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SHORT COMMUNICATION

Phylodynamic analysis of current *Porcine circovirus 4* sequences: Does the porcine circoviruses evolutionary history repeat itself?

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Abstract

Four porcine circoviruses (PCVs) have been discovered over time and seem to share a common history, particularly for PCV-2 and -3. Despite being reported as apparently new viruses, rapidly emerging as a threat for the worldwide swine industry, they were then proven to have been circulating and coexisting with domestic pigs undetected for decades, without causing relevant health issues. A similar scenario could be true for the most recently identified PCV-4. However, its detection in Asia only and the limited genetic variability could suggest a truly recent origin. To investigate which of the above-mentioned scenarios is more plausible, a phylodynamic analysis was performed on all available PCV-4 sequences for which adequate metadata were available to reconstruct the viral history and evolution. Obtained results suggest an ancient origin, at least decades ago, followed by a prolonged low-level circulation and a moderate increase in viral population size after the second half of the XX century, in parallel with a progressive rise in pig population and farming intensification. A relevant local geographical clustering was also highlighted. The reason behind such low spreading capacity and limited geographical distribution compared to other circoviruses is currently obscure and will require dedicated studies, involving a more extensive sampling and sequencing activity.

KEYWORDS
evolution, molecular epidemiology, origin, PCV-4, phylodynamics, phylogeography

1 | INTRODUCTION

Porcine circoviruses (PCVs) are members of the genus *Circovirus*, featured by a circular, single-stranded DNA genome of about 1.7–2.0 kb. The two major open reading frames, ORF1 and ORF2, encode the replicase protein (Rep) and capsid protein (Cap), respectively (Finsterbusch & Mankertz, 2009). *Porcine circovirus 1* (PCV-1) was first identified in the early 1970s and, although widespread in the swine population, is considered nonpathogenic (Opriessnig et al., 2020; Tischer et al., 1974). Conversely, *Porcine circovirus 2* (PCV-2) and, presumably, 3 (PCV-3) are associated with several multi-factorial syndromes of economical relevance, such as Porcine Circovirus Disease

(PCVD) and PCV-3 systemic (PCV-3-SD) and reproductive disease (PCV-3-RD) (Arruda et al., 2019; Chae, 2005; Gillespie et al., 2009; Klaumann et al., 2018; Saporiti et al., 2021; Trible & Rowland, 2012).

PCV-2 and -3 share a common history of recent detection and apparent sudden emergence, a picture thereafter disavowed by retrospective studies demonstrating a persistent, undetected circulation over several decades in domestic pigs. First identified in Canada in early 1990s, PCV-2 was thereafter detected globally, starting from archive samples collected in 1962 (Jacobsen et al., 2009). Similarly, the distantly related PCV-3 was first reported in the United States in 2015, and only later retrospectively detected in samples collected since 1967, testifying the ancient origin (Rodrigues et al., 2020). Moreover, molecular clock analyses have further backdated the origin of such viruses of decades or even centuries (Franzo et al., 2016; Franzo et al., 2019; Palinski et al., 2017; Rodrigues et al., 2020).

A distinct PCV species, designated as *Porcine circovirus 4* (PCV-4), has been recently identified in China from pigs with different clinical conditions (Tian et al., 2021; Zhang et al., 2019). The lack of detection in European countries like Italy and Spain, and epidemiological surveys showing its presence in several Chinese provinces and South Korea, apparently suggest a recent origin that might justify the currently restricted distribution (Chen et al., 2021; Franzo et al., 2020; Ha et al., 2021; Hou et al., 2021; Nguyen et al., 2021; Sun et al., 2021; Tian et al., 2021; Zhang et al., 2020). However, PCV-4 has been detected in samples from 2012, raising exciting questions on its real epidemiology and emergence (Hou et al., 2021). Moreover, the number of countries where its presence has been investigated is still limited, and caution should be applied in the inference of its actual distribution.

Because of the experience with PCV-2 and -3, this preliminary study attempts to investigate the origin and the evolution of the new PCV-4, based on currently available sequences.

2 | MATERIAL AND METHODS

2.1 | Data set preparation

All currently available PCV-4 full genome sequences and related metadata were downloaded from GenBank (accessed on 22 March 2022; Table S1). Each sequence was annotated with accession number, sampling country and province when available, host species and collection date. Complete ORF1 and ORF2 sequences data sets were also prepared. The sequence MK948416 was not included in the Rep-data set due to the significant difference in the protein length compared to the reference Rep-sequence; a sequencing error could thus not be reliably excluded.

2.2 | Alignment and phylogenetic analysis

Full genome, ORF1 and ORF2 sequences were aligned using the MUSCLE method implemented in MEGA X (Edgar, 2004; Kumar et al., 2018). Pairwise p-distances and phylogenetic trees were generated using the same software. The latters were inferred using the maximum likelihood method (ML) selecting the best substitution model based on the Bayesian Information Criterion (BIC) score calculated in MEGA X. The robustness of the inferred clades was assessed by performing 1000 bootstrap replicates.

2.3 | Recombination and selective pressure analysis

Recombination analysis was performed on complete genome, ORF1 and ORF2 using the Genetic Algorithm for Recombination Detection method (GARD) implemented in Datamonkey (Kosakovsky Pond et al., 2006; Weaver et al., 2018). Episodic diversifying selection was also investigated using Mixed Effects Model of Evolution (MEME) (Murrell et al., 2012).

2.4 | Phylodynamic analysis

Different population parameters, including time to most recent common ancestor (tMRCA), evolutionary rate and viral population dynamics, were independently estimated for the complete genome, ORF1 and ORF2, using the Bayesian serial coalescent approach implemented in BEAST 1.10. To this purpose, the best substitution model was selected based on the BIC calculated using JModelTest; molecular clock was selected by comparing the different models (strict vs. relaxed molecular clock) based on Bayesian Factor (BF), which was calculated through estimation of the marginal likelihood of the different models using the path sampling (PS) and stepping stones (SS) methods (Baele et al., 2012; Darriba et al., 2012). The non-parametric Skygrid model was selected to infer viral past population dynamics (i.e. Effective population time × generation time; $Ne \times t$) (Hill & Baele, 2019). The reconstruction of viral migration among provinces was simultaneously reconstructed on the ORF2 data set using the discrete-trait phylogeographic approach described by Lemey et al. (2009). A Bayesian stochastic search variable selection (BSSVS) was also implemented to allow the calculation of a BF test that identified the well-supported migration routes. All parameters were estimated performing a 200 million generation long Markov chain Monte Carlo (MCMC) sampling the parameters every 20 thousand generations. Runs' mixing and convergence were assessed using Tracer 1.6 and results were accepted only if the estimated sample size was greater than 200. Parameters were summarized in terms of mean and 95% Highest Posterior Density (HPD) after the exclusion of a burn-in equal to 20% of the run length. A maximum clade credibility tree was also reconstructed after removal of the first 20% of the trees using the treeannotator tool of the BEAST package.

3 | RESULTS

A total of 41 complete genome, 40 ORF1 and 47 ORF2 sequences were included in the data set. The only two non-Chinese sequences

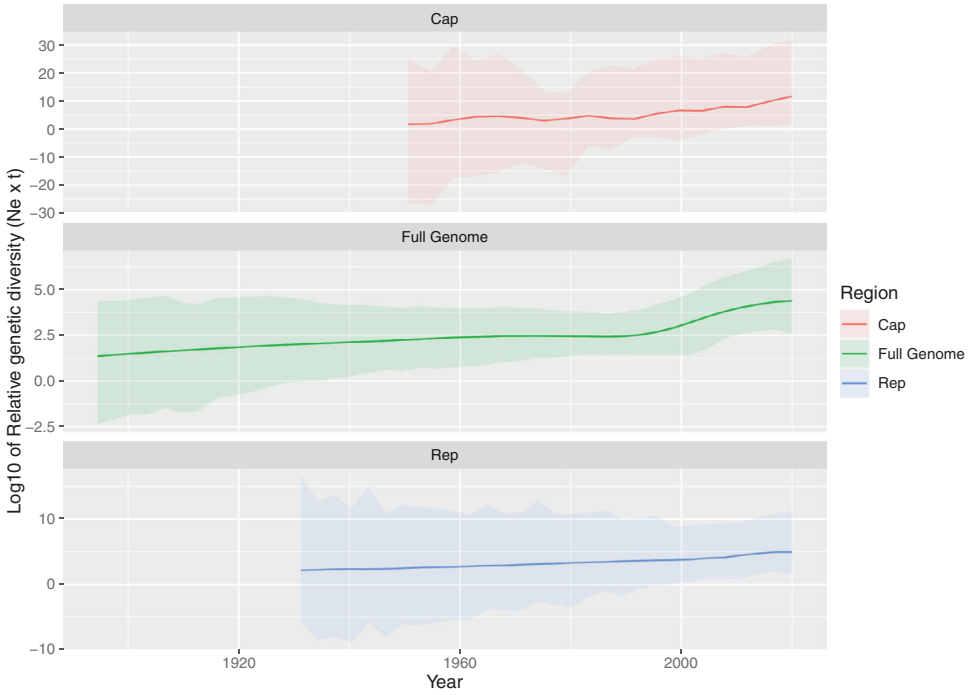

FIGURE 1 Skygrid plot reconstructing the viral relative genetic diversity ($Ne \times t$) over time. Results obtained based on complete genome, ORF1 and ORF2 are reported (colour-coded) in different panels. The mean value is represented by a continuous line while the 95HPD have been shaded

were detected in South Korea (one full genome sequence and one complete cap sequence). The remaining Chinese sequences originated from strains collected between 2012 and 2020 in five provinces: 18/47 (38.3%) from Henan; 14/47 (29.8%) from Hebei; 3/47 (6.4%) from Guangxi; 3/47 (6.4%) from Inner Mongolia; 4/47 (8.5%) from Jiangsu and 2/47 (4.3%) from Hunan.

The sampling province of one strain was not available. Regardless of the tested alignment, no evidence of recombination events was found by GARD.

The achieved alignment and phylogenetic trees show a certain genetic stability between strains over time, and congruent results were obtained for different regions (Figure S1).

The mean pairwise genetic distance was 0.96% [interval: 0.06%–1.81%], 0.84% [interval: 0.00%–1.81%] and 0.95% [interval: 0.00%–2.33%] for the full genome, ORF1 and ORF2, respectively. The corresponding estimated evolutionary rate was 5.83×10^{-5} [95HPD: 3.01×10^{-5} to 9.36×10^{-5}], 7.45×10^{-5} [95HPD: 4.32×10^{-5} to 1.87×10^{-4}] and 2.03×10^{-4} [95HPD: 2.7405×10^{-5} to 5.79×10^{-4}]. Moreover, no evidence of positive natural selection was detected on Rep or Cap proteins.

Despite the relatively high genetic homogeneity of PCV-4, the tMRCA, estimated through the coalescent Bayesian analysis performed on full genome, backdated PCV-4 origin before 1900 (i.e. 1894.52; 95HPD: 1832.96–1953.47), whereas ORF1 (1929.13; 95HPD: 1889.26–1956.87) and ORF2 (1952.48; 95HPD: 1907.77–1984.83) based origin estimations are around the first half of the 20th century, although with unavoidably broad 95HPD, due to the limited sample size and strain collection period. The analysis of viral population dynamics highlighted a long-term low-level circulation followed by a progressive rise, particularly in the second half of the XX century (Figure 1).

The phylogenetic analysis revealed an apparent geographical clustering (Figures 2 and S2). However, phylogeographic analysis demonstrated the presence of statistically supported migration rates between Henan and Guanxi, Henan and Hebei, Inner Mongolia, South Korea and Hunan, and Jiansgsu and Guangxi (Figure 3).

4 | DISCUSSION

The history of the better-known and ubiquitous PCV-2 and PCV-3 seems to conflict with that of the newly identified PCV-4, whose apparent geographical confinement raises several questions about its actual epidemiology and origin. Currently available PCV-4 sequences on GenBank provide a reliable baseline to start addressing these questions.

The phylogenetic approach adopted in this study shows a preliminary reconstruction of the evolutionary history of PCV-4 and results in a first estimation of its evolutionary parameters. This allows the comparison with other porcine circoviruses, opening to new speculative interpretations on its biology and impact.

In this regard, there are several elements that allow us to highlight a greater similarity of PCV-4 to PCV-3, rather than PCV-2.

The lack of recombination evidence resembles the PCV-3 pattern, contrasting with PCV-2 for which a marked tendency to recombine was demonstrated (Franzo et al., 2016; Franzo et al., 2019). However,

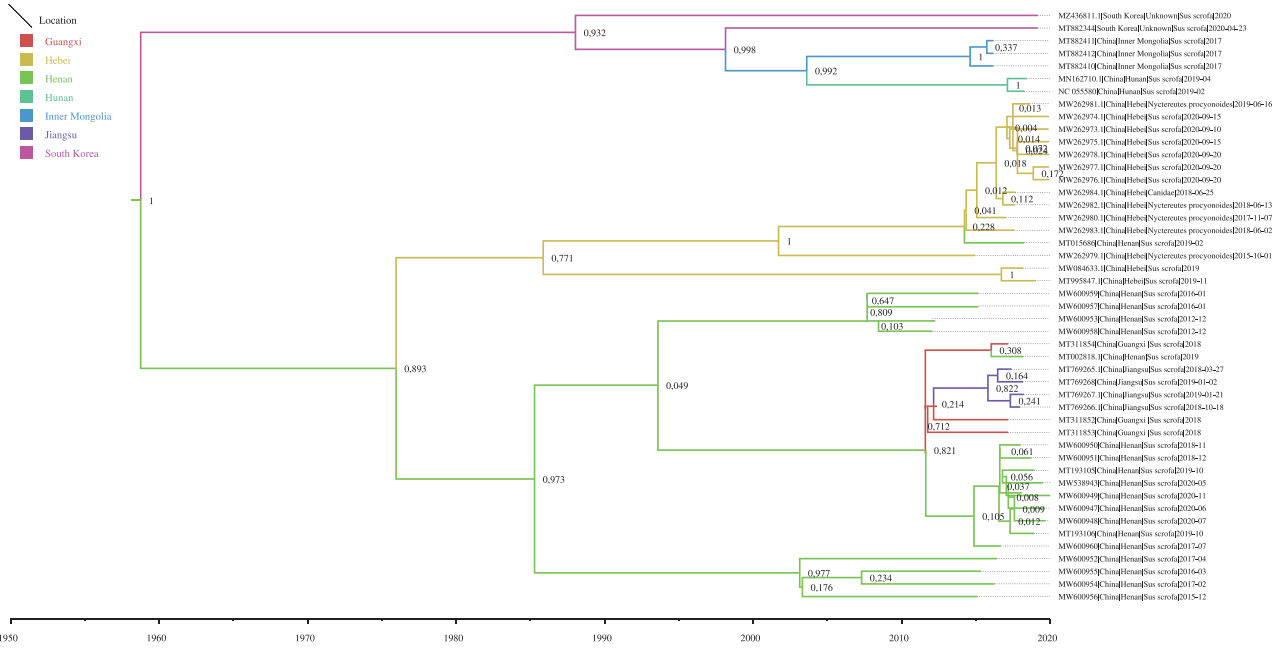

FIGURE 2 Maximum clade credibility tree estimated based on the ORF2 alignment. Branches have been colour-coded based on the strain sampling countries and predicted ancestral locations. The posterior probability of the inferred clade is reported nearby the corresponding node. The 95HPD of the estimated node age is reported as bars in the Figure S2.

it must be stressed that both PCV-3 and PCV-4 are featured by a lower genetic variability, which might hinder recombination detection and decrease the statistical power of the applied methods. Moreover, the limited PCV-4 sequence availability further complicates the results interpretation and future studies will be necessary to understand if the absence of recombination events reflects a biological viral feature or is ascribable to the considered data set.

The genetic stability between strains observed in previous studies has been confirmed by the achieved alignment and phylogenetic trees, pointing out another remarkable difference with PCV-2. Although the evolutionary rate is within the range featuring ssDNA viruses, it is lower compared to PCV-2 and of the same order of magnitude of PCV-3 (Duffy et al., 2008; Firth et al., 2009; Franzo et al., 2019). The reason behind such discrepancy remains obscure and different hypotheses could be advocated. A lower selective pressure acting on these species could be involved, potentially due to a less intense immune pressure, which has been repeatedly proven as one of the main driving forces of PCV-2 evolution (Correa-Fiz et al., 2020; Correa-Fiz et al., 2018; Franzo et al., 2016). While some evidence of PCV-3 pathogenicity has been reported (Saporiti et al., 2021), no consistent proof exists for PCV-4, potentially supporting a lower host immune system stimulation. Accordingly, no evidence of positive natural selection was detected on Rep or Cap proteins. However, also in this case, the limited sequence number decreased the methods' statistical power and robust results will require a more extensive and representative data set.

Furthermore, the possibility that the history of PCV-4 may retrace PCV-2 and PCV-3 steps deserves a few considerations. The PCV-4 detection in 2012 in Henan, which is the earliest sequence at present, demonstrates a circulation for at least 10 years in Chinese domestic pigs. As suggested by the analysis of viral population dynamics, similarly to other PCVs, an ancient origin followed by persistent but undetected circulation in the swine population can be claimed. The observed increase in the viral population size is likely associated with the intensification in the swine production system, creating more favourable conditions for viral replication and disrupting a long-lasting equilibrium. Nevertheless, the observed increase appeared less marked compared to what happened for PCV-2 and PCV-3 which might suggest a lower virulence/transmissibility (Franzo et al., 2016; Franzo et al., 2019). The smaller viral population size could also justify the reduced evolutionary rate and thus genetic variability observed for PCV-4.

On the other hand, the illustrated geographical clustering and the limited number of well supported migration rates among Chinese provinces, combined with the lack of PCV-4 detection in pivotal countries for swine farming, like Italy and Spain clashes with the ancient viral origin (Franzo et al., 2020). The currently scarce number of available sequences, both in China and at global level, and the short considered timespan hinder definitive conclusions. Nevertheless, we consider that if a wide PCV-4 circulation occurred, closely related strains should reasonably have been detected in different provinces and areas of the world.

Given such results, it comes natural to wonder what could be the factors justifying this intriguing contradiction between ancient origin and 'isolation' in such an interconnected world. Pathogen's related factors, like lower transmissibility, could be considered the main determinants since environmental and host's ones are shared with PCV-2 and PCV-3 that, in turn, display a worldwide distribution.

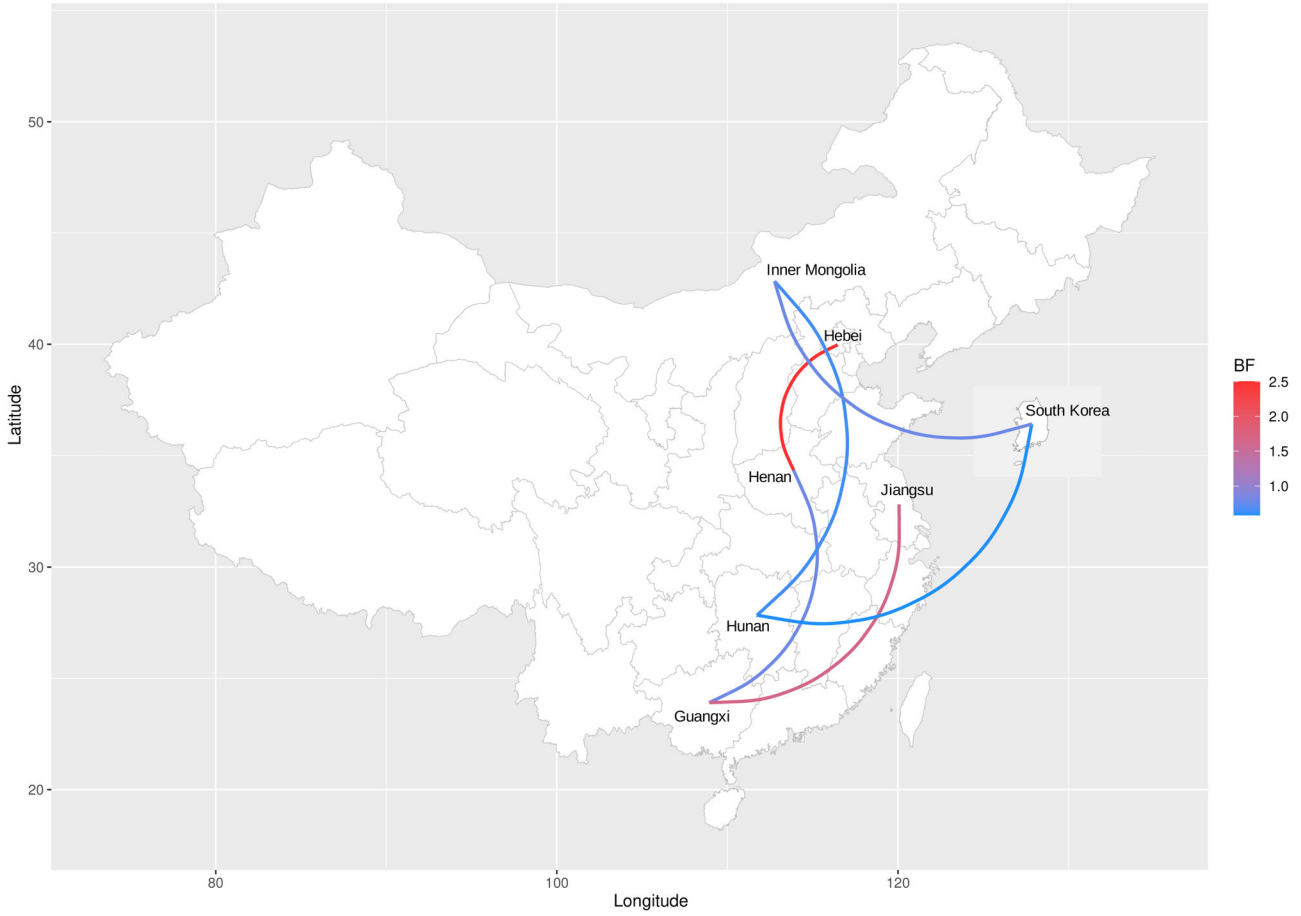

FIGURE 3 Map reporting the statistically supported migration rates among Chinese provinces. The connections are represented as lines colour-coded according to the Bayesian factor value

A less intensive diagnostic activity outside China cannot be excluded, although the high sample size tested in Franzo et al. (2020) disavows this hypothesis. Moreover, comparable and even smaller sample sizes did not preclude PCV4 detection in China and South Korea. Genetic heterogenicity in rapidly evolving viruses, like RNA and ssDNA ones, can decrease the diagnostic sensitivity of PCR-based assays due to the presence of primer-target genome mismatches.

An extreme geographical clustering leading to independent strain evolution and circulation of PCV-4 strains in Europe sufficiently heterogeneous to justify plausible mismatches between primers/probes and target cannot be excluded by current data. This hypothesis is nevertheless lessened by intensive pig flow among countries coupled with the apparent homogeneity of PCV-4 strains. Moreover, the higher genetic diversity of PCV-2 has never hindered its global identification.

Overall, the present results provide a preliminary glimpse into PCV-4 evolutionary history, fuelling the hypothesis of its long-lasting circulation. Nevertheless, the peculiarity of the described epidemiological scenario compared to other PCVs raises more questions than answers. Especially challenging is to understand the observed geographical isolation despite a likely ancient origin. The high uncertainty due to the limited number of sequences collected over a short period of time represents the main study limitation and confirms the scarce PCVs diagnostic and sequence sharing attitude featuring several countries. Of particular interest would be the implementation of retrospective studies, confirming the 'ancient origin hypothesis' and providing additional time-points for more accurate estimation.

These data could contribute to improve the understanding of PCV-4 epidemiology, origin and evolution, as well as genetic heterogeneity, and unmask the potential risk that PCV-4, similarly to the other more famous PCVs, may pose.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The present study is based on nucleotide sequences previously submitted in GenBank. No human or animal experiment was performed and no ethical approval was therefore required.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in GenBank at https://www.ncbi.nlm.nih.gov/nuccore. These data were derived from the following resources available in the public domain: GenBank, https://www.ncbi.nlm.nih.gov/nuccore/NC_055580, MT882410, MT882411, MT882412, MT882344, MW600947, MW600948, MW600949,MW600950, MW600951, MW600952, MW600953, MW600954, MW600955, MW600956, MW600957, MW600958, MW600959, MW600960, MW538943, MT193105, MT193106, MT311852, MT311853.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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