activity was tested on DNAse agar with methyl green (Oxoid). Catalase production was determined by the slide catalase test using hydrogen peroxide, and oxidase activity was determined using the filter paper spot method with 1% Kovács oxidase reagent. All five *S. borealis* strains were Gram-stain-positive cocci growing in clusters. They were non-motile on motility agar. All were oxidase-, DNAse-, coagulase- and clumping factor-negative, and catalase-positive. All five strains were

Table 6. Cell fatty acid–fatty acid methyl ester (CFA-FAME) analysis of *S. borealis* strains (CCUG 73747^T, CCUG 73748, CCUG 73749, CCUG 73750, CCUG 73751), showing the ECL (equivalent chain length), name of CFAs and the area per peak (%). Also shown are the corresponding CFA profiles of *S. aureus* (CCUG 1800^T), *S. devriesei* (CCUG 58238^T) and *S. haemolyticus* (CCUG 7323^T). TR denotes 'trace' which means a peak has been recorded, but too small to be integrated

ECL	13.618	14.621	14.711	15.626	16.000	16.629	16.722	17.724	17.769	18.000	18.633	18.729	20.000
Peak name of CFA	**C14:0 iso**	**C15:0 iso**	**C15:0 antesio**	**C16:0 iso**	**C16:0**	**C17:0 iso**	**C17:0 antesio**	**Summed feature***	**C18:1 ω9*c***	**C18:0**	**C19:0 iso**	**C19:0 antesio**	**C20:0**
Species													
S. aureus CCUG 1800^T	TR	7.3	55.1	1.6	2.0	3.9	19.5	3.3	1.4	2.6	TR	1.3	TR
S. devriesei CCUG 58238^T	0	3.2	52.1	TR	1.3	5.1	25.8	2.6	1.5	2.5	1.0	3.8	TR
S. haemolyticus CCUG 7323^T	1.1	6.9	42,7	1.3	1.8	6.8	18.8	1.2	0	8.5	2.8	5.7	2.5
S. borealis 51-48 CCUG 73747^T	1.1	10.0	61.8	TR	1.1	4.9	12.8	TR	1.0	2.3	1.0	1.9	TR
S. borealis 57-14 CCUG 73748	TR	12.0	64.3	TR	1.2	5.6	13.1	0	TR	2.4	TR	1.6	0
S. borealis 57-74 CCUG 73749	1.1	10.9	65.6	TR	TR	4.6	12.2	TR	TR	2.1	1.3	2.3	TR
S. borealis 58-22 CCUG 73750	1.2	10.8	61.1	1.0	1.2	5.5	13.3	TR	TR	2.6	1.1	1.7	TR
S. borealis 58-52 CCUG 73751	1.3	11.7	59.2	1.1	1.7	5.3	13.8	TR	TR	3.9	0	1.2	TR

facultative anaerobic, as determined by using the Brewer thioglycollate medium.

Scanning electron microscopy analyses were performed using a Zeiss Sigma scanning electron microscope (SEM; Zeiss). Whole cells were fixed with 2.5% glutaraldehyde and 4% formaldehyde in PHEM-buffer, before sedimentation onto poly-L-lysin coated coverslips. Samples were further processed according to the protocol of Cocchiaro using the Pelco Biowave (Ted Pella) [44]. Samples were dried in a Leica EM CPD300 and mounted on SEM stubs; gold/palladium was applied with a Polaron Range Sputter Coater. The bacterial cells had a round coccoid shape, appeared in clusters and had a diameter of 650 nm to 1.23 µm.

We tested temperature (4, 15, 30, 37, 42 and 45 °C) and NaCl tolerance (0, 0.5, 1.5, 3, 5, 7.5, 10 and 15%) according to the protocol by Freney *et al.* [8], using P-agar plates [1]. The haemolysis assay was performed on blood agar plates (Oxoid). *S. haemolyticus* CCUG 7323^T was included as a reference strain in all tests. All five *S. borealis* strains were able to grow at 30–42 °C, showed tolerance to NaCl up to 15% and displayed yellow pigmentation on P-agar plates (Fig. 3). After 24 h of aerobic incubation on horse blood agar at 37 °C, the *S. borealis* strains formed smooth, circular, raised or slightly convex colonies reaching 3–5 mm in diameter. A clear *β*-haemolysis (2 mm) was observed in the *S. borealis* strains and *S. haemolyticus* CCUG 7323^T (1.5 mm) on horse blood agar plates.

Metabolic profiling of the five *S. borealis* strains and *S. haemolyticus* CCUG 7323^T was performed. The CCUG STX phenotypic worksheet was followed using the API bacterial identification systems APIStaph, API 32 Staph and API Coryne test (bioMérieux; www.ccug.se/identification/worksheets), following the instructions of the manufacturer. The metabolic profiles are summarized in Table 5. Biochemically, the five *S. borealis* strains differed in three tests when compared with *S. haemolyticus* CCUG 7323^T. Briefly, all *S. borealis* strains were urease-positive, where *S. haemolyticus* is considered urease-negative. Urease production is one of the main phenotypic factors differentiating *S. haemolyticus* from *Staphylococcus warneri* and *Staphylococcus saprophyticus* [45]. All five *S. borealis* strains were positive for fermentation of fructose and mannitol, which is a variable trait in *S. haemolyticus* [46].

Cell fatty acid–fatty acid methyl ester (CFA-FAME) analysis was performed for the five *S. borealis* strains and the resultant profiles were contrasted with the type strain of *S. aureus* (type species of the genus *Staphylococcus*) and two closely related species (*S. haemolyticus* and *S. devriesei*) (Table 6). The strains were cultivated on Columbia blood agar base plus 5% defibrinated horse blood, at 37 °C, aerobically, overnight (18–24 h). An approximate biomass of 100 mg from each strain was harvested in early stationary phase to carry out fatty acid methyl ester (FAME) extraction. FAMEs were extracted and washed with alkaline solution after saponification and methylation of the cell biomass, following the protocol detailed by MIDI [47]. The CFA-FAME profile was determined using an HP 5890 gas chromatograph (Hewlett-Packard) and a standardized protocol similar to the MIDI Sherlock MIS system [47]

as described previously [48]. CFA-FAME analysis of the five *S. borealis* strains determined the major CFAs to be long-chain saturated fatty acids, C15:0 iso (11%), C15:0 anteiso (63%) and C17:0 anteiso (13%), while other CFAs observed included C17:0 iso (5%) and C18:0 (2.5%) (Table 5). Preponderance of uneven branched-chain fatty acid pairs with a difference of two carbons, iso/anteiso C15:0 and iso/anteiso C17:0, were present in all samples; of which the anteiso fatty acids, C15:0 anteiso (mean of approximately 63%) and C17:0 anteiso (mean close to 13%) had higher relative proportions than the iso fatty acids from the same pair, C15:0 iso (mean near to 11%) and C17:0 iso (mean of approximately 5%). Only a few straight chain fatty acids were identified, dominated by stearic acid, C18:0 (mean close to 2.5%). As fatty acids are highly preserved in the bacterial membrane, due to their role in the cellular structure, they are useful markers for bacterial differentiation [49]. Staphylococci exhibit specific CFA-FAME patterns at genus level [50], although characteristic fatty acids at species level [51]. The relative proportion of C15:0 anteiso is remarkably higher in *S. borealis* type strain CCUG 73747^T compared to *S. haemolyticus* CCUG 7323^T, showing 61.8 and 47% respectively. The increased amount of C15:0 anteiso may regulate membrane fluidity at lower temperatures [52, 53].

The determination of peptidoglycan structure was carried out by the German Collection of Microorganisms and Cell Culture GmbH (DSZM) identification service, as described by Schumann [54]. The total hydrolysate (100 °C, 4 N HCl, 16 h) of the peptidoglycan contained muramic acid (Mur) and the amino acids lysine (Lys), alanine (Ala), serine (Ser), glycine (Gly) and glutamic acid (Glu). Quantification of amino acids by GC/MS of *N*-heptafluorobutyric amino acid isobutylesters resulted in the following molar ratio: 0.9 Lys : 1.7 Ala : 0.7 Ser : 1.0 Glu : 2.4 Gly : 1.1 Mur. The identity of all amino acids was confirmed by agreement in the gas-chromatographic retention time with those of authentic standards and by characteristic mass-spectrometric fragment ions of the derivatives. After hydrolysis under milder conditions (100 °C, 4 N HCl, 0.75 h), the hydrolysate contained (in addition to the amino acids) the peptides Lys-Ala-Ala (backbone isomer), Lys-Ala (backbone isomer), Mur-Ala, Ala-Glu, Ala-Lys-Gly, Ala-Glu-Lys-Gly, Gly-Gly and Gly-Ser but no Gly-Gly-Gly or Lys-Ser peptide. From these data it was concluded that the strain *S. borealis* 51-48^T (CCUG 737547^T) displayed the peptidoglycan type A3α L-Lys–Gly–Gly–L-Ser–Gly (type A11.3, www.peptidoglycan-types.info).

Antimicrobial resistance testing was performed using the disc diffusion method and the microbroth dilution test according to the EUCAST guidelines [55]. Briefly, a 0.5 McFarland bacterial cell suspension was inoculated on Mueller–Hinton agar plates (Oxoid). Discs or MIC gradient strips were place on the inoculated agar plates and were incubated at 35±1 °C for 16–18 h, and zones of inhibition were measured. The five *S. borealis* strains were susceptible to the antimicrobial agents cefoxitin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), gentamicin (10 µg), linezolid (10 µg), rifampicin, tetracycline (30 µg), trimethoprim-sulfamethoxazole (1.25–23.75 µg), vancomycin (0.015–256 µg), clindamycin (0.125–256 µg) and lincomycin (0.125–256 µg). 51-48^T and 58-52 were resistant to erythromycin (both of which harboured the *ermC* gene) while 58-52 was also resistant to fusidic acid (harbouring the *fusC* gene). All strains showed resistance to the pleuromutilin antibiotic tiamulin according to the MIC breakpoints given by Frey *et al.* [56], which could be conferred by the *vga(A)* gene.

In conclusion, although the eight *S. borealis* strains share near-identical 16S rRNA gene sequences to *S. haemolyticus* NCTC 11042^T, and are phylogenetically closely related to *S. haemolyticus*, there are strong phenotypic and genomic justifications for assigning the strains to a novel species of the genus *Staphylococcus*, for which the name *Staphylococcus borealis* sp. nov. is proposed.

These justifications are:

(1) Phylogenetic distance, ANI <95% and inferred DDH <70%.
(2) Genome comparisons.
(3) Pigmented phenotype.
(4) Production of urease.
(5) Different cell fatty acid composition.

DESCRIPTION OF *STAPHYLOCOCCUS BOREALIS* SP. NOV.

Staphylococcus borealis (bo.re.a′lis. L. masc. adj. *borealis* related to the North, boreal).

Colonies are 3–5 mm in diameter, round, smooth and have a yellow tint. The difference in pigmentation between typical *S. haemolyticus* and *S. borealis* colonies is particularly evident on different supplemented P-agars (non-supplemented, full fat milk and horse blood) after 48 h at 37 °C. Cells are Gram-stain-positive, coccoid, 650 nm to 1.23 µm in diameter and form clusters. They are facultative anaerobic, coagulase-negative and catalase-positive. They are biochemically negative for fructose and mannitol and positive for production of urease. The major fatty acids are branched fatty acid pairs C15:0 iso, C15:0 anteiso and C17:0 anteiso and C17:0 iso, while the straight-chain fatty acid C18:0 is present in a much lower amount. The peptidoglycan type is type A11.3.

The five *S. borealis* strains are deposited to the Culture Collection University of Gothenburg (CCUG), with the following numbers: 51-48^T=CCUG 73747^T, 57-14=CCUG 73748, 57-74=CCUG 73749, 58-22=CCUG 73750 and 58-52=CCUG 73751. Two strains are deposited to the Spanish Type Culture Collection (CECT) with the following identifiers: 51-48^T=CECT 30011^T and 57-14=CECT 30010.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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