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Streptococcus suis strains with novel and previously undescribed capsular loci circulate in Europe

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ABSTRACT

Streptococcus suis (*S. suis*) causes serious diseases in pigs, and certain serotypes also pose a risk to humans. The expression of capsular polysaccharides (CPS) is considered an important virulence property of the pathogen. Recently, some serotypes have been reclassified as other organisms, while novel *S. suis* serotypes are being described. Although the CPS can be typed by serological methods using antisera, the presence of unique sequences for each capsular polysaccharide synthesis locus (*cps* locus) enables convenient PCR-based serotyping. In this study, we characterized 33 non-serotypeable *S. suis* strains obtained from diseased pigs in the Czech Republic by sequencing and analyzing the *cps* locus. Phylogenetic analysis of *cpn60* confirmed that all isolates belong to the *S. suis* species. Four isolates had *cps* loci similar to the previously described reference *S. suis* serotypes. Eleven isolates were classified as recently described novel *cps* loci (NCLs). Nine isolates had substitutions, insertions and/or deletions in their *cps* loci and showed only partial similarity to the already described NCLs. Another eight isolates had previously undescribed *cps* locus structures and were proposed as novel NCLs. One isolate had lost the genes encoding capsule biosynthesis. Only four sequence types (ST) had two isolates each; the rest had unique STs. Two isolates harbored the classical virulence associated genes (VAGs) *mrp* and *sly*. Another isolate had only the *mrp* gene, while a different isolate harbored only the *sly* gene. This study provides insight into untypeable isolates in the Czech Republic, highlighting the genetic diversity and potential for novel serotype identification.

1. Introduction

Streptococcus suis (*S. suis*) is an encapsulated Gram-positive bacterium that is a natural inhabitant of the upper respiratory tract, the urogenital tract, and the gastrointestinal tract of healthy pigs (Marois et al., 2007; Segura et al., 2016). However, pathogenic strains of the organism can cause opportunistic infections, which usually include meningitis, septicemia, endocarditis, and arthritis in suckling, weaned and growing pigs. These infections collectively represent a major problem for the swine industry (Staats et al., 1997; Neila-Ibáñez et al., 2021). *S. suis* is also an emerging zoonotic agent. In humans, especially in populations in close contact with pigs and in individuals consuming foods containing raw pork or blood (mainly in Thailand and Vietnam), the bacterium can cause meningitis, sepsis, skin lesions, arthritis, and streptococcal toxic shock-like syndrome (Hughes et al., 2009; Huong et al., 2014; Kerdsin et al., 2022).

Protection of *S. suis* against phagocytosis is provided by a polysaccharide capsule that surrounds the organism (Segura et al., 2004). Synthesis of the capsular polysaccharide (CPS) is carried out via a Wzx/Wzy-dependent pathway (Yother, 2011; Okura et al., 2013; Zheng et al., 2015) and involves the binding of the initial monosaccharide to the membrane lipid carrier using the initial glycosyltransferase and the subsequent attachment of the monosaccharides using specific transferases. The resulting synthesized polysaccharide units are then

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transported from the inner surface of the cytoplasmic membrane to the outer surface via Wzx flippase. On the outer face of the cytoplasmic membrane, the repeat unit is polymerized by Wzy polymerase. The final capsular polysaccharide is translocated to the cell wall peptidoglycan (Yother, 2011; Islam and Lam, 2014; Zheng et al., 2015). The genes encoding the enzymes involved in the synthesis of the CPS are clustered at the *cps* locus, located between the conserved *orfZ-orfX* gene region and either the *aroA* gene or the UDP-galactopyranose mutase gene (*glf*) (Smith et al., 1999; Smith et al., 2000; Okura et al., 2013).

Based on an antigenic reaction directed against the CPS, 35 *S. suis* serotypes have been described, but 6 serotypes were later reclassified as other streptococcal species (Okura et al., 2016). In addition to the 29 "authentic" serotypes, non-serotypeable strains of *S. suis* are commonly isolated from both clinically healthy and diseased animals. In recent years, novel *cps* loci were identified and designated as "novel *cps* synthesis loci" (NCL) (Zheng et al., 2015; Qiu et al., 2016; Zheng et al., 2017; Huang et al., 2019), as well as serotypes "Chz" (Pan et al.,2015) and "Chz-2" (Qiu et al., 2016).

Strains of *S. suis* are also classified using a multilocus sequence typing (MLST) scheme (King et al., 2002). Similar to other pathogens, the MLST scheme enables investigation of the genetic diversity and global distribution of *S. suis*, including the determination of the population structure of the organism, as well as its evolution and spread (Ibarz Pavón and Maiden, 2009).

In our previous work, among 582 isolates collected in 2018–2020 in the Czech Republic, we found that a substantial number of isolates were untypeable (i.e., their serotype could not be determined) (Zouharová et al., 2022). Here, to better characterize these untypeable strains, we analyzed the organization of the *cps* locus and other genetic characteristics of these organisms.

2. Materials and methods

2.1. Identification of bacterial strains and growth conditions

We used 33 untypeable *S. suis* isolates recovered in the Czech Republic in the period 2018–2020. The organisms were recovered from different pig organs, as well as from nasal, tracheal, and vaginal swabs obtained from dead or diseased pigs showing clinical symptoms of *S. suis* infection. The list of strains and the site of isolation are provided in the Supplement 1. Isolates were tested biochemically using the commercial STREPTOTEST 24 (ERBA LACHEMA, Brno, Czech Republic) and subsequently confirmed as "authentic" *S. suis* or as one of the novel species formerly considered to be *S. suis* by using the Matrix-Assisted Laser Desorption/Ionisation–Time of Flight method (MALDI-TOF). *S. suis* isolates were cultivated overnight at 37°C on blood agar plates containing 5 % ram's blood (LabmediaServis, Jaroměř, Czech Republic). Bacteria were grown overnight in BHI broth at 37°C with shaking (250 rpm). Further confirmation that the isolates belonged to the *S. suis* species was carried out using a PCR test targeting the *recN* gene, encoding a recombination/repair enzyme (Ishida et al., 2014).

2.2. Serotyping

The multiplex PCR described by Kerdsin et al. (2014) was used for serotype determination of all isolates. Thirty-three isolates, identified as non-serotypeable, were subjected to further analysis. Additionally, conventional serology techniques were applied to these isolates, namely co-agglutination reactions using antisera directed against the reference strains of the 29 *S. suis* serotypes (Mittal et al., 1983). Briefly, one drop of the *S. suis* co-agglutination agent was mixed with one drop of bacterial suspension on a glass slide. The slide was then manually swiveled from side to side. The results were deemed positive if co-agglutination was visible within one minute; otherwise, they were classified as negative. Five strains (NK001, NK006, NK014, NK028, NK031), representing isolates with *cps* loci structures different from the 29 *S. suis* serotypes (NCLs and potential novel serotypes, see results), were selected to immunize rabbits. Immunization was performed according to Mittal et al., 1983. Experimentation on animals was approved by the Ministry of Agriculture, Czech Republic (Approval number MZe 2188). Antisera were prepared from these rabbits and used as described above in co-agglutination assays.

2.3. Transmission electron microscopy (TEM) for assessment of capsule production

Capsule production was determined using transmission electron microscopy (TEM) with the negative staining method. Briefly, washed bacterial cell suspensions were covered with a grid coated formvar film (Sigma-Aldrich, Czech Republic) and carbon (Agar Scientific, Austria). The grid was removed from the suspension after 5 minutes and the residual water was dried with a strip of filtration paper. A drop of 1 % aqueous ruthenium red (Sigma-Aldrich, Czech Republic) was placed onto the grid for a few seconds, then excess stain was dried with filtration paper. The samples were observed under a Philips 208 S Morgagni electron microscope (FEI, Czech Republic) at 7,500–36,000× magnification and an accelerating voltage of 80 kV. The presence of capsule was observed.

2.4. Whole-genome sequencing and de novo assemblies

Genomic DNA of the 33 untypeable isolates was extracted using the E-Z 96™ Tissue DNA Kit (Omega Bio-tek, Inc., GA, USA) according to the manufacturer's instructions and subsequently quantified using a Qubit Fluorometer (Thermo Fisher Scientific, USA). Sequencing libraries were prepared with Nextera XT DNA Library Preparation Kits (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The libraries were sequenced as pair-end reads (2×150 bp) using an Illumina NextSeq 550 instrument. Quality control of the obtained sequence reads was performed using FastQC (Andrews, 2010). *De novo* genome assemblies were performed using the A5-miseq pipeline (Coil et al., 2015). Genome sequenced data have been deposited into the NCBI's Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) with accession number PRJNA1051809.

2.5. In silico serotyping, MLST determinations, and analysis of the cps locus

The pipeline described by Athey et al. (2016) streamlined the *in silico* characterization of the *S. suis* isolates in terms of serotype, MLST, and classical virulence-associated genes (VAGs; namely genes *mrp*, *epf*, *sly*) content. Briefly, the pipeline uses SRST2 (Inouye et al., 2014) to align whole-genome sequencing (WGS) short-read data to the sequence of the *recN* gene, thus permitting species confirmation (Ishida et al., 2014). Next, the pipeline identifies the serotype of the strains by read alignment to a custom *cps* database for 27 *S. suis* serotypes, followed by the use of custom scripts to discriminate between serotype pairs (1 and 14, as well as 2 and 1/2) whose *cps* loci are identical except for a single nucleotide polymorphism (SNP) in the *cpsK* gene. MLST determinations are carried out by read alignment to the MLST database maintained by PubMLST (Jolley et al., 2018; https://pubmlst.org/organisms/streptococcus-suis) which we downloaded on May 1, 2024. The pipeline also uses read alignment to a custom VAG database to detect the presence of classical VAGs in the *S. suis* strains.

Using custom scripts, we extracted the sequence corresponding to the *cps* locus from the *de novo* assembled genomes of the 33 isolates and used Prokka (Seemann, 2014) to identify and annotate open reading frames (ORFs). We used BLAST (http://www.ncbi.nlm.nih.gov) and ClustalW2 (Larkin et al., 2007) for the alignment of these sequences to those of NCL serotypes. Visualization of the data was accomplished using the Artemis Comparison Tool (ACT) (Carver et al., 2005) and EasyFig (Sullivan et al., 2011).

2.6. Phylogenetic analysis

The *cpn60* gene, encoding a 60 kDa chaperonin protein, provides high resolution for bacterial species identification and phylogenetic analysis (Hill et al., 2005; Links et al., 2012). To determine the phylogenetic relationship of our strains with *S. suis*, the sequences of the *cpn60* gene for reference serotypes were retrieved from the GenBank, following the approach outlined by Brousseau et al. (2001). The *cpn60* gene sequences of our isolates were extracted from their respective genome assemblies. The *cpn60* DNA sequences were aligned using ClustalW2 with default parameters. Next, a phylogenetic tree was constructed, using MEGA-X software (Kumar et al., 2018) and the neighbor-joining method, with bootstrapping parameters (N=500), p-distance, and complete gap deletion. The interactive Tree of Life (iTOL; Letunic and Bork, 2021) tool was used for visualization. An important means of classifying novel serotypes is phylogenetic analysis of Wzy polymerase (Okura et al., 2013; Pan et al., 2015). The phylogenetic analysis of Wzy polymerase was performed similarly to the phylogenetic analysis of the *cpn60* gene. The data retrieved for constructing the phylogenetic trees are listed in Supplement 3.

3. Results

3.1. Identification of bacteria and serotyping

MALDI-TOF assigned all 33 strains analyzed here to the *S. suis* species. Consistently, all strains were positive for the presence of the species-specific *recN* gene (Ishida et al., 2014), confirmed by both PCR and *in silico* analysis of WGS data. However, we failed to detect any amplicons for these 33 isolates when using a multiplex PCR permitting indirect serotyping of 29 *S. suis* serotypes and a group of other bacterial species formerly thought to belong to 6 additional *S. suis* serotypes. Moreover, the 33 strains showed non-agglutination or poly-agglutination when tested in the co-agglutination test using antisera directed against the reference *S. suis* serotypes. Based on these results, the 33 isolates were deemed untypeable. To differentiate between organisms not producing a capsule from those producing novel capsular types, we next investigated capsule expression by using transmission electron microscopy (Supplement 1). Of 33 untypeable isolates, 27 expressed a thick capsule visible after staining (Fig. 1A presents a typical TEM result for this group of isolates), while 6 other strains NK038, NK048, NK049, NK098, NK172, and NK242, did not appear to possess capsular material (Fig. 1B provides an example of this phenotype). We then selected 5 strains, including previously described NCLs (NK001 – NCL5, NK006 – NCL26) and newly proposed NCLs (NK014 – NCL28, NK028 – NCL29, NK031 – NCL27), all of which express a capsule. We performed co-agglutination tests using antisera raised in rabbits and directed against the capsular material from these 5 strains (Table 1) to demonstrate that the novel *cps* can induce the formation of antigen-specific antibodies. All 5 strains showed positive homologous agglutination reactions. Bacterial suspensions of strains NK001, NK006, and NK014 reacted only with their corresponding antisera. One-way cross-reactions occurred for the NK001 antiserum with the NK028 and NK031 antigens, while the NK014 antiserum reacted with NK031 antigen. In addition, the NK031 antiserum reacted with the NK028 antigen in a one-way cross-reaction.

3.2. Analysis of the cps loci

We next interrogated the *de novo* assembled genomes for the presence of *cps* genes and whether, if found, they were organized in a *cps* locus. The *cps* gene clusters are located between the *orfZ-orfX* region and either the *aroA* or *glf* gene. Except for strain NK038, which exhibited a loss of genes encoding the synthesis of CPS, we found that strains possessed several *cps* genes organized in *cps*-like loci. Notably, genes *cpsA-cpsD*, putatively involved in the regulation of the biosynthesis of the CPS, were present in all untypeable strains. The NK028 isolate had *cpsA-cpsE* located on a different contig than the rest of the *cps* genes, but this may be an assembly artifact. The gene encoding the initial glycosyltransferase, Wzx flippase, as well as the gene encoding the Wzy polymerase were also present in all strains. Different isolates had in their *cps* loci genes encoding different modifying enzymes (e.g., aminotransferases, acyltransferases, epimerases) that may be involved in CPS synthesis (see graphic visualization in Supplementary Material Figures).

Four isolates had *cps* loci very similar to those previously described for the 29 *S. suis* serotypes. In detail, one isolate (NK061) demonstrated a *cps* locus highly similar to that of reference serotype 24, albeit with several nucleotide substitutions throughout the locus (96 % nucleotide identity). Another isolate (NK148) showed 97 % nucleotide identity to

Table 1
Reactivity of antisera induced by *S. suis* isolates.

antisera isolate	NK001	NK006	NK014	NK028	NK031
NK001	+	-	-	-	-
NK006	-	+	-	-	-
NK014	-	-	+	-	-
NK028	+	-	-	+	+
NK031	+	-	+	-	+

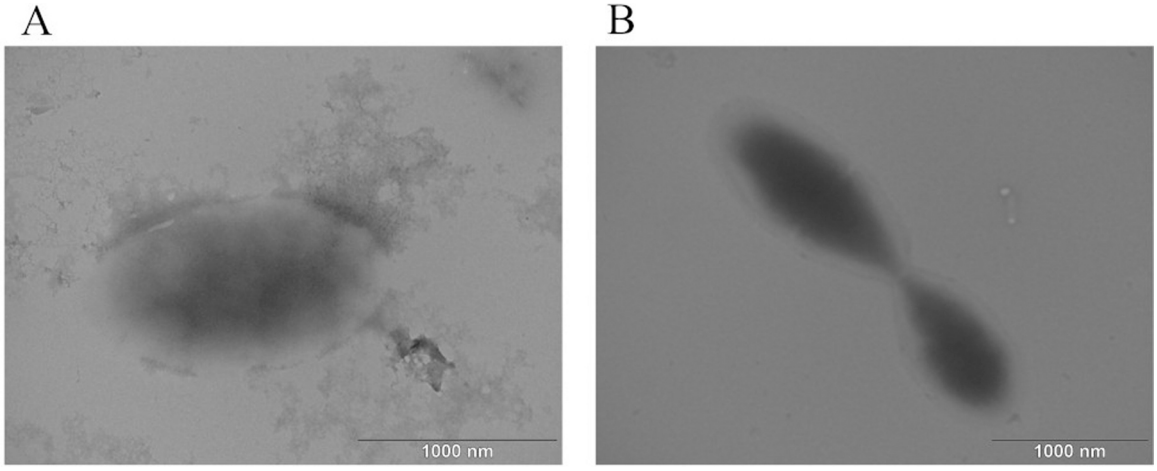

Fig. 1. Visualization of the *S. suis* capsule using transmission electron microscopy. (A) The NK028 isolate represents a group of isolates producing novel capsular types; (B) The NK098 isolate demonstrates the typical TEM result for a group of isolates without capsule material.

the *cps* locus of the reference serotype 13, but with a different variant of the gene encoding the Wzy polymerase. The third isolate (NK084) had a *cps* locus closely resembling that of the reference serotype 27 but the 5' end (*cps27A-cps27E* genes, 95 % nucleotide identity) and the 3'end (*cps27M-cps27S*, including sialic acid synthesis genes, 95 % nucleotide identity) of the locus. However, it differed in the central region (*cps27F-cps27K*; with only 81 % nucleotide identity). In addition, NK084 had insertions of genes encoding transposases downstream of the *cps27E* gene, along with insertions of a transposase and a N-acylneuraminate cytidylyltransferase downstream of the *cps27K* gene, compared to the reference serotype 27. The fourth isolate (NK083) had a *cps* locus that was similar to that of serotype 9 in the 3'region (LicD family protein *cps9I*, Wzy polymerase *cps9J*, and *cps9L-cps9N* nucleotidyl transferase, epimerase, and Wzx flippase, respectively; 96 % nucleotide similarity). However, gene *cps9K* was absent, and the 5'region of the *cps* locus (*cps9A-cps9E*) showed only 83 % nucleotide identity. In addition, four

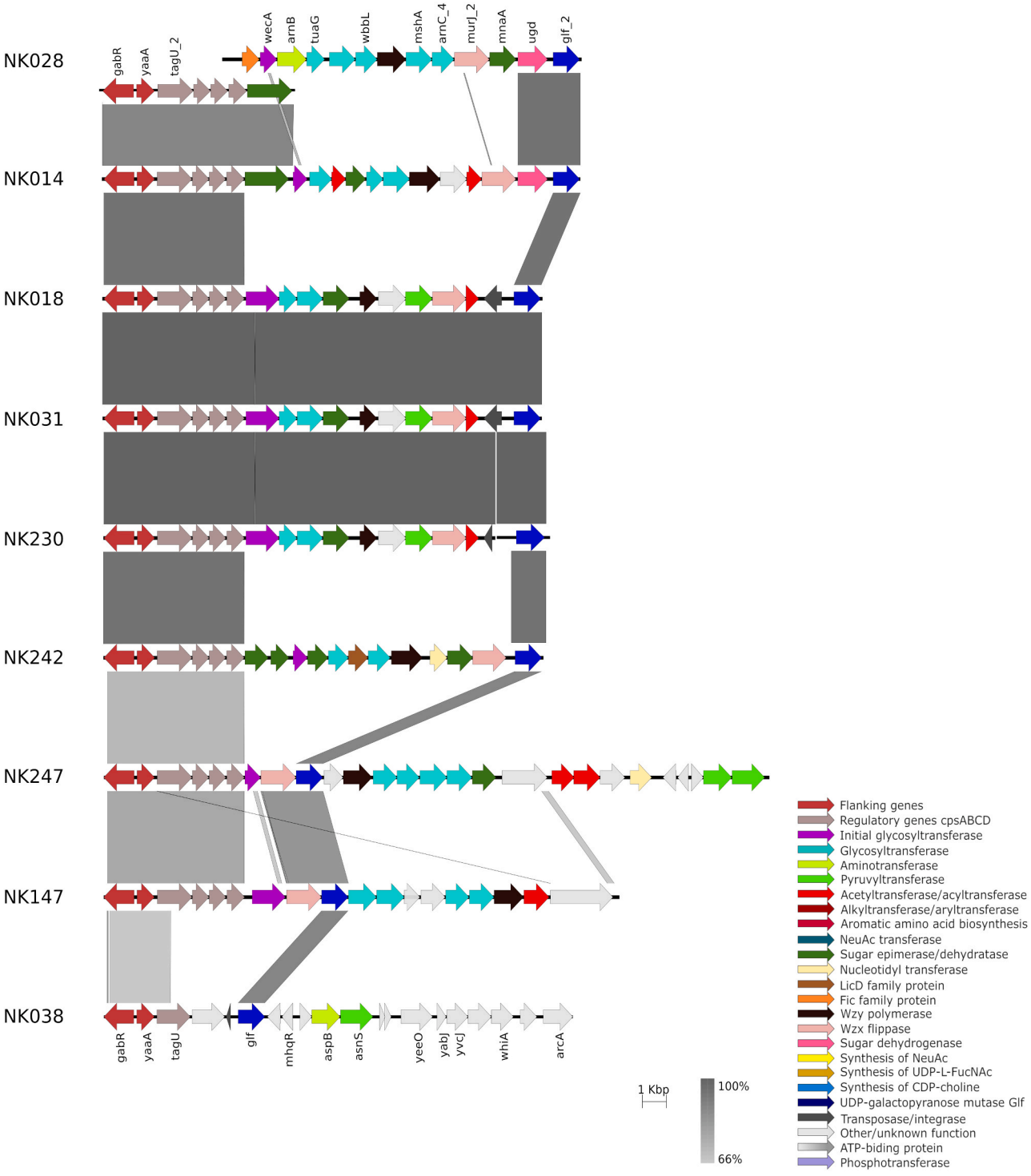

Fig. 2. Genetic organization of the *cps* loci of untypeable strains. Included are 8 strains that we propose represent previously undescribed NCLs, as well as one strain that has lost cps genes (NK038). The *cpsA–cpsE* genes of isolate NK028 and the *glf* gene of isolate NK230 were found on different contigs. Arrows represent the genes, with their predicted functions shown in the legend. Gene names were assigned after annotation by the PROKKA (Seemann, 2014).

genes in the central region were different.

Eleven strains had *cps* loci identical to those found in *S. suis* serotypes collectively known as NCL. Most of them had an NCL3–1 *cps* locus (isolates NK048, NK049, NK093, NK121, NK172, NK258, NK259, n=7), followed by those with a NCL11–1 locus (isolates NK157, NK236, n=2). Two isolates had NCL5, and NCL25 *cps* loci (NK001, NK006, respectively).

Nine additional strains had *cps* loci similar to loci in known NCLs but with substitutions, deletions, and/or insertions of some genes. The *cps* loci found in three isolates (NK071, NK229, NK233) were similar to that of NCL7. One isolate (NK071) had a *cps* locus identical (99 %) to that of NCL7–1, but unlike NCL7–1, the isolate did not have a transposase present in its *cps* locus downstream of *cpsS*. The *cps* loci of two other isolates (NK229, NK233) showed partial similarity to the *cps* locus of NCL7–1, with a similar Wzx flippase and Wzy polymerase but different modifying enzymes in the central part of the locus, while one isolate (NK233) additionally had a different hypothetical protein in the 3'region. The *cps* locus of the NK098 isolate was split across two contigs. One contig contained *cpsA-cpsR,* which were highly similar to those in NCL1–1 (95 % nucleotide identity), while the other contig contained hypothetical proteins and *glf,* with 99 % identity to NCL1–2. One isolate (NK235) had a *cps* locus similar to that of NCL2, but with two hypothetical proteins instead of the hypothetical protein *cpsT*. Isolate NK243 had an NCL8-like *cps* locus, but with the insertion of an epimerase upstream of *cpsH* and different glycosyltransferase *cpsH*. Unlike the NCL9 reference strain, the *cps* locus of isolate NK238 had insertions of four hypothetical genes between the *cpsX* and *glf* genes. Isolate NK234 had a different hypothetical protein after the sugar dehydratase *cpsU* in its *cps* locus compared to the reference strain NCL11–1. The *cps* locus of NK219, unlike the NCL25 *cps* locus, had two transposases inserted downstream of *cpsM* (Wzy polymerase), and there were insertions of a transposase, an aminotransferase, and two ATP-binding proteins upstream of *aroA*. Based on the similarity of *cps* loci, including Wzy polymerases and Wzx flippases, to different known NCLs but the presence of alternative modifying genes, we propose to classify these isolates as subgroups of known NCLs: NCL1–8 (NK098), NCL2–4 (NK235); NCL7–3 (NK071), NCL7–4 (NK229), NCL7–5 (NK233); NCL8–4 (NK243); NCL9–2 (NK238); NCL11–6 (NK234); and NCL25–2 (NK219).

The remaining eight strains (NK014, NK018, NK028, NK031, NK147, NK230, NK242, NK247) exhibited different gene compositions in their *cps* loci, indicating that they carry novel, previously undescribed NCLs. Three of these isolates (NK018, NK031, NK230) shared an identical *cps* locus. Based on previous publications, we propose to name this group as NCL27. The other five isolates each had unique *cps* loci (Fig. 2). We propose to name the *cps* loci of NK014 and NK028 as serotypes NCL28 and NCL29, respectively. Interestingly, two isolates (NK147, NK247) had Wzy polymerase and CPS modifying enzymes downstream of the UDP-galactopyranose mutase-encoding gene *glf*. We propose to classify them as NCL30 and NCL31, respectively. Isolate NK242 had genes encoding nucleotidyltransferase, epimerase, and Wzx flippase in its *cps* locus 99 % identical to the *cpsM-cpsO* genes of the reference strain NCL4. However, the central region, including the Wzy polymerase, was different from NCL4. Additionally, the sequence of the *cpsE-cpsL* gene region in the *cps* locus was 90 % identical to the *cpsE-cpsK gene* region in the *cps* locus of the reference strain NCL26, where the Wzy polymerase of NK242 was 84 % identical to the Wzy polymerase of NCL26 (*cpsK*). The NCL26 *cpsL-cpsN* gene region was 98 % identical to the *cpsM-cpsO* region of the NK242 *cps* locus. This may indicate horizontal gene transfer between the NCL4 and NCL26 strains, leading to the emergence of a recombinant NCL, which we propose to name as NCL32.

Based on the analysis of the *cps* loci, we selected a gene unique to the newly proposed NCL and Wzy polymerase sequences for already known NCLs (Supplement 2), which, when complemented with the database for the *Ssuis*Serotyping_pipeline (available at https://github.com/streplab/SsuisSerotyping_pipeline), permit BLASTN-based *in silico* serotyping using the *S. suis* seroblast script (available at https://github.com/matiajan/seroblast). Within our limited validation, the *S. suis* seroblast script thus could identify all known *S. suis* serotypes, including both the previously known NCLs and NCLs we identified in this study.

3.3. MLST and classical VAG content

Known sequence types were identified in 11 isolates. Seven isolates had new combinations of alleles, and their STs were obtained from PubMLST. Fourteen isolates had new allele sequences, profiles, and STs. One isolate (NK038) had an *aroA* sequence that was too dissimilar and less than 90 % in length; therefore, its allelic profile and ST could not be determined. The most frequent sequence types were ST792 (NK258, NK259 – both NCL3), ST2089 (NK048, NK049 – both NCL3), ST2096 (NK018, NK031 – both proposed NCL27), and ST2892 (NK093, NK172 – both NCL3). Sequence types ST801 (NK028), ST2078 (NK084), ST2083 (NK098), ST2095 (NK006), ST2466 (NK238), ST2852 (NK014), ST2865 (NK061), ST2866 (NK121), ST2867 (NK148), ST2868 (NK219), ST2869 (NK230), ST2870 (NK242), ST2889 (NK001), ST2890 (NK071), ST2891 (NK083), ST2893 (NK147), ST2894 (NK157), ST2896 (NK229), ST2897 (NK233), ST2898 (NK234), ST2899 (NK235), ST2900 (NK236), ST2901 (NK243), and ST2902 (NK247) occurred only once.

Out of 33 untypeable isolates, 29 isolates were *epf-, mrp-, sly-*, 2 isolates (NK006, NK219) were *epf-, mrp+, sly+*, 1 isolate (NK038) was *epf-, mrp+, sly-* and 1 isolate (NK028) was *epf-, mrp-, sly+*. The results, including allelic profiles, STs, and VAGs, are shown in Supplementary Material 1.

3.4. Phylogenetic analysis

Phylogenetic analysis of the *cpn60* gene confirmed the relatedness of the isolates used in this study to *S. suis* (Fig. 3). This analysis shows that our 33 isolates cluster with conventional strains of *S. suis* and novel NCLs, while *S. orisratti*, *S. parasuis*, and *S. ruminantium* form their own distinct clusters. Phylogenetic analysis of Wzy polymerase amino acid sequences revealed similarities among different polymerases, which may implicate phenotypic characteristics such as CPS synthesis mechanisms and polysaccharide structures. The phylogenetic tree of serotype-specific Wzy polymerase indicates that eight isolates (NK014, NK148, NK018, NK031, NK230, NK028, NK247, NK147) may represent unique serotypes. In contrast, the *wzy* genes from 24 other isolates clustered with known reference serotypes or known NCLs (Fig. 4).

4. Discussion

In a previous study (Zouharová et al., 2022), the epidemiological situation of *S. suis* in the Czech Republic was evaluated in 2018–2022 and showed that the most prevalent serotype was serotype 7 (14.96 %). Other serotypes represented fewer than 10 % of the analyzed isolates, but a relatively high percentage of isolates (18.94 %) were non-serotypeable. Although the percentage of non-serotypeable isolates varies between studies, our previous findings were nonetheless unsurprising (Prüfer et al., 2019; Estrada et al., 2019, Scherrer et al., 2020, Yang et al., 2021). Indeed, our previous findings and these global reports describe the existence of an important population of *S. suis* strains that cannot be characterized by current serotyping methods. Similarly, the growing number of new sequence types in the *S. suis* MLST database highlights the ongoing discovery of previously unrecognized *S. suis* genetic diversity.

To address the knowledge gap concerning the characteristics of this *S. suis* population of untypeable strains circulating in Europe, we conducted an analysis of the *cps* locus of non-serotypeable *S. suis* isolates recovered in our previous study (Zouharová et al., 2022) from clinically diseased pigs. This analysis utilized whole-genome sequencing and phenotypic analysis. However, we limited the analysis to untypeable isolates that tested positive for the presence of the *recN* gene, widely recognized as confirming the *S. suis* species. Further confirmation that

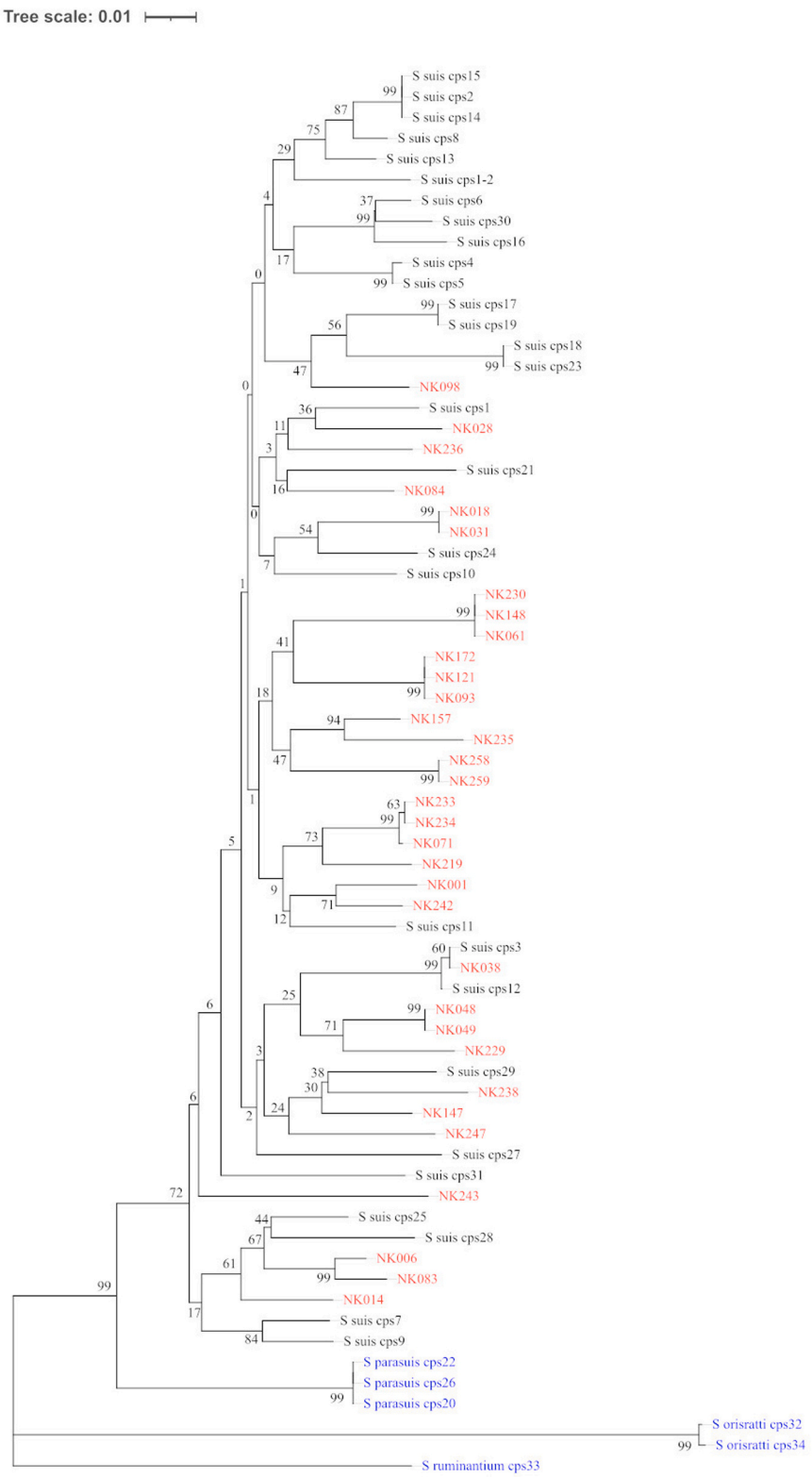

Fig. 3. Phylogenetic relationships of the strains based on the sequence of the *cpn60* gene. The unrooted phylogenetic tree, visualized with iTOL, is based on ClustalW2 alignment of *cpn60* sequences and constructed in MEGA-X software (Neighbor-joining; 500 bootstraps; p-distance; complete gap deletion). The *cpn60* of the 33 isolates analyzed in this study are marked in red, while *S. orisratti*, *S. parasuis,* and *S. ruminantium* are marked in blue. The numbers at the nodes represent bootstrap values.

the 33 isolates belonged to the *S. suis* species was provided through sequence comparison of the gene encoding the 60 kDa chaperonin (Glazunova et al., 2009; Okura et al., 2016).

Analysis of the *cps* loci of these isolates revealed that eleven isolates belonged to known NCLs. Seven of them belonged to the NCL3–1 group. Although this NCL was initially reported in tonsil swabs of healthy pigs (Zheng et al., 2015), all of our isolates were recovered from diseased pigs: one from the brain, three from tracheal swabs, and three from nasal

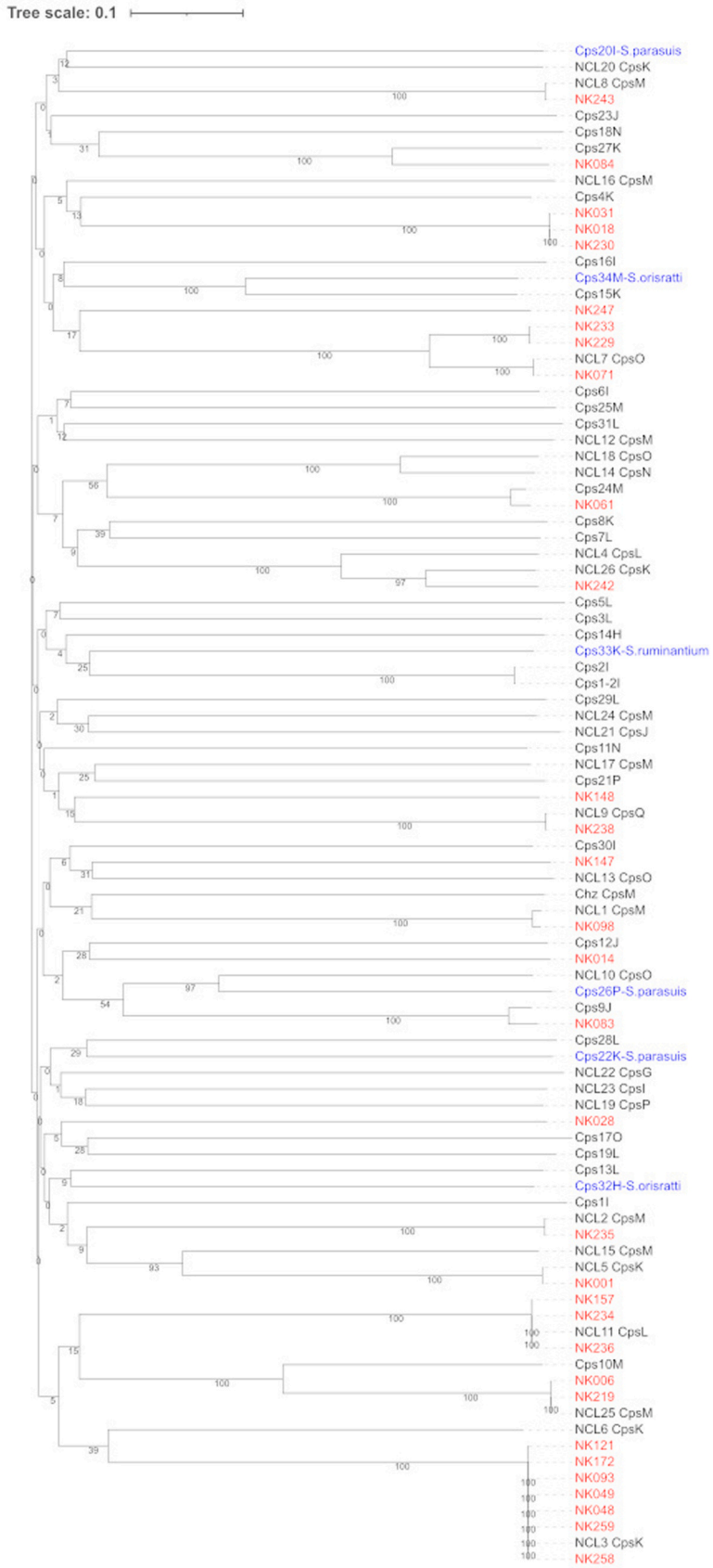

Fig. 4. Phylogenetic relationships based on Wzy of *S. suis* reference serotypes, known NCLs, and the 32 strains used in this study. The unrooted phylogenetic tree was visualized with iTOL based on ClustalW2 alignment of Wzy amino acid sequences and constructed in MEGA-X software (Neighbor-joining; 500 bootstraps; p-distance; complete gap deletion). The Wzy of 32 isolates analyzed in this study are marked in red, while *S. orisratti*, *S. parasuis*, and *S. ruminantium* are marked in blue. Strain NK038 was not included due to the loss of capsule synthesis genes. The numbers at the nodes represent bootstrap values.

swabs. The remaining four isolates belonged to NCL5, NCL11–1, and NCL25, all originally reported in China among healthy pigs (Zheng et al., 2015; Qiu et al., 2016; Huang et al., 2019). However, our 4 isolates were recovered from the upper respiratory tract, urogenital tract, and lower respiratory tract, suggesting potential virulence of these strains.

Previously, these NCLs were only documented in samples from China and Canada (Zheng et al., 2015; Qiu et al., 2016; Zheng et al., 2017; Huang et al., 2019). However, novel candidate NCLs had also been identified in Europe (Bojarska et al., 2020). The prevalence of untypeable strains frequently reported worldwide suggests that NCLs and new serotypes could be the norm. Considering that seven of our untypeable isolates belonged to NCL3–1, we speculate that, at least, NCL3–1 is relatively well established in Czech farms and may even be native to Europe. Notably, there is no evidence of extensive importation of raw pork or even livestock pigs from China or other Asian countries into the Czech Republic in the last few decades. On the other hand, breeding animals and weaned piglets have been extensively imported from Western Europe, particularly from Germany, the Netherlands and Denmark (Matiašovic et al., 2021).

We found that some isolates had *cps* loci similar to those of previously reported NCLs (Zheng et al., 2015; Qiu et al., 2016; Zheng et al., 2017; Huang et al., 2019; Bojarska et al., 2020), but exhibited only partial similarity due to gene substitutions and insertions/deletions. We propose that these variants represent novel types/subtypes that we named NCL1–8, NCL2–4, NCL7–3, NCL7–4, NCL7–5, NCL8–4, NCL9–2, NCL11–6, and NCL25–2.

Eight isolates had *cps* loci that did not correspond to any previously reported serotypes or NCLs. One isolate (NK242) had a *cps* locus similar to the reference strain NCL26, and based on the Wzy sequence (Huang et al., 2019), it was phylogenetically relatively closely related to NCL26. Moreover, the *cpsM-cpsO* sequences were similar to the NCL4 group. Although the *cps* locus seems to be complete, TEM showed that this isolate was not encapsulated, indicating a complex regulation of CPS synthesis (Xiao et al., 2017). The remaining seven isolates expressed a capsule, as confirmed by TEM. Among them, three isolates (NK018, NK031, NK230) had identical unique Wzy polymerase sequences, while the other four isolates had different unique Wzy polymerases. The presence of several transferases, Wzx, and Wzy indicates that the resulting saccharide repeating unit structures could be divergent (Wang et al., 2011, Okura et al., 2013). None of these isolates reacted with reference coagglutination reagents, indicating that their antigenic saccharide repeat units are unique. We hypothesize that these isolates may represent novel serotypes.

Interestingly, two isolates (NK006, NK219) whose *cps* loci were identical or similar to NCL25 possessed the classical VAGs *mrp* and *sly*. Although isolate NK038 lacked the genes for regulation and synthesis of capsular polysaccharides in the *cps* locus, it possessed the VAG *mrp*. Only one isolate (NK028) from the proposed novel NCL29 possessed the VAG *sly*, while the other proposed NCL isolates were negative for all classical VAGs (*epf, mrp, sly*). The fact that all isolates studied here were recovered from dead or diseased pigs further exemplifies the wide redundancy of *S. suis* virulence factors (Segura et al., 2017, Tram et al., 2021).

A different isolate in our collection (NK038) had lost the genes encoding capsule synthesis. The loss of capsule expression was confirmed by TEM. Interestingly, this isolate was sampled from the heart of a pig. The loss of the *S. suis* capsule has been shown to enable better adherence to platelets and caused porcine endocarditis (Lakkitjaroen et al., 2011).

5. Conclusion

In conclusion, we propose appending 9 subgroups to the already known NCL subgroups: NCL1–8 (NK098), NCL2–4 (NK235); NCL7–3 (NK071), NCL7–4 (NK229), NCL7–5 (NK233); NCL8–4 (NK243); NCL9–2 (NK238); NCL11–6 (NK234); and NCL25–2 (NK219). Furthermore, based on the analysis of *cps* loci and coagulation tests, we propose creating new NCL groups for six potentially new serotypes named NCL27 (NK018, NK031, NK230), NCL28 (NK014), NCL29 (NK028), NCL30 (NK147), NCL31 (NK247), and NCL32 (NK242).

CRediT authorship contribution statement

Natálie Králová: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft. **Nahuel Fittipaldi:** Conceptualization, Methodology, Software, Formal analysis, Writing – Original Draft, Funding acquisition. **Monika Zouharová:** Resources, Writing – Review & Editing. **Kateřina Nedbalcová:** Resources, Writing – Review & Editing. **Katarína Matiašková:** Resources, Writing – Review & Editing. **Jan Gebauer:** Resources, Writing – Review & Editing. **Pavel Kulich:** Investigation, Writing – Original Draft. **Bronislav Šimek:** Resources, Writing – Review & Editing. **Ján Matiašovic:** Conceptualization , Software, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.110265.

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