IN SILICO DEVELOPMENT OF HIGH-RESOLUTION MLVA TYPING SCHEME FOR *ENTEROCOCCUS FAECIUM*

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ABSTRACT

Emergence of enterococci as nosocomial pathogens is frequently associated with hospital outbreaks. Vancomycin resistance is especially perturbing as it limits the possible therapeutic options. Based on vast *in silico* analysis, we introduce a new multiple-locus variable-number tandem repeat analysis (MLVA) scheme for genotyping of *Enterococcus faecium* isolates, parameters of which are comparable to these of pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The scheme was tested *in silico* on all available at this time *E. faecium* genomes in NCBI Genbank. Searching for suitable variable number of tandem repeats (VNTR) loci was conducted with a set of free access applications. Ten VNTR loci were selected according to their polymorphic structure and stability using the Primer-BLAST utility of NCBI. Primers were designed to be compatible in a multiplex reaction and the method was adapted for high resolution separation techniques. As a result, a total of 60 MLVA profiles and 35 MLST profiles were generated from the analysis of 114 sequenced genomes. Minimum spanning trees were created for both MLVA and MLST in order to analyze the genetic relatedness between isolates. Hunter Gaston discriminatory index was measured for both MLVA (0,959) and MLST (0,926). Typeability was also measured for both methods (MLVA – 85.9%; MLST – 89.4%). These results suggest that the new MLVA scheme is suitable for epidemiological studies of hospital-adapted *E. faecium* isolates.

KEYWORDS:

Enterococcus faecium, MLVA, MLST, VNTR

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ABBREVIATIONS

VRE – vancomycin-resistant enterococci
PFGE – pulsed-field gel electrophoresis
MLST – multilocus sequence typing
TRs – tandem repeats
VNTR – variable number of tandem repeats
MLVA – multiple-locus variable-number tandem repeat analysis
MT – MLVA type
TRF – Tandem Repeats Finder
TRDB – Tandem Repeats Database
WGS – whole-genome sequencing
ST – sequence type
HGDI – Hunter Gaston discriminatory index
CI – confidence interval
MST – minimum spanning tree

INTRODUCTION

The genus *Enterococcus* consists of gram positive, facultatively anaerobic cocci. They are usually found in human and animal intestinal tract as part of the normal microflora. However, they also appear as important nosocomial pathogens causing infections in immunocompromised patients. *Enterococcus faecalis* and *Enterococcus faecium* are the two most common pathogens among the enterococcal species (1). Enterococci have intrinsic resistance to penicillin and cephalosporins due to expression of low affinity penicillin-binding proteins (2). *E. faecium* and *E. faecalis* have innate resistance to aminoglycosides due to reduced membrane permeability (3, 4). *E. faecium* possess an enzyme called aminoglycoside 6'acetyltransferase (AAC(6')-Ii) which confers resistance to tobramycin and kanamycin (5). Another *E. faecium*-specific enzyme is the *efmM*-encoded 16S rRNA m^5C methyltransferase which confers resistance to dibekacin, tobramycin and kanamycin (6).

The antimicrobial agents to which enterococci may possess resistance include macrolides, tetracyclines, lincosamides, chloramphenicol, vancomycin, quinolones, linezolid, quinupristin/dalfopristin. Vancomycin resistance is especially perturbing (1).

Vancomycin-resistant enterococci (VRE) are reported for the first time in the late 1980s in USA and Europe. Vancomycin resistance is usually associated with the acquisition of *vanA* or *vanB* gene cluster (7, 8). The frequency of this type of resistance is much higher for *E. faecium* than for any other enterococci. A correlation between the higher rates of acquisition of vancomycin resistance and the higher rates of infections caused by *E. faecium* was observed (9).

Most enterococci usually do not have the classic virulence factors, which is the reason they cause infections primarily in seriously ill or immunocompromised patients.

In a hospital setting enterococci can be transmitted from patients to other patients or even to the personnel. Enterococci are not necessarily causing infections. They can colonise the intestinal tract of a healthy person making him a carrier who can further transmit them. If no action is taken, this can become a prerequisite for an outbreak. Nosocomial enterococcal pathogens can be isolated from personnel's gloves, medical equipment, different surfaces and passively contaminated environment with feces and urine. Some of the risk factors for acquisition of nosocomial enterococcal infections are: severe or chronic illness; prolonged hospital stay; transplantations; urinary or vascular catheters; residence in the intensive care unit; renal insufficiency; post-antibiotic therapy; neutropenia (1).

The emergence of enterococci as a cause of hospital outbreaks defines the need for reliable and accessible method for genotyping which can easily discriminate between different strains and between pathogen and wild strains.

Pulsed-field gel electrophoresis (PFGE) is considered as the "gold standard" in molecular typing of clinically relevant isolates. This is due to its high discrimination ability, typeability and reliable epidemiological concordance it provides. It is an inexpensive method with good intra- and inter-laboratory reproducibility. There are standardised protocols and international fingerprinting database (Pulsenet) which gives the opportunity to detect and monitor the dissemination of pathogen strains among different countries. However, this method has its disadvantages. It is technically demanding and requires qualified personnel. Furthermore, fragments with lengths differing by 5% are not discriminable. Also its portability is still an issue (10).

Multilocus sequence typing (MLST) is another method for genotyping of different isolates. It is based on sequencing and its discriminatory power is comparable to that of PFGE. There is an international standardised nomenclature and free access databases (http://pubmlst.org and www.mlst.net) which contain information about the allele sequences and sequence types (ST). Thus, the results generated by this method are less likely to be ambiguous. These databases also provide an online tool (eBURST) for determining genetic relatedness between isolates. Unfortunately, this method also has its disadvantages. It is expensive, time-consuming and labor-intensive (11). Along with these, a lower discriminatory power regarding some isolates is observed which makes this method non-applicable for routine screening and monitoring of hospital outbreaks.

In 2004, J.Top et al. published detailed multiple-locus variable-number tandem repeat analysis scheme (MLVA-6) for genotyping of *E. faecium*, which was developed and tested on total of 392 isolates, from which 126 with clinical origin (blood, urine, wounds), 68 from clinical studies, 111 isolates from 25 documented hospital outbreaks, 17 from society-derived infections, and 70 isolates from different environments, animals and foods.

Searching for tandem repeats (TRs) in the genomic sequences, that were not yet completed due to the lack of reliable whole-genome sequencing (WGS) technologies at the time, is accomplished with specialised software called Tandem Repeats Finder (TRF) (12). Initially the scheme consisted of 10 loci which were selected based on the following criteria: TRs minimum length – 20 bases; conservation of TRs – (>90%); location of TRs – in non-coding regions.

Later, 4 of the loci are excluded, leaving the scheme with only 6 loci (13).

The aim of this study is to introduce a new and reliable MLVA scheme (MLVA10) for typing of *E. faecium* with parameters comparable to these of PFGE and MLST.

MATERIALS AND METHODS

Five referent genomes were randomly selected and downloaded from NCBI GenBank: 1. *E. faecium DO* (CP003583.1); *2. E. faecium Aus0004* (CP003351.1); *3. E. faecium ATCC 8459 = NRRL B-2354* (CP004063.1); *4. E. faecium Aus0085* (CP006620.1); 5. *E. faecium AUSMDU00004055* (CP027506.1). Searching for suitable loci in these genomes was conducted with a set of specialised softwares.

Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html) was used first. The search of TRs was performed according to the following criteria: pattern of TRs – 1 to 100 bases; number of TRs – minimum 2; alignment parameters (match, mismatch, indel) – (2,7,7). The referent genomes were uploaded for analysis in the form of FASTA files. Subsequently we came across another web application – Polloc V (14) which was able to search for TRs in 3 genomes simultaneously. It also gave an opportunity for sequence comparison which makes selection of polymorphic loci easier. Searching criteria used were as follows: Region length – unlimited; Unit length – 3-60 bp; Copy number of TRs – 2 or more; Alignment parameters – match/mismatch/indel (-2;-7;-7); Similarity percentage – >90%.

Another web application described in publication of Lee et al. („pSTR Finder: a rapid method to discover polymorphic short tandem repeat markers from whole-genome sequences") was tested. pSTR Finder is searching for TRs with lengths 1-10bp (15). The search criteria were as follows: Alignment Score (match, mismatch, indel) – 2,7,7; Min Align Score – 50; Max Repeat Unit – 10; 5' & 3' Flanking Sequence Size – 15.

Tandem Repeats Database, described in „TRDB— The Tandem Repeats Database", was also used. Concatenation of the referent genomes was done and the search of VNTR loci was performed according to the following criteria: Alignment Parameters (match, mismatch, indels) - 2, 7, 7; Minimum Alignment Score To Report Repeat – 50.
Another software for genome processing and analysis, developed by Biomatters, used in our study, was Geneious Prime.
Primers were designed according to all criteria for primer design for each locus with Geneious Prime and Oligo7 (Table 1).
Analysis for determination and confirmation of the polymorphic structure of the VNTR was performed on all the available at this time sequenced genomes in NCBI Genbank using the web application of NCBI – Primer-BLAST.

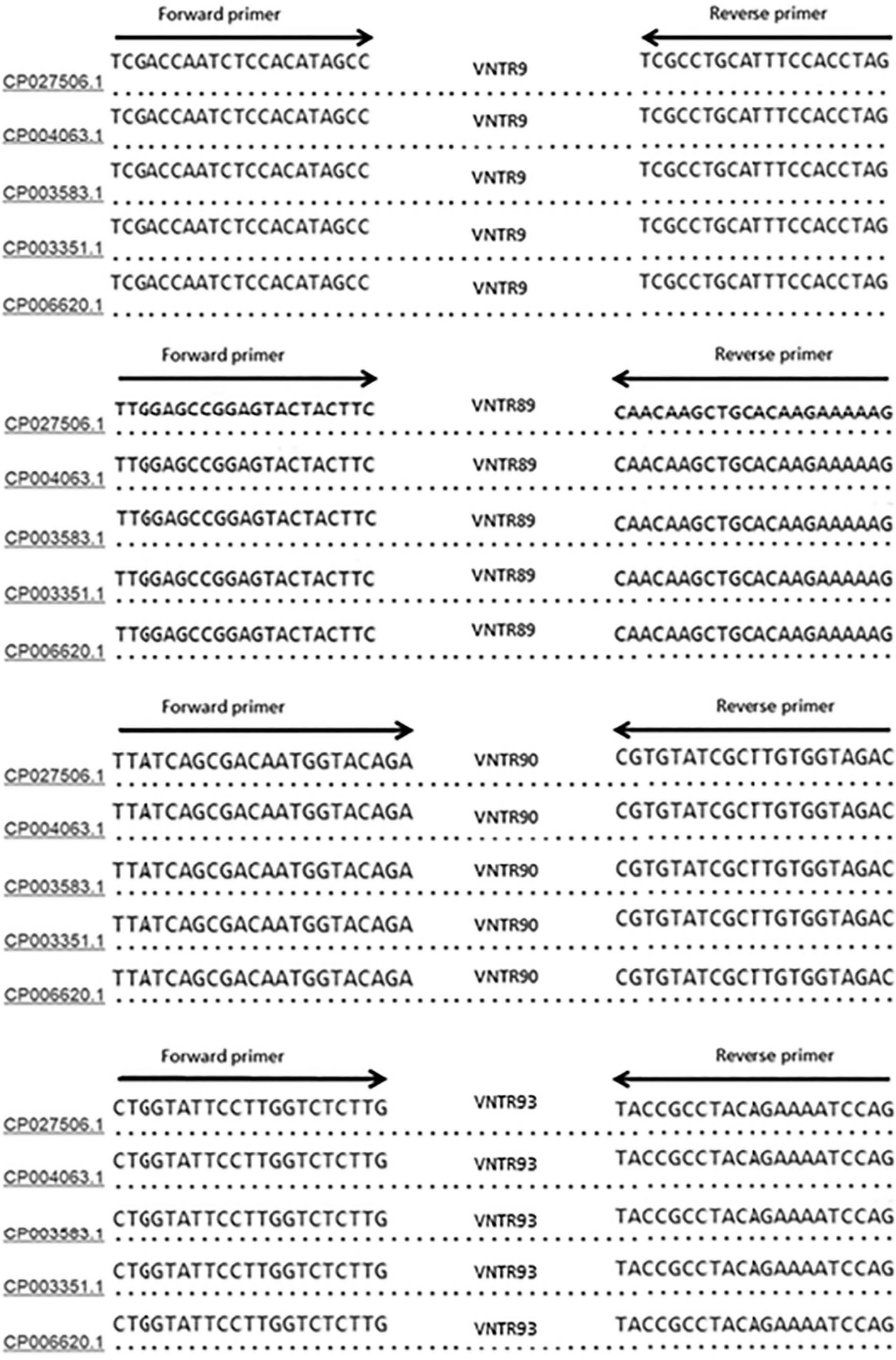
RESULTS AND DISCUSSION

We selected 9 original loci based on their variability and stability, and designed primers for each of them. VNTR-9 from MLVA6 was included, but with new original primers and different amplicon lengths in order to use it in multiplex PCR. MLVA10 was adapted for high resolution separation techniques like capillary electrophoresis and chip-based technologies, which allow separation of fragments differing by ~5 bp.

Table 1. Primer sequences for each VNTR locus.

VNTR	Primers
VNTR9	Forward: TCGACCAATCTCCACATAGCC
	Reverse: TCGCCTGCATTTCCACCTAG
VNTR89	Forward: TTGGAGCCGGAGTACTACTTC
	Reverse: CAACAAGCTGCACAAGAAAAAG
VNTR90	Forward:TTATCAGCGACAATGGTACAGA
	Reverse: CGTGTATCGCTTGTGGTAGAC
VNTR93	Forward: CTGGTATTCCTTGGTCTCTTG
	Reverse: TACCGCCTACAGAAAATCCAG
VNTR94	Forward: AATCGCKTATATYCGTTTTGC
	Reverse: AGCATTTTCCAAGGAAATGCC
VNTR96	Forward: CAAGCAGGAAATCGAGGCTA
	Reverse: CCCAGCCTCTTCTTTTTTTATAG
VNTR121	Forward: CTGCACGAACAAACTATGGAC
	Reverse: CAGCCAAKGATATGAATGTTAACG
VNTR148	Forward: CCAGAACGATCACCACAAAA
	Reverse: AAGAAAAGAGTAACAATCGCT
VNTR152	Forward: CATCGGCTGGAATATTTCGTC
	Reverse: GCAGCTGAAAGTCTAGTTGTC
VNTR153	Forward: TTTTACGTCGTTCTGCTCT
	Reverse: AACGACGGACCTTGAAGC

It was inconvenient that TRF works only with 1 genome at a time and the data it provides cannot be filtered, which makes the process difficult and time consuming. It was also not possible to differentiate between polymorphic and non-polymorphic loci which was essential for the purposes of this study.
The web application "Polloc V", developed by Luis-Miguel Rodriguez-R and Ralf Koebnik, which was used, contains integrated TRF algorithm (12). The role of Polloc V is to arrange the loci in groups based on the rate of similarity and their flanking regions. Then the groups can be visualized with a software for genomic processing like Geneious (16).
pSTR Finder (pSTR) is developed to analyze multiple samples of genome sequences for the presence of STRs. That application is also TRF based and it is available for non-commercial use (15). The user must provide sequences as FASTA files and use one of them as referent. Additionally, the length of the 5′ and 3′ flanking regions can be specified which is useful for subsequent sequence comparison analyzes. Unfortunately, we did not achieve any success with this application. Concatenating the referent genomes was necessary for the following clustering and analysis of the potential polymorphic loci. However, processing of concatenated genomes turned out to be beyond the capabilities of this software.
This issue was resolved by using TRDB. In 1999 Benson developed TRF – the algorithm which is now the basis for most of the available softwares for tandem repeats searching. TRDB consists of 2 parts: 1. public information about TRs, their nature and location in a set of different genomes; 2. the researchers work table where the user can chose between a set of different tools for genome processing.
Initially, TRDB contain information about 22 genomes. Later, more genomes are added as sequence information became available. TRDB gives the user an opportunity to search and filter certain TRs; to cluster TRs based on sequence identity; to predict polymorphic loci based on basic mutation patterns; to construct primers with interface based on Primer3 application (17); to download and save information in different formats; to visualize dynamic histograms and scatterplots of TRs characteristics; to align and visualize TRs in a separate browser (18).
The combination of two Geneious plugins - Mauve (19) and Phobos (20) played a key role in the detection of VNTRs. Mauve was used to make multiple alignment of the five referent genomes, and the other plugin – Phobos, was used to search for VNTRs directly in the aligned genomes, which facilitated the differentiation between polymorphic and non-polymorphic loci. Primers and their templates in each referent genome are shown on Figure 1.


Forward primer
Reverse primer
CP027506.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
CP004063.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
CP003583.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
CP003351.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
CP006620.1 TCGACCAATCTCCACATAGCC VNTR9 TCGCCTGCATTTCCACCTAG
Forward primer
Reverse primer
CP027506.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
CP004063.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
CP003583.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
CP003351.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
CP006620.1 TTGGAGCCGGAGTACTACTTC VNTR89 CAACAAGCTGCACAAGAAAAAG
Forward primer
Reverse primer
CP027506.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
CP004063.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
CP003583.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
CP003351.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
CP006620.1 TTATCAGCGACAATGGTACAGA VNTR90 CGTGTATCGCTTGTGGTAGAC
Forward primer
Reverse primer
CP027506.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG
CP004063.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG
CP003583.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG
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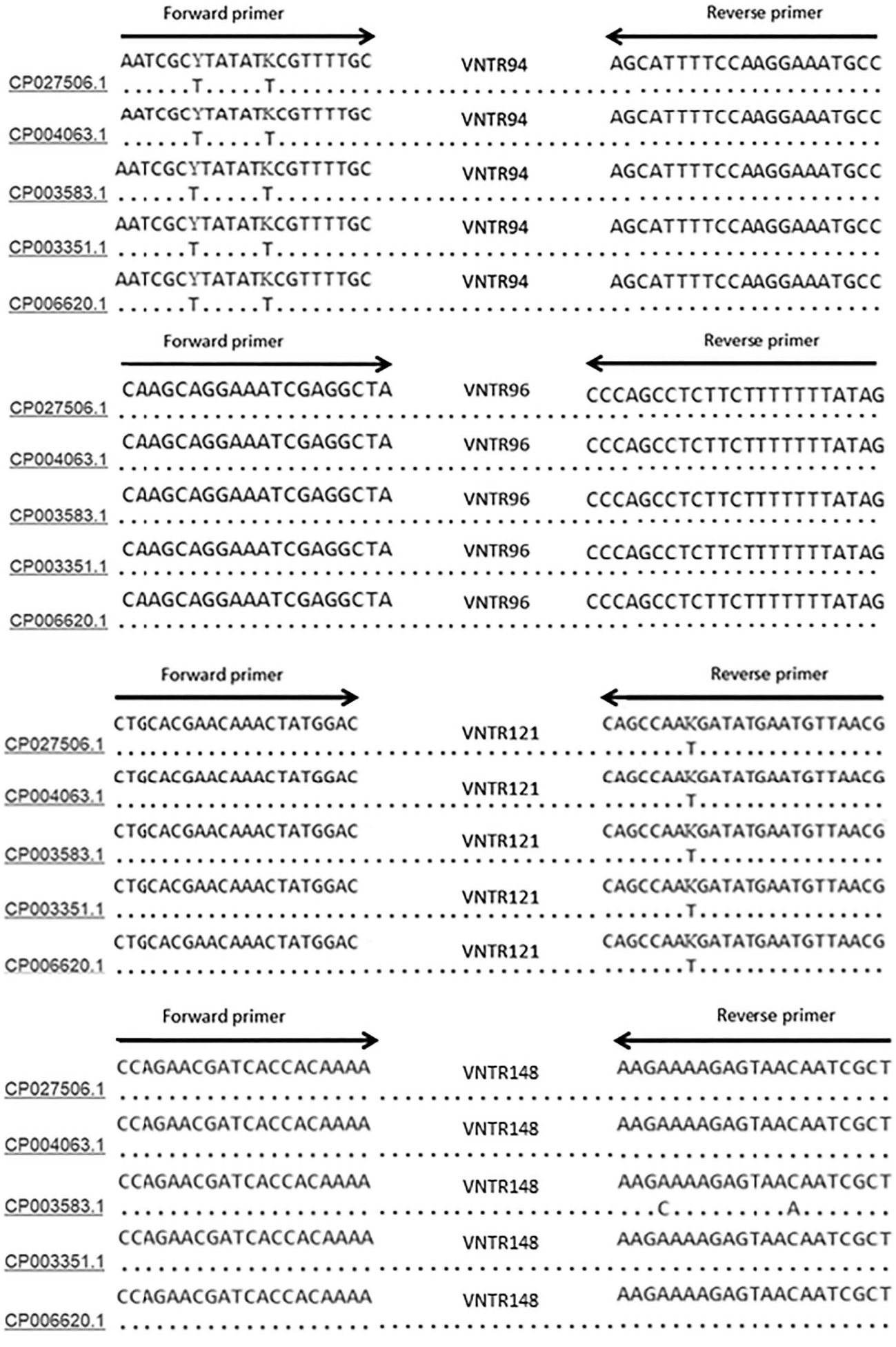
CP006620.1 CTGGTATTCCTTGGTCTCTTG VNTR93 TACCGCCTACAGAAAATCCAG


Forward primer
Reverse primer
AATCGCYTATATKCGTTTTGC
VNTR94
AGCATTTTCCAAGGAAATGCC
CP027506.1
CP004063.1
CP003583.1
CP003351.1
CP006620.1
Forward primer
Reverse primer
CAAGCAGGAAATCGAGGCTA
VNTR96
CCCAGCCTCTTCTTTTTTTATAG
CP027506.1
CP004063.1
CP003583.1
CP003351.1
CP006620.1
Forward primer
Reverse primer
CTGCACGAACAAACTATGGAC
VNTR121
CAGCCAAKGATATGAATGTTAACG
CP027506.1
CP004063.1
CP003583.1
CP003351.1
CP006620.1
Forward primer
Reverse primer
CCAGAACGATCACCACAAAA
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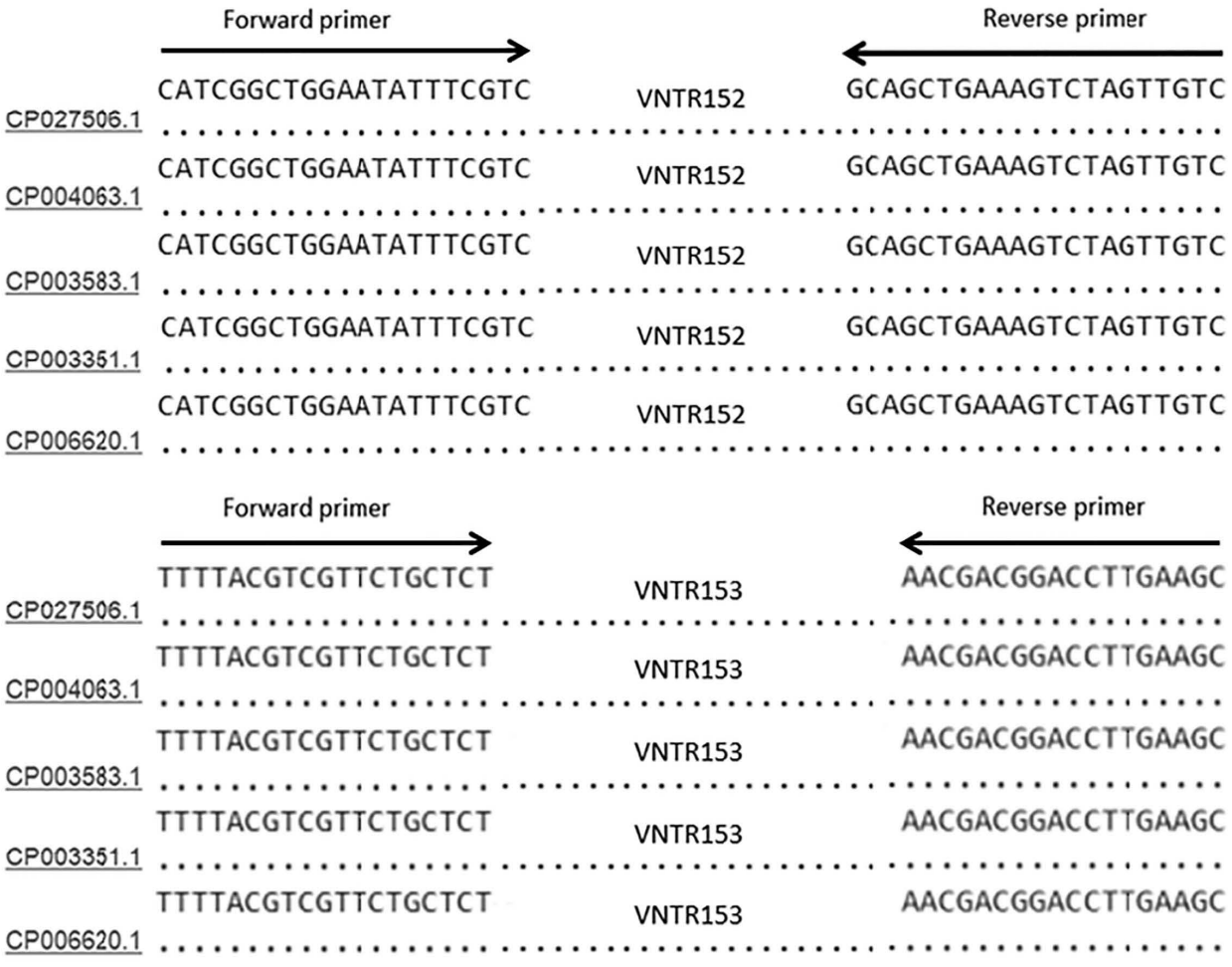

Figure 1. Used primers and the corresponding templates. Red letters correspond to ambiguous nucleotide bases (K=T/G; Y=C/T). Dots correspond to complete complementarity. At the left accession numbers of the referent genomes in NCBI Genbank are shown.

Hunter Gaston discriminatory index (HGDI) was measured for every single locus used (Table 2).

Table 2. HGDI for every locus of the new scheme.

VNTR	TR length (bp)	No. of Alleles	Fragment Length min/max (bp)	HGDI
VNTR9	121	0-2	666-911	0,509
VNTR89	33-36	2-7	298-475	0,479
VNTR90	57	6-18	331-1048	0,707
VNTR93	24	2-13	201-465	0,647
VNTR94	12	2-9	164-248	0,225
VNTR96	10	1-2,5	129-148	0,34
VNTR121	12	1-2	128-140	0,282
VNTR148	5	1-2	90-95	0,119
VNTR152	6	1-2	106-112	0,45
VNTR153	12	1-2	56-68	0,310

A total of 59 MLVA types (MTs) were generated from the *in silico* analysis of 114 isolates.

Some of the new loci showed lower discriminatory index than others. However, they were essential for discrimination between some initially indistinguishable sequence types. ST21, ST121 and ST456 were placed in one MLVA type. The addition of VNTR148 in the scheme resulted in dividing ST121 from ST21 and ST456. MLVA10 was unable to divide several variants of ST16 and ST17. ST796 and ST192 were also indistinguishable and the addition of VNTR153 solved this issue. ST5 was successfully separated from ST6 and ST1147, and also ST16 from ST282 by VNTR96. Another indistinguishable pair was ST412 and ST656. Two variants of ST412 shared the same MLVA profile with ST656. One of them was successfully separated by VNTR90, but the other remained bound to ST656. ST398 was separated from ST598 by VNTR148 and VNTR153. One variant of ST203 also remained indistinguishable from ST266.

Minimum spanning tree (MST) based on the in silico analysis was created for MLVA profiles. Eight of all the MLVA profiles contained 2 MLST profiles each (Fig.2). The opposite was also observed. Some MLST profiles (e.g. ST117, ST203) were divided and fell into different MLVA types, which is probably due to their high variability. MLVA10 failed to type 16 isolates, from which 8 were untypeable by both methods. The other 8 were successfully typed by MLST. The inability of MLVA10 to genotype some isolates is probably due to mutational events in some of the target loci

A total of 35 sequence types were acquired from the analysis in PubMLST (Public databases for molecular typing and microbial genome diversity) (21). Twelve of all the 114 isolates were not typeable by MLST. Four of these 12 were successfully typed by MLVA10. The rest 8 isolates were not typeable by both methods as mentioned above.

In order to get clearer and complete picture we analyzed the reverse option too. Minimum spanning tree based on MLST profiles was also created (Fig. 3). Sequence types which are divided into 2 or more MLVA types are pointed out by arrows. ST203, ST117 and ST17 turned out to be highly variable. ST203 is broken down into 7 MLVA types, ST117 – 10 MLVA types, ST17-7 MLVA types. These results show that MLVA10 is highly discriminatory against the variations in some of the sequence types.

With the current set of VNTR loci, MLVA10 was unable to discriminate between the following STs: ST21 and ST456; ST16 and ST17; ST6 and ST1147; ST117 and ST203; ST412 and ST656; ST117 and ST78; ST203 and ST266.

MLVA6 issues were similar – inability for discrimination of the following STs is observed: ST18, ST282, ST17, ST280 - MLVA type 1; ST65, ST16 - MLVA type 5; ST18, ST202 - MLVA type 7; ST78, ST117 - MLVA type 12; ST78, ST192, ST203, ST283 - MLVA type 159; ST279, ST314, ST307 - MLVA type 231. Table 3 summarizes the problems of the both schemes.

Table 3. Problems of MLVA6 and MLVA10

	MLVA type	Sequence type
MLVA6	1	ST18; ST282; ST17; ST280
	5	ST65; ST16
	7	ST18; ST203
	12	ST78; ST117
	159	ST78; ST192; ST203; ST283
	231	ST279; ST314; ST307
MLVA10	31	ST6; ST1147
	36	ST117; ST203
	38	ST21; ST456
	39	ST656; ST412
	41	ST203; ST266
	46	ST117; ST78
	50	ST16; ST17

ST78 and ST117 remained an issue for both the modified and the original scheme.

Discriminatory power of MLVA10 and MLST based on the *in silico* analysis was measured with the web app Comparing partitions (http://www.comparingpartitions.info/?link=Home). HGDI value for MLVA10 was 0.960 with confidence intervals 95% (CI95%) from 0.932 to 0.988. HGDI value for MLST was 0,928 with CI95% from 0.902 to 0.954. In order to get accurate result, isolates untypeable by one or both the methods, were excluded.

Comparison of the typeability between MLVA10 and MLST was also made. MLVA showed 85.9% typeability and MLST – 89.4%. MLVA10 was unable to type 16 of all 114 isolates, while MLST failed to type 12 of 114 isolates.

Some of the untypeable isolates are probably wild strains which are quite divergent and their genome can differ slightly from nosocomial

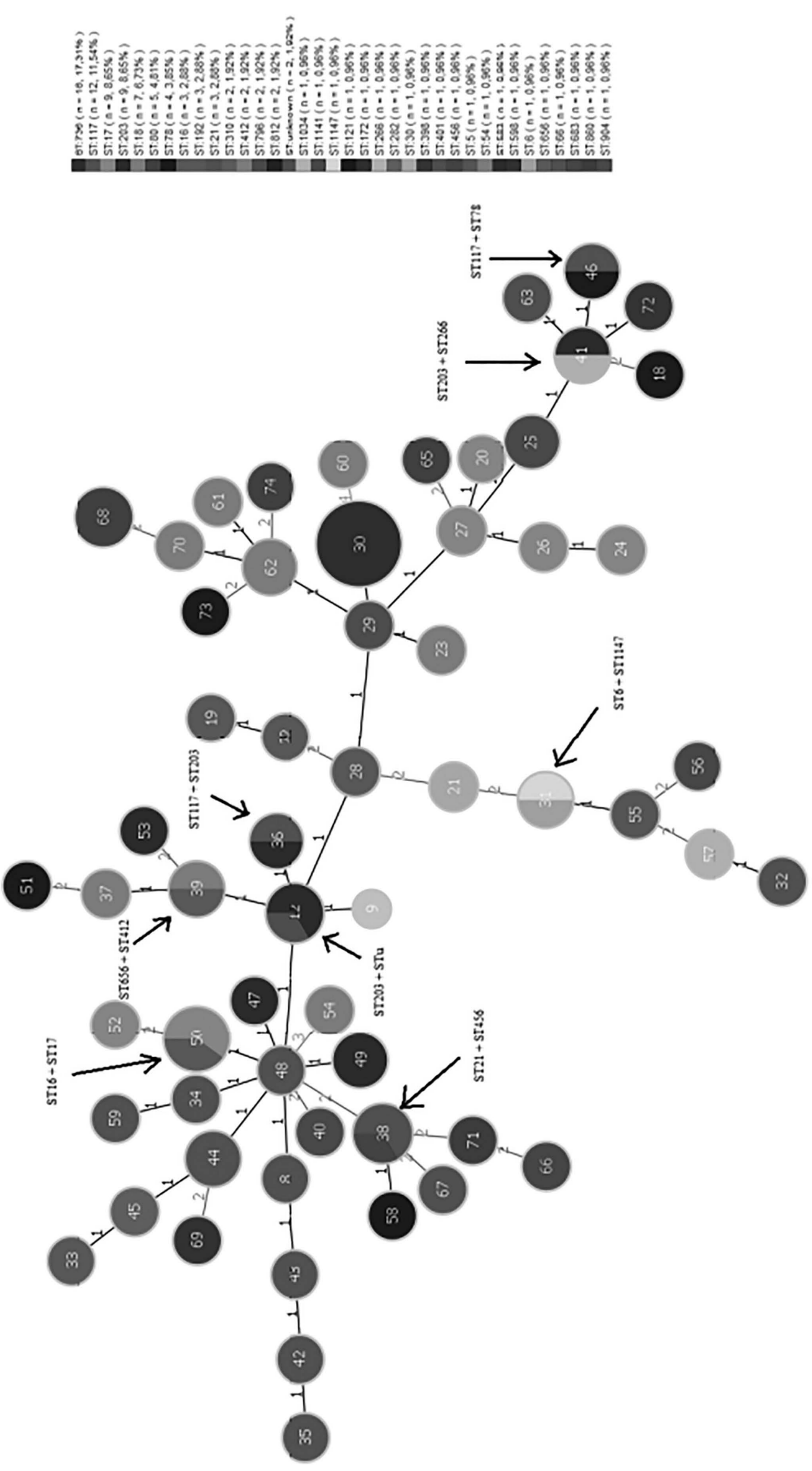

Figure 2. Minimum spanning tree (MST) based on 10 locus MLVA allele profiles of 114 *E. faecium* isolates used in *in silico* analysis. Relationship between different MLVA types is shown as line with an index of the number of loci by which they diverge. The arrows point out the MLVA types which contain more than 1 sequence types. STu corresponds to unknown sequence type. Isolates untypeable by both methods were excluded.

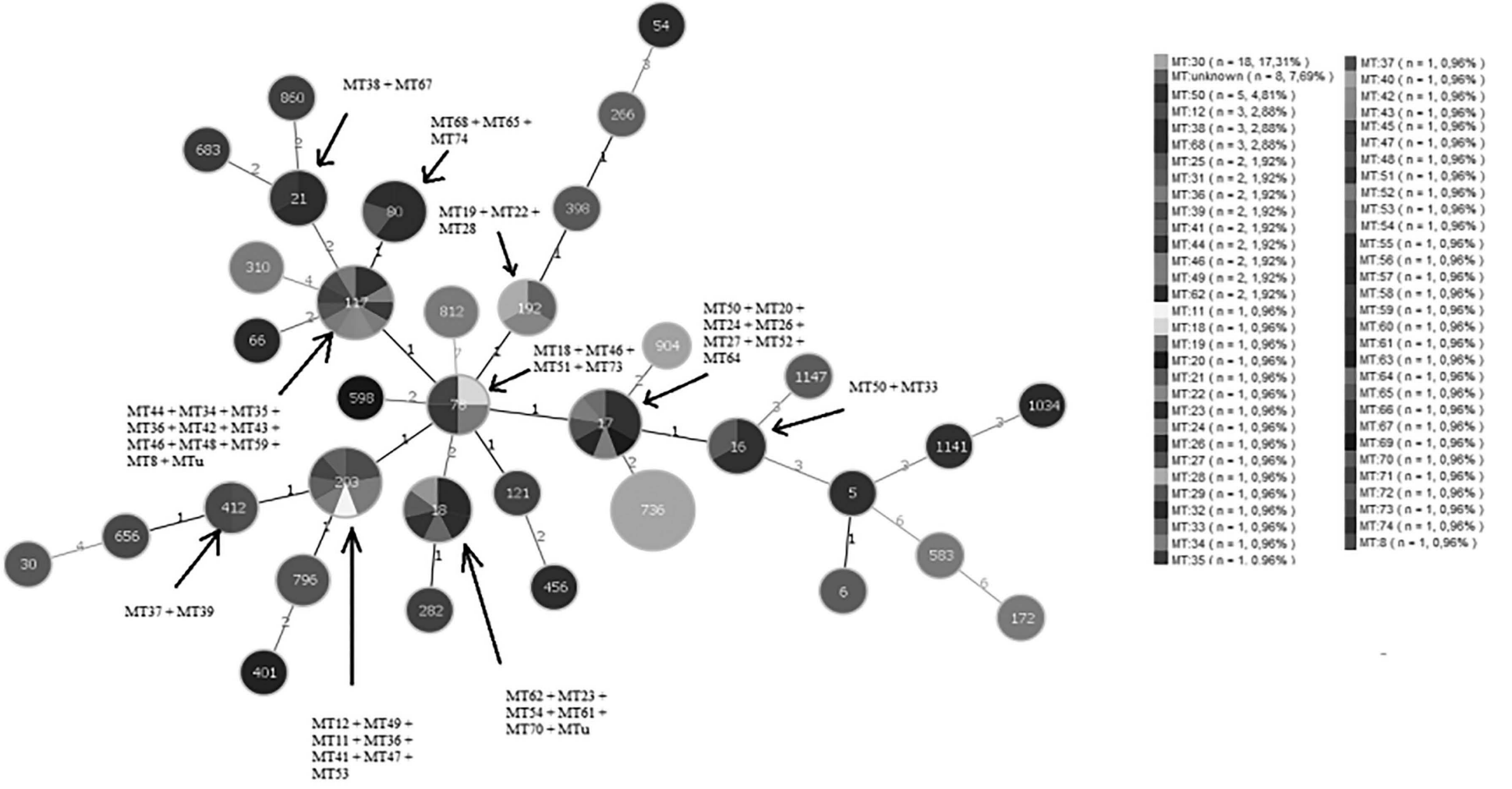

Figure 3. Minimum spanning tree (MST) based on MLST allele profiles of 114 *E. faecium* isolates used in *in silico* analysis. Relationship between different sequence types is shown as line with an index of the number of loci by which they diverge. The arrows point out the sequence types which are divided into more than 1 MLVA types. MTu corresponds to unknown MLVA type. Isolates untypeable by both methods were excluded.

strains. The inability of MLVA10 to genotype some isolates may be due to mutational events in some of the target loci.

CONCLUSIONS

Further examination and addition of more polymorphic loci compatible with the new multiplex PCR scheme can result in better discriminatory power.

MLVA shows higher discriminatory power than MLST and allows the prediction of sequence type with high accuracy.

MLVA10 is suitable for epidemiological studies of hospital-adapted isolates. Also it allows establishing of phylogenetic relatedness between geographically divergent strains.

MLVA can be performed within 4 hours (by capillary electrophoresis) per ~36 isolates, which is significantly faster than MLST and PFGE. It is cost-effective and easy to implement which makes it suitable for routine screening.

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