Full genome sequencing of porcine circovirus 3 fields strains from Denmark, Italy and Spain demonstrates a high within-Europe genetic heterogeneity

Running title:

PCV3 in Europe

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Summary

Porcine circovirus 3 (PCV3) is a new species of the *Circovirus* genus, which has recently been associated with different clinical syndromes. Its presence has been reported in different countries of North and South America, Asia and recently also Europe (Poland). However, differently from the other continents, no European PCV3 sequence is currently available in public databases. There is a strong need of epidemiological data and full genome sequences from Europe because of its relevance in the understanding of PCV3 molecular epidemiology and control. To fill this lack of information, samples collected in Denmark, Italy and Spain in 2016 and 2017 were screened for PCV3. Of the Danish samples, 36/38 of the lymph nodes, 6/20 serum samples and 2/20 lung

samples tested positive. Similarly, 10/29 lungs, 20/29 organ pools, 6/33 sera and 1/8 nasal swabs tested PCV3 positive in Italy. Fourteen out of 94 serum pools from 7/14 Spanish farms were also positive. Despite the convenience nature of the sampling prevents any precise prevalence estimation, the preliminary screening of the data from three European countries confirmed a rather wide PCV3 distribution in Europe. Furthermore, the analysis of the six obtained complete European PCV3 genomes and their comparison with the public available sequences seems to support a remarkable worldwide PCV3 circulation. These results underlines once more the urgency of more extensive epidemiological studies to refine the current knowledge on PCV3 evolution, transmission, spreading patterns and impact on pig health.

Keywords

Porcine circovirus 3, Europe, Full genome, Molecular epidemiology

Introduction

The *Circovirus* genus includes non-enveloped viruses with a single stranded circular genome of approximatively 2kb. The tropism of these viruses was traditionally considered limited to a restricted number of avian species and to swine (Todd, 2004). More recently, circoviruses have been proven to infect several host species, belonging to different animal classes. Nevertheless, their causative role in overt clinical disease is still unclear or marginal in most instances (Delwart and Li, 2012). The main exception is represented by the porcine circovirus 2 (PCV2), which has emerged as one of the most widespread and devastating diseases affecting swine farming (Segalés, 2012; Franzo et al., 2016). In 2016, a new circovirus species, named *Porcine circovirus 3* (PCV3) was identified by deep sequencing in the USA (Palinski et al., 2017). Since then, several reports have described its presence in China (Zheng et al., 2017), Poland (Stadejek et al., 2017) and Korea (Kwon et al., 2017), supporting a worldwide distribution. Similarly to PCV2, PCV3 has been associated with various clinical outcomes and lesions, including porcine dermatitis and nephropathy syndrome (PDNS), reproductive disorders, respiratory signs (Palinski et al., 2017; Ku et al., 2017; Shen et al., 2017) and myocarditis (Phan et al., 2016). Nevertheless, its presence in asymptomatic animals has also been reported (Zheng et al., 2017) and definitive evidences of its virulence are still lacking.

Being a single stranded DNA virus, PCV3 is expected to display a high evolutionary rate and therefore the knowledge of its molecular epidemiology is of pivotal importance. In fact, the availability of viral genome sequences represents a fundamental substratum for the understanding of viral spreading patterns and for planning adequate control measures (Kühnert et al., 2011; Scotch et al., 2011).

Thus, even though PCV3 has been reported in a single country in Europe (Stadejek et al., 2017), no European PCV3 sequences are currently publicly available. To fill this gap, the present study reports the first European PCV3 complete genome sequences, obtained by the joined efforts of three

laboratories located in Denmark, Italy and Spain, further supporting the wide distribution of this virus in Europe.

Materials and Methods.

Samples

A total of 271 samples were included in the study consisting of, 78 Danish (20 lungs, 20 serum and 38 lymph nodes), 99 Italian (29 lungs, 29 organ pools, 33 sera and 8 nasal swabs) and 94 Spanish samples (serum pools).

Italian samples were randomly selected archived samples delivered to the Veterinary Infectious Disease laboratory (Dept. Animal Medicine,Production and Health, Padua University, Italy) for routine diagnostic purposes between 2016 and 2017. Samples originated from sows and gilts (13 samples), nursery (45 samples) and growing and finishing (41 samples) pigs. The Danish samples (lymph nodes and placenta of sows, lungs from pigs (age unknown) and serum from pigs) were delivered for different diagnostic purposes, including the evaluation of decreased farrowing rates presence of respiratory disease and PCV2 viral load quantification. The Spanish samples were part of a longitudinal study in which 4-6 serum pools per farm were obtained from pigs at the end of the nursery and/or beginning of fattening periods (starting collection at 7 or 8 weeks of age and finishing it between 12 and 14 weeks of age). Each pool corresponded to 10 animals of the same age, collected longitudinally on a weekly basis, from a total of 15 farms. All studied farms were considered healthy (no evident clinical signs) and selection of pigs to be bled was performed randomly.

PCV3 diagnosis and sequencing

Italian and Spanish samples were extracted using the ExtractSpin TS kit (Bio-Cell, Rome, Italy) and tested for PCV3 using the real-time PCR described by Franzo et al., 2017 (Submitted).

Briefly, 2µL of extracted DNA were added to a standard mix composed by 1X DyNAmo Flash Probe qPCR Master mix, 0.6 µM and 0.3 µM of PCV3 specific primers and probe, respectively

(Table 1), 0.4 µM and 0.2 µM of internal control (IC) primers and probe (Hoffmann et al. 2006), respectively and 5 pg of IC plasmid. Sterile nanopure water was added to bring the final volume up to 10 µL. The cycling parameters were 95° C for 7 min followed by 45 cycles of 95°C for 10 sec and 60°C for 30 sec. The fluorescence signal was acquired at the end of each cycle extension phase. Danish samples were extracted as described elsewhere (Hjulsager et al., 2009) and tested for PCV3 using the assay described by Palinski et al. (2017).

Full genome sequencing was attempted on all samples with a Cp lower than 30 (corresponding to a viral titer of 100 copies/µL). Three primer pairs (Table 1) were used to amplify and sequence the whole PCV3 genome by three overlapping amplicons. Two µL of extracted DNA were added to a standard mix composed of 1X Phusion®High-Fidelity mix, 200 µM dNTPs, 0.6µM of each primer and 0.5 units of Phusion DNA Polymerase. Sterile nanopure water was added to bring the final volume up to 25 µL. The following thermal protocol was selected: 98° C for 30 sec followed by 45 cycles of 98°C for 10 sec, 64°C for 20 sec and 72°C for 45 sec. A final extension phase of 5 min at 72°C was also performed. Amplification and specificity of bands were visualized using a SYBR safer stained 2% agarose gel. DNA sequencing was performed at Macrogen (Macrogen Europe, Amsterdam, Netherlands).

Data analysis

All chromatograms were visually inspected with Finch TV program. 1.4.0 (2004–2006 Geospiza Inc) and consensus were obtained using the CromasPro (CromasPro Version 1.5; Technelysium Pty Ltd, South Brisbane, Australia; **http://technelysium.com.au/wp/chromaspro/**). The sequences were aligned with all PCV3 complete genomes available in GenBank (accessed 25/08/2017) using MAFFT (Standley, 2013), and tested for recombination using GARD (Kosakovsky Pond et al., 2006). Finally, a phylogenetic tree was reconstructed using the Maximum likelihood method implemented in PhyML (Guindon et al., 2010) selecting as substitution model the one with the lowest AIC calculated using JmodelTest (Posada, 2008). The robustness of the clade reliability was evaluated by performing 1000 bootstrap replicates.

The raw genetic distance among strain pairs was calculated using MEGA6 software (Tamura et al., 2013).

Results and discussion

Of the Danish samples, 36 out of 38 of the lymph nodes collected from sows were PCV3 positive, as well as 6 out of 20 serum samples and 2 out of 20 lungs. Similarly, 10 out of 29 lungs, 20 out of 29 organ pools, 6 out of 33 sera and 1 out of 8 nasal swabs tested PCV3 positive in Italy. Fourteen out of 94 (15%) serum pools from 7 (50%) out of 14 tested Spanish farms were also positive (ranging from 1 to 4 positive pools, depending on the farm). Despite the convenience nature of the sampling prevented any precise prevalence estimation, the results confirmed a rather wide PCV3 circulation in Europe, since it was initially detected in Poland (Stadejek et al., 2017). The virus was detected in several tissues as well as in placenta-associated lymph nodes, supporting the broad and systemic organ tropism of PCV3 (Palinski et al., 2017).

All six complete genome sequences (Acc.Numbers MF805719-MF805724) were 2000 nt long and displayed two ORFs coding for 296 (*Rep*) and 214 aa (*Cap*) proteins, as previously described (Palinski et al., 2017).

The sequences displayed p-distance distances from the USA isolates (Acc.Number KT869077) ranging from 0.007 to 0.01. The Italian clade (mean within-clade genetic distance = 0.001) was more closely related to strains collected in South Korea and Brazil (p-distance = 0.003) (KY996341, KY996343, KY996341 and MF079254) while the Spanish sample demonstrated a higher similarity with South Korean strains (p-distance = 0.003) (KY996341 and KY996343). The two Danish sequences (mean within-clade genetic distance = 0.004) revealed the closest relationship with strains detected in South Korea and China (p-distance = 0.006) (KY996338, KY996341, KY996338 and KY075986). A heat Map reporting the p-distance calculated between different sequence pairs of the analyzed sequences is displayed in Figure 1.

The phylogenetic tree based on the complete genome alignments demonstrated a tendency of the sequences obtained in the present study to cluster according to the country of sampling. Nevertheless, a more comprehensive analysis of the phylogenetic tree demonstrated a quite different scenario characterized by a broad mixing of strains collected in different countries and even continents. Particularly, even if the definition of a genetic cut-off is challenging and probably misleading (Franzo et al., 2014), at least two groups can be potentially defined (Figure 2), both including strains collected in North and South America, Asia and Europe. Remarkably, while Danish sequences form a quite independent clade part of the B group (Figure 2), Italian and Spanish ones were part of the A Group. Import of living pigs to Denmark is almost non-existing whereas a large number of sows have been exported to other countries, including Korea and China. This may explain the different grouping of the Danish sequences.

Based on these results, a single PCV3 introduction event is an unlikely justification for the European PCV3 heterogeneity and for the phylogenetic relationship herein described. As already described for PCV2 (Franzo et al., 2015), a worldwide PCV3 circulation leading to multiple introduction events in different European countries followed by independent local evolution appears a more likely scenario. . This is further supported by the demonstration of the PCV3 infection in asymptomatic animals, which, together with the recent PCV3 identification, could have favored an undetected and uncontrolled viral circulation.

Unfortunately, the paucity of currently available information hampers any definitive statement and further studies and more data will be necessary to clarify PCV3 molecular epidemiology, its origin, its impact on pig health and its transmission in and between countries and continents.

Conflict of interest statement

All authors have declared no conflict of interest.

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Primers/probes	Oligonucleotides	Assay
PCV3_353_F	5’-TGACGGAGACGTCGGGAAAT-3’	qPCR
PCV3_465_R	5’-CGGTTTACCCAACCCCATCA-3’	
PCV3_418_probe	5’-FAM-GGGCGGGGTTTGCGTGATTT-BHQ1-3’	
PCV3_74_F	5’-CACCGTGTGAGTGGATATAC-3’	PCR1 (Palinski et al.,2017)
PCV3_927_R	5’-CAAACCCACCCTTAACAG-3’	
PCV3_1303_F	5’-ACCGGAGGGGTCAGATTTAT-3’	PCR2
PCV3_541_R	5’-GAGCTGCTGCTTGAAGATCC-3’	
PCV3_817_F	5’-GTTATAATGGGGAGGGTGCT-3’	PCR3
PCV3_1647_R	5’-GCCTGGACCACAAACACT-3’	

Table 1

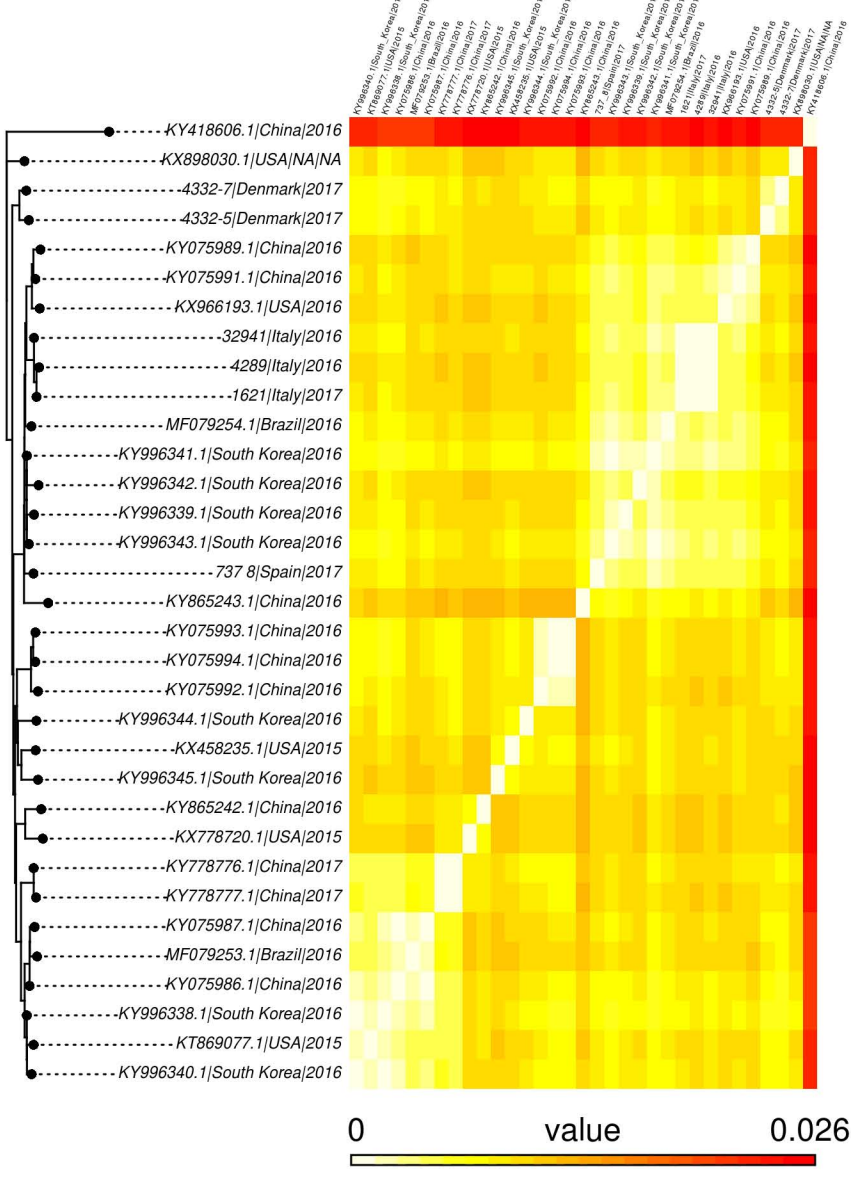
Primers and probes implemented in the assays described in this study.

Captions

Figure 1) Heat Map reporting the p-distance calculated between different sequence pairs. The relationship among strains is displayed through the maximum likelihood phylogenetic tree based on the full PCV3 genome.

Figure 2) Maximum likelihood phylogenetic tree based on the full PCV3 genome. The branch support is displayed in grayscale with darker black indicating higher bootstrap values. Danish, Italian and Spanish sequences are highlighted in blue, red and yellow, respectively.

254
255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KY778777.1|China|2017
KY778776.1|China|2017
KX778720.1|USA|2015
KY865242.1|China|2016
KY996345.1|South_Korea|2016
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737 8|Spain|2017
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KX898030.1|USA|NA|NA
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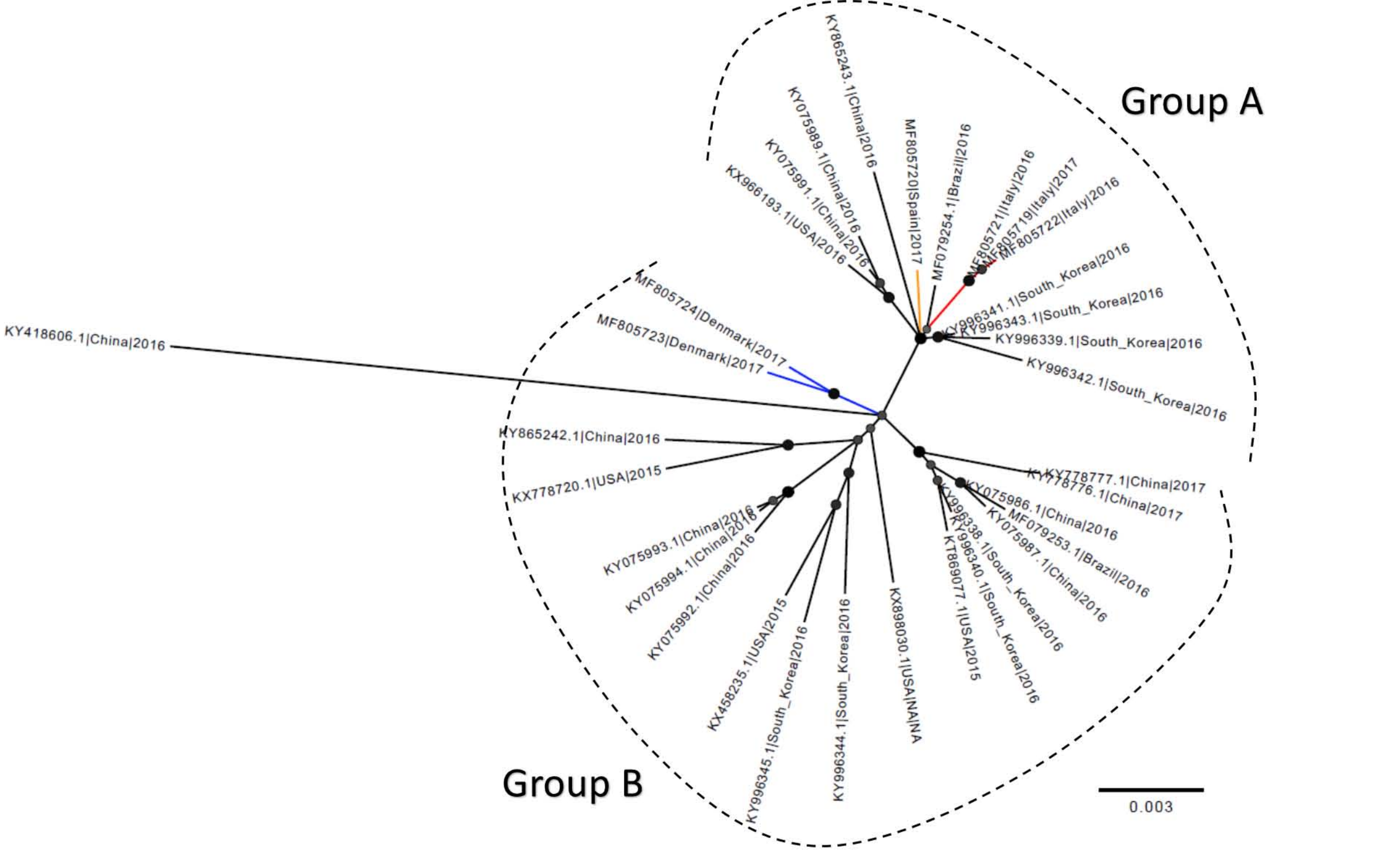
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