Abortion storm of Yili Horses is Associated with Equus Caballus Papillomavirus 2 Variant Infection

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Abstract

Background: Nine different species of *Equus caballus papillomavirus* (EcPV) and three bovine papillomavirus (BPV) have been reported to infect horses, however, there are so far no describing such infections in China. In January 2021, an abortion storm occurred in Yili horses, as a result of which 50 out of 93 aborted fetus samples were found to be negative for equid herpesvirus (EHV) and equine arteritis virus (EAV).

Results: In our pioneer study with Chinese horses, we first found EcPV-2 in the nasal swabs (4/230, 1.7%) of Yili horses, and semen (3/18, 16.7%) of the Thoroughbred horses. This indicated that EcPV can be indeed hosted by horses in China, and that EcPV-2 might be transmitted though breeding. Further detection of EcPVs in the lung tissues of aborted fetus in Yili horses, which were originally negative for equid herpesviruses, established that EcPV-2 was positive in 19 of 50 samples, thereby indicating that EcPV-2 might be a new pathogen causing of abortions. Thereafter, the sequence analyses for L1 genes sequences of 26 China's EcPV-2 were performed which indicated that EcPV-2, that primarily infected the horses in China, shared 98.3%-99.9% nt identity with the already published sequences for EcPV-2. These observations indicated that EcPV-2 identified in the current study were highly similar variants of the previously identified strains of EcPV-2. Phylogenetic analysis based on L1 genes in GenBank showed that EcPV-2, found in the Chinese horses, was closely related to and clustered together with an already known EcPV-2a lineage.

Conclusion: Our study provides the first evidence related to EcPV-2 infection in the Chinese horses, which can serve as a causative agent for Yili horse abortions, and thus can possibly lay the foundation for a systematic and detailed epidemiological study of this infection in the Chinese horses.

Background

Papillomaviruses (PVs) are small, non-enveloped viruses which harbor 8 kb of circular and double-stranded DNA genomes, that are prevalent amongst a wide range of the host species, including mammals, birds, reptiles, and fish [1]. They can be classified into the different species of PVs according to the diversity of nucleotide sequences in the open reading frame (ORF) of the L1 gene [2–4]. Moreover, now, more than 650 distinct animal and human papillomavirus (HPV) types have been identified, and the 440 HPV types have been discovered within the human population. Among other 210 PV types, nine different species of *equus caballus papillomavirus* (EcPV-1 to EcPV-9), and three species of bovine papillomavirus (BPV-1, BPV-2 and BPV-13) have been reported to be capable of infecting the horses worldwide [1, 3–8], however, there is no information available about the potential EcPV and BPV infection in the horses within China.

We have designed this present study to detect EcPVs and BPV infections in the horses of North Xinjiang, which is one of the major horse-producing regions in China, and found a possible association between the viruses detected and diseases in the horses.

Results

EcPV and BPV detection

In the present study, we have used PCR method to potentially detect EcPV and BPV infections in the Chinese horses. Interestingly, the PCR results confirmed the presence of EcPV-2 in the nasal swabs of Yili horses of farm A located in Yili city (Table 1), with the percentage of the positive cases for each strain being 5.4% (4/37) (Table 1), thus indicating that EcPV can effectively circulate in the horses of Xinjiang. Further investigation revealed that only EcPV-2 was detected in the three semen of 18 thoroughbred stallions in farm A and farm D belonged to Yili city and Changji city respectively (Table 1), which clearly suggested that EcPV-2 might possess a potential vertical transmission pathway, and possibly has a distinct geographical distribution. In order to validate our speculation, we detected the presence of EcPV-2 in 50 aborted fetus lung tissues without EHV as well as EAV, and PCR amplification revealed that EcPV-2 could be detected in 19 of 50 aborted samples. These findings supported our hypothesis that EcPV-2 could possibly be spread by the vertical transmission route, and might be a new pathogen responsible for abortions.

EcPV-2 sequencing

The fragments of EcPV-2 complete genome sequences were thereafter amplified using the primers listed in Table S1 and Table S2. After sequence assembling, the complete genome of the EcPV-2 obtained from the thoroughbred stallion named as XJ-YLKS1391 (accession numbers MW410986) was found to be 7802 nt in length which was rather similar to other EcPV-2 strains. In addition, the L1 genes of other 25 Chinese EcPV-2 were sequenced, and then deposited into the GenBank database under accession numbers: MW429199-MW429203 and OK362333-OK362352.

Comparison and phylogenetic analysis

Based on L1 gene-phylogenetic characteristics, published EcPV-2 have been assigned to EcPV-2a and EcPV-2b. The complete genome sequences of the EcPV-2, which was identified in the horse semen (7802 nt), named XJ-KS1391, (GenBank accession no. MW410986), shared a 99.87% sequence identity with EcPV-2a (GenBank accession no. EU503122) and a sequence identity of 99.05% with EcPV-2b (GenBank accession no. HM461973) thereby indicating that EcPV-2 XJ-KS1391 and EcPV-2a share a relatively closer genetic relationship. E6, E7, E1, E2, E4, L1 and L2 ORF of EcPV-2 XJ-KS1391 share high nucleotide (98.3-100%) and high amino acid identity (98.4-100%) with EcPV-2 reference strains. The L1 genes in 26 different strains of EcPV-2 from these horses possess a nucleotide identity of 99.8-100% (GenBank accession nos. MW410986, MW429199-MW429203), thereby indicating that these strains found in China are highly similar in their genetic makeup. In addition, these identified sequences were also compared with those of EcPV-2 reference strains Zurich_2009 (accession no. HM461973), IZS PLVA_tomach (accession no. MT063185), IZS PLVA_vulva (accession no. MT063186), and UK isolate (accession no. EU503122), with the results indicating a percentage similarity level of 98.3%-99.9% for the DNA sequences.

Phylogenetic tree based on L1 genes analysis of EcPV-1 to 9 revealed that EcPV-2 from the Chinese horses and EcPV-2a reference strains (accession no. EU503122) could potentially form an independent branch, and were closely related to EcPV-2b reference strains (accession no. HM461973, MT063185 and MT063186). These EcPV-2 were clustered into the lineages of *Dyoiota*, which were divergent from the lineages of *Treiskaapa*, *Zata* and *Dyorho* (Fig. 1).

Discussion

A number of previous studies that were conducted in different countries have documented that EcPV and BPV can infect the horses [1, 3–8]. However, before this study, no one has described about infections caused by EcPV and BPV in the Chinese horses. In the current study, we first investigated the existence of these two viruses in the different samples of Chinese horses. EcPV2 has been earlier detected in the penile, vulvar, clitoral and oro-pharyngeal papillomas, and the tissues of squamous cell carcinomas [10–13]. Our study is the first of its kind to report the presence of EcPV-2 in the nasal swabs of Chinese horses without display of any significant clinical signs (Table 1), and whether Chinese EcPV-2 can potentially cause papillomas and squamous cell carcinomas (SCC) of horses needs further epidemiological investigation. Moreover, similar to the previous studies which have reported the existence of EcPV-9 in the semen from a Thoroughbred Stallion with a Penile Lesion [6], and identification of PV nucleic acids in the semen of healthy cattle [14], we first detected the presence EcPV-2 in the semen from three clinically normal Thoroughbred Stallions (Table 1). These results suggested that the primary route for transmission of EcPV-2 could be possibly through the sexual activities in horses, and that the occurrence of EcPV-2 in the semen of Thoroughbred stallions should be cause of potential concern. In the present study, the semen, collected from Yili and Changji, was found to be positive for EcPV-2, thereby indicating that the virus could have been in circulation in the different regains of North Xinjiang (Table 1). in addition, to the nasal swabs of Yili horses and the semen of Thoroughbred stallions in farm A positive for EcPV-2, and aborted samples of Yili horses without EHVs and EAV in the same farm, a single prior study has found the existence of EcPV-2 DNA in the genital, oral mucosa, eyelid and skin from muzzle of dead equine fetuses due to dystocia [13]. Based on these initial observations, we decided to examine these aborted samples for the presence of EcPV-2 DNA. PCR results clearly showed that the EcPV-2 DNA was significantly amplified in 19 out of 50 lung tissues. Our data first detected the presence of EcPV-2 in aborted samples, thereby clearly indicating that the virus could contribute to Yili horse abortions. This finding is not surprising given that vertical transmission of HPV and BPV has been documented earlier [15, 16]. A number of the previously published studies have shown that EHV-1, EHV-2, EHV-4, EHV-5, EHV-8 and EAV can cause equine abortions [17–20]. The results of our current study, also indicated that the EcPV-2 could possibly be considered to be a new equine abortion virus.

Moreover, upon the sequencing and comparison of the L1 gene in the EcPV-2 strains in the Chinese horses showed a 98.3-99.9% nucleotide sequence identity with the reported EcPV-2 strains. According to EcPV taxonomy, that follows the general criteria established by the International Committee on the Taxonomy of Viruses (ICTV), an EcPV strain is potentially recognized as a variant if the nucleotide sequence of the L1 ORF differs by less than 2% homology with the closest known EcPV type [2–4]. Based

on this criteria, it can be concluded that the EcPV-2 strains in the Chinese horses could be indeed considered as legit variants of referenced EcPV-2 strains.

Based on L1 gene-phylogenetic characteristics, Lange et al proposed that EcPV-2 in the GenBank could be clustered into the lineages of EcPV-2a and EcPV-2b [4]. The results of the current study indicated that the Chinese EcPV-2 derived from the nasal swabs, semen and aborted fetuses were clearly related to EcPV-2 reference strain of EcPV-2a lineage (Fig. 1). In fact, L1 genes of EcPV-2 found in the Chinese horses share higher nucleotide (99.6-99.9%) with EcPV-2a reference strain than the known EcPV-2b ones (98.3-98.8%), these results support that Chinese EcPV-2 and EcPV-2a reference strain form a lineage.

Conclusion

In summary, EcPV-2 was detected from the samples of aborted fetuses, and as such, it was shown that the virus could be the probable cause behind the abortion storm. Further characterization of the L1 genes provided substantial evidence that Chinese EcPV-2 belonged to a known EcPV-2 variant. It is expected that the findings of this study will create awareness about the possible role of EcPV-2 in the horse abortions and its detection could be used for the differential diagnosis of abortions in the horses worldwide.

Methods

Sample collection

During July 2020 to May 2021, a total of 298 different samples, including 230 nasal swabs, 18 semen, and 50 aborted fetus lung tissues of Yili horses negative for both equid herpesvirus (EHV) and equine arteritis virus (EAV), were collected from the four farms located in Yili, Changji and Urumqi of North Xinjiang (Table 1). All the samples were, therefore, transferred to a tube containing 10 mL phosphate buffer for storage at -80°C until required for analysis. The bodies of aborted fetuses were treated by the farm's veterinarian using harmless treatment (Nong Yi Fa [2017] No. 25).

Viral nucleic acids extraction and sequencing

For nucleic acid extraction, each sample was first ground, then vortexed and after centrifugation, viral nucleic acids were extracted from 200 μL of sample supernatant based on the manufacturer's instructions (Geneaid Biotech Co., LTD). The presence of EcPVs and BPVs DNA in these nucleic acids was subsequently detected by PCR using the primers listed in table S1. PCR was carried out using the 2× *TransStart® FastPfu* Fly PCR SuperMix (TransGen Biotech Co., LTD) with the following PCR conditions: An initial denaturation at 95°C for 2 minutes, followed by 35 cycles, and with a denaturation step at 95°C for 20 seconds, as well as both an annealing and an extension steps at 72°C for 30 seconds each respectively. Eventually a final extension at 72°C for 5 minutes was performed (TransGen Biotech Co., LTD). The positive PCR amplicons were thereafter ligated into a *pEASY*®-Blunt T vector (TransGen Biotech Co., LTD) for transforming *E. coli* DH5a competent cells (TransGen Biotech Co., LTD) and ten

distinct clones of each amplicon were eventually selected for the Sanger sequencing (Sangon Biotech Co., LTD).

Comparison of the multiple sequences and phylogenetic analyses

A detailed information (GenBank accession numbers) about the sequences used in this study has been provided in Figure 1. All the nucleotide sequences of EcPV-2 which were generated in this study were submitted to GenBank, with the following accession numbers: MW410986, MW429199-MW429203 and OK362333-OK362352. The sequences were thereafter analyzed by MegAlign software in Lasergene v7.1 and phylogenetic trees of all the different target sequences were generated using the maximum-likelihood method based on the Tamura–Nei model. The accuracy of the tree topologies was also evaluated using 1,000 bootstrap replicates [9].

Abbreviations

EcPV
Equus caballus papillomavirus
BPV
bovine papillomavirus
EHV
Equid herpesvirus
EAV
equine arteritis virus
nt
Nucleotide
ORF
Open reading frame
SCC
squamous cell carcinomas

Declarations

Ethics approval and consent to participate

All experimental procedures involving animals were approved by the Animal Care and Use Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China under animal protocol number: 2020012, and performed according to the Animal Ethics Procedures and Guide-lines of the Ministry of Agriculture of China. Lung tissues of aborted fetuses were collected by the farm's veterinarian according to the approved procedures. The owner gave informed written consent for his lung tissues of aborted fetus's inclusion in the study. The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

No applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files. Sequences of EcPV-2 from aborted fetuses generated in this study have been submitted to GenBank

under accession nos. MW410986, MW429199-MW429203 and OK362333-OK362352.

Competing interests

The authors have declared no competing interests.

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Authors' contributions

P.T. and J.X. performed the research, analyzed the data, and drafted the manuscript. R.D., X.S., H.D., N.P., L.D., C.J., S.T., E.Y., W.M., J.W., L.K. contributed to the collection of samples and detection of PCR. P.T., L.K. and J.X. revised the manuscript. J.X. conceived the study, carried out additional analyses and finalized the manuscript. All authors have contributed to the editing of the manuscript. The authors have also read and approved the final manuscript.

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References

1. Della Fera AN, Warburton A, Coursey TL, Khurana S, McBride AA. Persistent Human Papillomavirus Infection. Viruses. 2021;13(2):321.
2. Bernard HU, Burk RD, Chen ZG, van Doorslaer K, zur Hausen H, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology. 2010;401(1):70–79.
3. de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology. 2004;324(1):17–27.

4. Lange CE, Vetsch E, Ackermann M, Favrot C, Tobler K. Four novel papillomavirus sequences support a broad diversity among equine papillomaviruses. J Gen Virol. 2013;94(Pt 6):1365–1372.
5. Peters-Kennedy J, Lange CE, Rine SL, Hackett R P. Equus caballus papillomavirus 8 (EcPV8) associated with multiple viral plaques, viral papillomas, and squamous cell carcinoma in a horse. Equine Vet J. 2019;51(4):470–
6. Li CX, Chang WS, Mitsakos K, Rodger J, Holmes EC, Hudson BJ. Identification of a novel equine papillomavirus in semen from a thoroughbred stallion with a penile lesion. Viruses. 2019;11(8):713.
7. Otten N, von Tscharner C, Lazary S, Antczak DF, Gerber H. DNA of bovine papillomavirus type 1 and 2 in equine sarcoids: PCR detection and direct sequencing. Arch Virol. 1993;132(1-2):121–131.
8. Lunardi M, de Alcantara BK, Otonel RA, Rodrigues WB, Alfieri AF, Alfieri AA. Bovine papillomavirus type 13 DNA in equine sarcoids. J Clin Microbiol. 2013;51(7):2167–2171.
9. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–1874.
10. Sykora S, Samek L, Schönthaler K, Palm F, Borzacchiello G, Aurich C, Brandt S. EcPV-2 is transcriptionally active in equine SCC but only rarely detectable in swabs and semen from healthy horses. Vet Microbiol. 2012;158(1-2):194–
11. Bogaert L, Willemsen A, Vanderstraeten E, Bracho MA, De Baere C, Bravo IG, Martens A. EcPV2 DNA in equine genital squamous cell carcinomas and normal genital mucosa. Vet Microbiol. 2012;158(1-2):33–
12. Kainzbauer C, Rushton J, Tober R, Scase T, Nell B, Sykora S, Brandt S. Bovine papillomavirus type 1 and Equus caballus papillomavirus 2 in equine squamous cell carcinoma of the head and neck in a Connemara mare. Equine Vet J. 2012;44(1):112–
13. Greenwood S, Chow-Lockerbie B, Epp T, Knight C, Wachoski-Dark G, MacDonald-Dickinson V, Wobeser B. Prevalence and prognostic impact of equus caballus papillomavirus type 2 infection in equine squamous cell carcinomas in Western Canadian Horses. Vet Pathol. 2020;57(5):623–
14. Lindsey CJ, Almeida ME, Vicari CF, Carvalho C, Yaguiu A, Freitas AC, Beçak W, Stocco RC. Bovine papillomavirus DNA in milk, blood, urine, semen, and spermatozoa of bovine papillomavirus-infected animals. Genet Mol Res. 2009;8(1):310–318.
15. Skoczyn´ski M, Goz´dzicka-J´ozefiak A, Kwas´niewska A. Risk factors of the vertical transmission of human papilloma virus in newborns from singleton pregnancy—preliminary report. J Matern Fetal Neonatal Med. 2014;27(3):239–242.
16. Stocco Dos Santos RC, Lindsey CJ, Ferraz OP, Pinto JR, Mirandola RS, Benesi FJ, Birgel EH, Pereira CA, Beçak W. Bovine papillomavirus transmission and chromosomal aberrations: an experimental model. J Gen Virol. 1998;79(pt 9):2127–2135.
17. Marenzoni ML, Lepri E, Casagrande Proietti P, Bietta A, Coletti M, Timoney PJ, Passamonti F. Causes of equine abortion, stillbirth and neonatal death in central Italy. Vet Rec. 2012;170(10):262.
18. Leon A, Laugier C, Foucher N, Tapprest J, Sevin C. A 24-Year retrospective study of equine abortion in normandy (France). J Equine Vet Sci. 2011;31:116–123.

19. Marenzoni ML, Bietta A, Lepri E, Proietti PC, Cordioli P, Canelli E, Stefanetti V, Coletti M, Timoney PJ, Passamonti F. Role of equine herpesviruses as co-infecting agents in cases of abortion, placental disease and neonatal foal mortality. Vet Res Commun. 2013;37(4):311–317.
20. Garvey M, Suárez NM, Kerr K, Hector R, Moloney-Quinn L, Arkins S, Davison AJ, Cullinane A. Equid herpesvirus 8: Complete genome sequence and association with abortion in mares. PLoS One. 2018;13(2):e0192301.

Tables

Table 1 Information of equine samples included in this study

Sampling place	Location	N/S/L	M/F	Breed	Average age	EcPV-2
Yili	A	37/0/0	6/31	Yili horse	5.4(3-10)	4(10.8%)
		0/6/0	6/0	Thoroughbred stallion	6.3(5-8)	1(16.7%)
		0/0/50	21/29	Yili horse	<0	19(38%)
	B	96/0/0	30/66	Thoroughbred horse	10(0-13)	0
Changji	C	41/0/0	7/34	Thoroughbred horse	6.3(4-12)	0
		0/12/0	12/0	Thoroughbred stallion	8.1(6-12)	2(16.7%)
Urumqi	D	56/0/0	10/46	Akhal-teke horse	4.4(0-8)	0
	Total	298	92/206		7.2(3-13)	26(2.5%)

M/F: Female/Male, N/S/L: Nasal swab/Semen/Lung tissue of aborted fetus

Figures

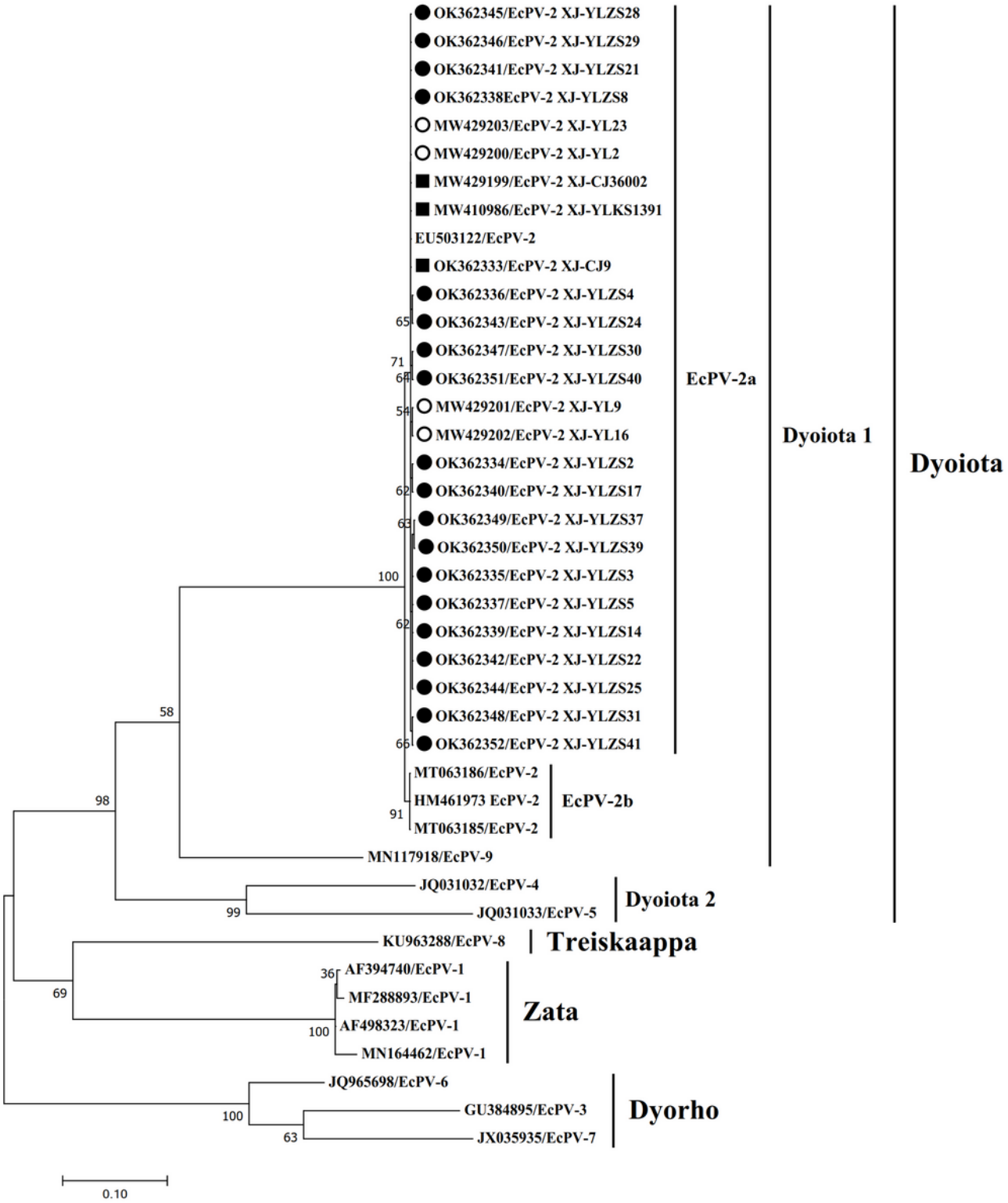

Figure 1

Phylogenetic tree of EcPV-2 strains found in China based on partial L1 gene sequences. The black circles indicate the EcPV-2 strains identified in aborted fetus lung tissues of Yili horses; The black boxes indicate the EcPV-2 strains identified in semen of Thoroughbred stallions; The open circles indicate the EcPV-2 strains identified in the nasal swabs of Yili horses.

Supplementary Files

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- TableS1.docx
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