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RESEARCH ARTICLE

Molecular Investigation of Important Protozoal Infections in Yaks

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ABSTRACT

Theileria, Babesia and Cryptosporidium are important parasitic pathogens in cattle. Herein, we carried out this survey to reveal the prevalence and genetic characteristics of four important protozoa in yaks in western China. A total of 1091 serum samples were totally acquired from yaks on the high remote plateau and were assayed for antibodies against Theileria and Babesia by pilot commercial ELISA kits. Eighty-five blood samples and 57 feces were obtained and PCR analyses were performed. The results revealed that the overall prevalence of *Theileria*, *Babesia* and mixed Theileria-Babesia infections in yaks was 15.12, 21.54 and 6.51%, respectively. Four out of 85 blood samples were observed to be PCR-positive for *Babesia bigemina* (*B. bigemina*) with 99.77% similarity to the MK587709.1 isolate. Among the 57 fecal samples, five samples tested positive for *C. parvum* (IId genotype), while one sample tested positive for *C. hominis*. Our study provides a solid theoretical basis for the need to prevent infections of animals by those protozoa in order to improve yaks' productivity on the Qinghai Tibetan plateau.

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INTRODUCTION

The widely distributed Tick-borne hemoprotozoan parasites such as Theileria and Babesia (phylum Apicomplexa, order Piroplasmida) are endemic in tropical and subtropical climates causing serious parasitosis with great impact on livestock production (Luo *et al.*, 2017; Zhou *et al.*, 2017). These two intracellular protozoan parasites may lead to severe diseases and death of the hosts (Masatani *et al.*, 2017). A complicated and crucial pathogen named Theileria causing serious anemia, jaundice, and anorexia in cattle (*Bostaurus*, *Bos indicus* and *Bubalus bubalis*) (Al-Fahdi *et al.*, 2017; Gomes *et al.*, 2017; Park *et al.*, 2017). Although, Babesia infection may be asymptomatic, clinical characteristics including fever, anaemia, haemoglobinuria, jaundice have been reported (Ozubek and Aktas, 2017). Further, some *Babesia* species are zoonotic and are of concern as emerging infections in humans (Lempereur *et al.*, 2017; Ozubek and Aktas, 2017). The zoonotic intestinal protozoan of *Cryptosporidium spp.* are important pathogens in humans and wide range of animals which cause diarrhea (Li *et al.*, 2019; Li *et al.*, 2020; Zhang *et al.*, 2019). Besides serious diarrhea in ruminants like cattle, cryptosporidiosis can lead to obvious delayed growth, weight loss, and high mortality with great economic loss (Ma *et al.*, 2014; Li *et al.*, 2019).

The Himalayan long-haired yak (Bos grunniens) is an invasive species (Li *et al.*, 2014; Li *et al.*, 2016). China possesses roughly 90% of the world's yak population, according to statistics (Li *et al.*, 2018). Yaks are particularly valued to local herders due to their great economic value (Li *et al.*, 2018). This animal is prized for its milk, meat, and hide. As a result, yaks are particularly vital to the high plateau's local herders (Li *et al.*, 2014; Li *et al.*, 2020). As a result, any yak sickness that affects animal output could result in numerous losses for the indigenous people (Li *et al.*, 2017; Li *et al.*, 2020).

Theileria and Babesia had previously been found in cattle, buffalo, yaks, sheep, and goats in Asia-Pacific countries such as China (Fujisaki *et al.*,1994; Yin *et al.*, 2004; Tian *et al.*, 2013; Li *et al.*, 2016b; Jirapattharasate *et al*., 2016; Al-Fahdi *et al*., 2017; Li *et al*., 2017; Masatani *et al*., 2017; Park *et al*., 2017; Ozubek and Aktas 2017). Ruminant Theileriosis has been reported as a severe and even lethal disease, which severely limiting the development of the cattle business in northwest China (Li *et al*., 2016b; Luo *et al*., 2017). Previous researches have shown Cryptosporidium spp. in yaks in Qinghai and Tibet (Ma *et al*., 2014; Zhang *et al*., 2019; Li *et al*., 2-020), but nothing is known about yaks in Sichuan's Hongyuan. On the Qinghai-Tibet Plateau, we conducted this study to determine the prevalence and genetic characteristics of four key protozoa in yaks.

MATERIALS AND METHODS

Ethics statement: The procedures were carried out with the consent of Huazhong Agricultural University's ethics committee.

Serum samples: Blood samples from yaks on the Qinghai-Tibet Plateau (n=1091) were collected from 2013 to 2016. (Fig. 1, Table 1). The gender and age of the yaks were also thoroughly documented. The samples were then centrifuged, and the resulting sera were preserved at -20°C for future research. Eighty-five blood samples were obtained from yaks in Sichuan in 2018 (Solarbio, China) and stored in sterile tubes with EDTA. Fifty-seven fresh feces were obtained from yaks in Hongyuan, Sichuan in 2019. The fecal samples were shipped on dry ice to the laboratory in Huazhong Agricultural University, Wuhan, China.

Determination of antibodies against Theileria in yaks: The Theileria Ag were examined using the ELISA Kit (Jianlun Biological Pharmaceuticals Co., Ltd, Guangzhou, China).

Determination of antibodies against Babesia in yaks: According to the manufacturer's instructions, all sera samples were screened for Babesia antibodies using a commercial ELISA kit (Cattle Babesia Ag ELISA Kit, Yikang Biological Pharmaceuticals Co., Ltd, Guangzhou, China).

DNA extraction and *18S rRNA* amplification: A commercial Gentra Puregene DNA purification kit was used to extract genomic DNA (gDNA) from blood and feces samples (Qiagen, China). The amplification of *18S SSU rRNA* of *Babesia*/*Theileria* spp. via PCR (Casati *et al*., 2006), and *Cryptosporidium* spp. via nested PCR (Li *et al*., 2020) were also carried out as previous reported. After electrophoresis, 1.5 percent agarose gels with ethidium bromide were used to examine the PCR amplified products. Purified DNA fragments were sequenced for further study using the Hi-TIANgel Midi Purification Kit (Tiangen Biotech CO., LTD, Beijing, China).

***C. parvum* subtype analysis:** As previously mentioned, all C. parvum-positive samples were subtyped by PCR and sequenced for the 60-kD glycoprotein (gp60) gene (Feng *et al*., 2019). All of the PCR products were electrophoresed and sequenced using the same strategies.

Phylogenetic analysis: To identify the species of *Babesia* and *Cryptosporidium* found in the samples, phylogenetic analysis was performed by MEGA (Version 7.0) using the neighbor-joining method. While distances between the species were determined using the NJ method. References utilized for *Cryptosporidium spp*. were *C. bovis* (MN696246, MN696243, MF142033.1, KY809003.1), *C. baileyi* (L19068.1), *C. parvum* (L16996.1, MK252646.1), *C. muris* (L19069.1), *C. hominis* (KP280061.1, MK801121.1), *C. aviaum* (JQ246415.1), *C. felis* (AF093013.1), *C. meleagridis* (AF381169.1). For *C. parvum* subtype, the reference genes were *C. parvum* IIa (GU214364.1), IIc (GU214366.1), IId (MK252647.1, GU214367.1, KX397563.1, AB777903.1), IIe (GU214369.1). For *Babesia 18S rRNA* were: *B. bigemina* (MK587709.1, MH257709.1), *B. microti* (AB190459.1), *B. canis* (AY072925.1), *B. bovis* (KF928959.1), *B. ovis* (KY867435.1),

Statistical analysis: Statistical investigation was conducted out using IBM SPSS Statistics 20.0 via chi-square scale. Statistical significance was described as a probability value of less than 0.05.

RESULTS

Infections with Theileria, Babesia, and mixed Theileria-Babesia were found in 15.12, 21.54, and 6.51 percent of yaks, respectively (Table 1). The prevalence of Theileria infection in yaks ranged from 5.33 percent to 21.89 percent, with major regional variations (χ2=28.238, P<0.001) (Fig. 1). Babesia infection rates in yaks ranged from 10.14 percent to 28.43 percent, with major variations between regions (χ2=34.736, P<0.001) (Fig. 1). The prevalence of Theileria and Babesia co-infection in yaks ranged from 0 to 13.80%, with major variations between regions (χ2=44.154, P<0.001) (Fig. 1).

Theileria infection in yaks ranged from 7.32 percent to 23.86 percent, with a substantial variation between years (χ2=38.232, P<0.001) (Fig. 2). While, Babesia infection in yaks ranged from 10.98% to 28.61%, with a substantial variation between years (χ2=30.651, P<0.001) (Fig. 2). The prevalence of Theileria and Babesia infection in yaks ranged from 7.28 to 10.78%, with a distinct difference between the years (χ2=20.799, P<0.001) (Fig. 2).

Theileria infection was found in 13.23 percent of male yaks and 16.36 percent of female yaks, respectively (Fig. 3). Babesia infection was found in 16.71 percent of male yaks and 24.70 percent of female yaks, respectively, with a significant difference between the genders (χ2=9.853, P=0.002<0.01) (Fig. 3). The prevalence of co-infection of Theileria and Babesia in yaks was 6.73 and 6.36% in male and female yaks, respectively (Fig. 3).

Table 1: The seroprevalence of Theileria and Babesia infection in yaks on the Qinghai Tibetan plateau

Sample No.	Theileria	Babesia	Theileria+Babesia
	Positive No. (Prevalence)	Positive No. (Prevalence)	Positive No. (Prevalence)
1091	165 (15.12%)	235 (21.54%)	71 (6.51%)

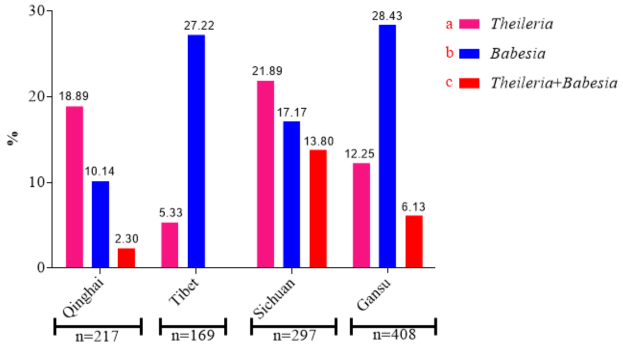

Fig 1: The seroprevalence of Theileria and Babesia infection in yaks in different regions on the Qinghai Tibetan plateau. ^{a}The prevalence of Theileria infection in yaks was significant between different regions on the Qinghai Tibetan plateau ($\chi2=28.238$, $P<0.001$). ^{b}The prevalence of Babesia infection in yaks was significant between different regions on the Qinghai Tibetan plateau ($\chi2=34.736$, $P<0.001$). ^{c}The prevalence of co-infection of Theileria and Babesia in yaks was significant between different regions on the Qinghai Tibetan plateau ($\chi2=44.154$, $P<0.001$).

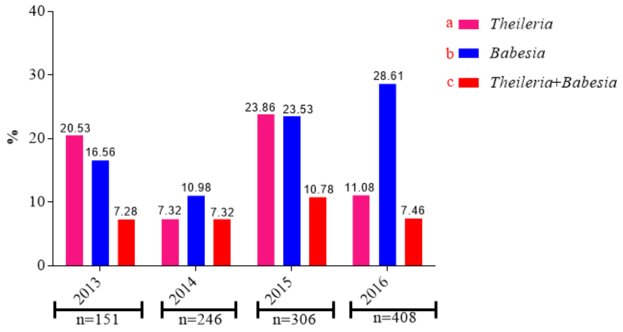

Fig 2. The seroprevalence of Theileria and Babesia infection in yaks in different years on the Qinghai Tibetan plateau. ^{a}The prevalence of Theileria infection in yaks was significant between different years on the Qinghai Tibetan plateau ($\chi2=38.232$, $P<0.001$). ^{b}The prevalence of Babesia infection in yaks was significant between different years on the Qinghai Tibetan plateau ($\chi2=30.651$, $P<0.001$). ^{c}The prevalence of co-infection of Theileria and Babesia in yaks was significant between different years on the Qinghai Tibetan plateau ($\chi2=20.799$, $P<0.001$).

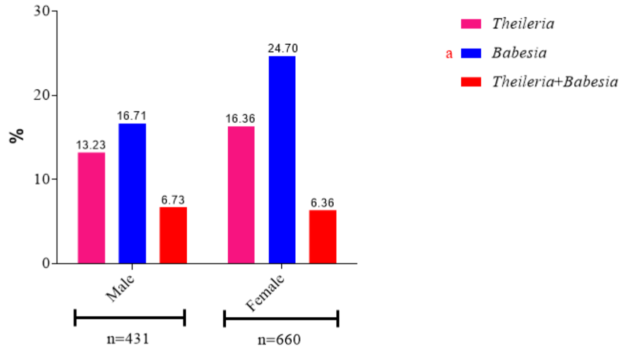

Fig 3. The seroprevalence of Theileria and Babesia infection in yaks in different genders on the Qinghai Tibetan plateau. ^{a}The prevalence of Babesia infection in yaks was significant between male and female yaks on the Qinghai Tibetan plateau ($\chi2=9.853$, $p=0.002<0.01$).

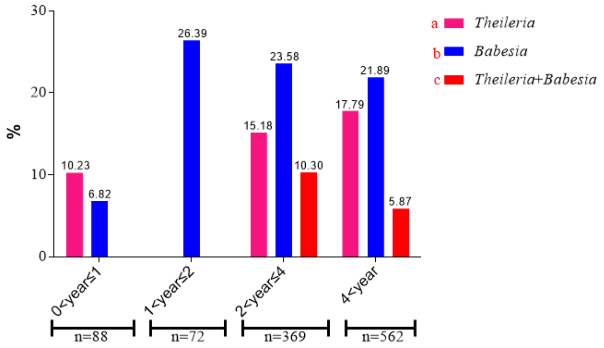

Fig 4: The seroprevalence of Theileria and Babesia infection in yaks in different ages on the Qinghai Tibetan plateau. ^{a}The prevalence of Theileria infection in yaks was significant between different years on the Qinghai Tibetan plateau ($\chi2=17.595$, $p=0.001<0.01$). ^{b}The prevalence of Babesia infection in yaks was significant between different years on the Qinghai Tibetan plateau ($\chi2=13.233$, $p=0.004<0.01$). ^{c}The prevalence of co-infection of Theileria and Babesia in yaks was significant between different years on the Qinghai Tibetan plateau ($\chi2=20.224$, $P<0.001$).

Theileria incidence rates ranged from 0% to 17.79 percent depending on age, with a large disparity between the ages of yaks ($\chi2=17.595$, $p=0.001<0.01$) (Fig. 4). Babesia incidence rate ranged from 6.82 percent to 26.39 percent, with a large variation in age ($\chi2=13.233$, $p=0.004<0.01$) (Fig. 4). The positive prevalence of Theileria and Babesia co-infection ranged from 0 to 10.30 percent, with a large differential between ages ($\chi2=20.224$, $P<0.001$) (Fig. 4).

From all the 85 samples, 4 (4.71%) PCR-positive samples were found and the representative corresponding sequence was deposited in NCBI database with GenBank accession number: MW040568. The existence of B. bigemina in yaks was found by phylogenetic tree research (Fig. 5). The homology of MW040568 was 99.77% to MK587709.1 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). From the 57 fecal samples, both *C. parvum* (n=5) and *C. hominis* (n=1) were observed (Fig. 6A). The representative corresponding sequences were deposited in NCBI database with GenBank accession number: MN810061 and MN810072. The current *C. parvum* isolate was identified to IId through gene subtype analysis (Fig. 6B).

DISCUSSION

Cattle production is the third largest national agricultural commodity in China, with over 100 million cattle raised annually, thanks to the country's socioeconomic development over the last two decades (Li *et al*., 2016a; Li *et al*., 2017; Li *et al*., 2020). As a result, epidemiological surveys of economically important diseases in domestic animals are extremely important and necessary (Li *et al*., 2017).

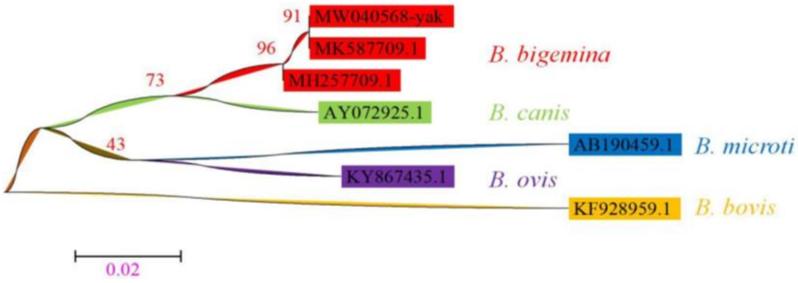

Fig 5: Phylogenetic tree for *Babesia spp.* based on partial *18S rRNA* gene sequences was constructed using the neighbor-joining method with Kimura two-parameter analysis and bootstrap analysis of 1000 replicates. The numbers on the branches indicate the percentage of replicates that reproduced the topology for each clad. The black cattle indicate the sequences acquired from the current study.

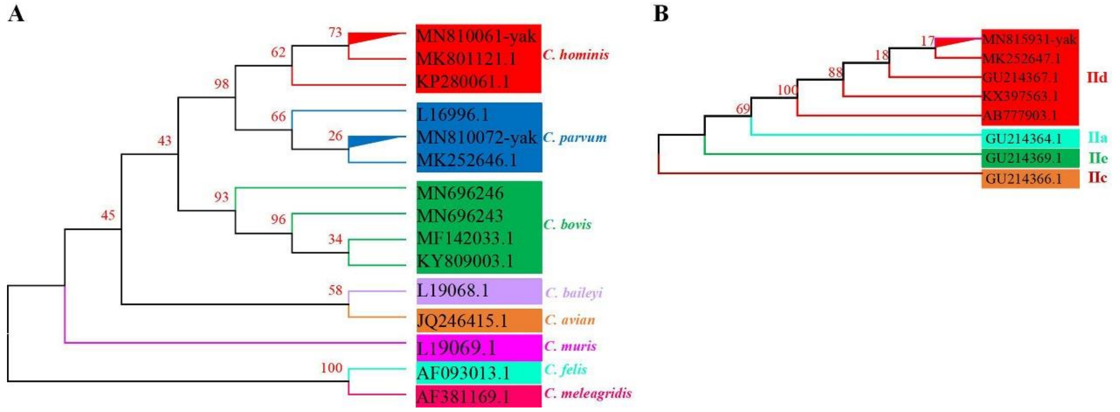

Fig 6: Phylogenetic tree for *Cryptosporidium spp.* based on partial *18S rRNA* gene sequences (A) and *C. parvum* genotypes based on gp60 gene sequences (B) were constructed using the neighbor-joining method with Kimura two-parameter analysis and bootstrap analysis of 1000 replicates. The numbers on the branches indicate the percentage of replicates that reproduced the topology for each clad. The black cattle indicate the sequences acquired from the current study.

The high morbidity and mortality in calves and exotic cattle make bovine theileriosis a significant impediment to the cattle industry's growth (Jirapattharasate *et al*., 2017). The prevalence of bovine theileriosis (15.12%) in the current study was comparable to that of theileriosis in cattle in Tanzania (14.2%) (Kerario *et al*., 2017) and West Azarbaijan, Iran (18.65%). (Narimani *et al*., 2017). However, the prevalence in this study was significantly lower than that seen in 11 Chinese provinces, ranging from 84.1 percent to 98.3 percent (Li *et al*., 2016b), and 69.2 percent in Malaysian cattle (Kho *et al*., 2017). The prevalence of Theileria infection in yaks in different regions was significantly different ($P<0.001$) (Fig 1.), This is in line with the findings in Tanzanian cattle and Chinese goats (Kerario *et al*., 2017; Luo *et al*., 2017). The prevalence of Theileria infection in yaks differed significantly between years ($P<0.001$) (Fig. 2), indicating a gradual rise in infection rate. There was no substantial difference in Theileria infection prevalence between genders ($p>0.05$) (Fig. 3), which was consistent with findings in cattle in Tanzania and goats in China (Luo *et al*., 2017; Gunasekara *et al*., 2019). The current and previous findings may indicate that there is no connection between Theileria prevalence and animal gender. There was a major difference in Theileria infection prevalence in yaks of different ages ($P<0.01$) (Fig. 4), which differs from the findings recorded in cattle in Tanzania (Kerario *et al*., 2017). The prevalence of Theileria was higher in sexually mature yaks, according to the current research, and the prevalence of Theileria was higher in animals over the age of two years.

The overall prevalence of Babesia infection in yaks was 21.54 percent in this study, which was similar to the prevalence of Babesia infection in Sri Lankan cattle (19.0 percent) (Gunasekara *et al*., 2019), white yaks (17.76 percent) (Qin *et al*., 2015), and cattle (20.7 percent) in China (Niu *et al*., 2015). However, the prevalence rate of Babesia in yaks is higher than in sheep and goats in China (12.9 percent), wild animals in northern Australia (5.3 percent), and cattle in northern and north-eastern Thailand (9.1 percent), according to the current analysis (Niu *et al*., 2016; Jirapattharasate *et al*., 2017; Barbosaa *et al*., 2017). Babesia infection in yaks was found to be different in each of the four regions studied (Fig. I). It's possible that the cause is due to variations in geography, environment, and tick behavior (Luo *et al*., 2017; Li *et al*., 2017). From 2013 to 2016, the incidence of Babesia infection in yaks increased dramatically (Fig. 2), indicating that the disease was circulating in yaks. Female yaks had a significantly higher incidence of Babesia infection than male yaks (Fig. 3). Babesia was found to be more prevalent in yaks after weaning, while Theileria was found to be more prevalent in animals aged one year and up (Fig. 4). The prevalence of Theileria and Babesia co-infection in yaks was 6.51 percent, which was similar to the prevalence in cattle in Thailand (3.0%). (Jirapattharasate *et al*., 2017). There were no major variations in Theileria and Babesia co-infection in yaks across provinces, ages, or years (Fig. 1, 2, 4).

In the current study, only *B. bigemina* (4/85) was identified (Fig. 5), which may be due to the limited number of samples, and also those samples were collected in winter when the vector ticks were inactive (Barbosaa *et al*., 2017). Previous studies reported *B. bigemina* in white yaks (Qin *et al*., 2015), *B. bovis* in black yaks (Niu *et al*., 2015), so we confirmed that *B. bigemina* also infects black yaks. The infected yaks may recover but remain carriers of Theileria and Babesia, thus act as reservoirs for tick infection (Cortés *et al*., 2017). The gregarious yaks are in habitants with abundant wild animals such as Pika, Tibetan sheep, snow leopard, argali, sika deer etc. (Li *et al*., 2014) that may contribute to the spread of Theileria and Babesia on the high plateau, with enormous economic losses to livestock and serious threat to wildlife in those areas. Ticks are abundant on the plateau, with a previous study reporting that 32.9% (5 600/ 17 019) of Tibetan sheep were infested with ticks resulting in mortality of 43.2% (Chen *et al*., 2015). The increase in the prevalence of Theileria and Babesia among free-range yaks may be related to increased activity of ticks due to increasing climatic temperatures on the plateau.

Previously, *C. bovis, C. ubiquitum, C. andersoni, C. ryanae,* and *C. xiaoi* were mostly found in yaks on plateau (Ma *et al*., 2014; Zhang *et al*., 2019; Li *et al*., 2020), with one study reporting *C. parvum* and *C. hominis* in yaks in Qinghai (Wang *et al*., 2018). *C. parvum* and *C. hominis* were discovered in yaks in Hongyuan, Sichuan, that were phylogenetically similar to previous isolates (Fig. 6). *C.*

parvum is one of the most common pathogens that causes diarrhea in young children and livestock. Infected yaks may potentially spread parasites to herders and other animals through contaminated water and food in shared plateau areas (Li *et al*., 2019; Li *et al*., 2020).

The findings of our serologic survey and molecular detection support the need to avoid Theileria, Babesia, and Cryptosporidium infections in yaks on the Qinghai Tibetan plateau in order to increase yak productivity. To keep the yak industry developing, effective and timely steps to control diseases should be performed.

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Authors contribution: Conceived and designed the experiments: YFL and KL. Analyzed the data: YFL, KL and KM. Contributed reagents/materials/analysis tools: YFL and KL. Wrote and revised the paper: YFL, KL and KM.

Supporting Information: The *18S rRNA* gene of *B. bigemina, C. parvum* and *C. hominis* have been deposited in the GenBank under the Accession Number: MW040568, MN810061 and MN810072.

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