First report on molecular identification of *Eimeria sp.* from captive forest musk deer

Ziwei Ren
Sichuan Agricultural University
Dong Yu
Sichuan Agricultural University
Wei Zhao
Sichuan Agricultural University
Yan Luo (lycjg@163.com)
Sichuan Agricultural University
Jianguo Cheng
Sichuan Institute of Musk Deer Breeding
Yin Wang
Sichuan Agricultural University
Zexiao Yang
Sichuan Agricultural University
Xueping Yao
Sichuan Agricultural University
Wei Yang
Sichuan Agricultural University
Xi Wu
Sichuan Agricultural University
Yimeng Li
Sichuan Agricultural University

Research

Keywords: Captive forest musk deer, coccidiosis infection, Eimeria sp., molecular identification, 18S rRNA

Posted Date: August 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-58019/v1

Abstract

Background: Forest musk deer (*Moschus berezovskii*) is a national first-level protected and endangered wild animals in China. The intestinal coccidiosis of captive forest musk deer is one of the most important diseases. However, few studies have been conducted to quantify *Eimeria* sp. infection and to identify its molecules. Thus, the objective of this study was to investigate the *Eimeria* sp. infection in the intestinal tract of forest musk deer in Sichuan and Shaanxi, China, and to identify the 18S rRNA gene fragment of *Eimeria* sp. , which provides scientific basic experimental data for the molecular epidemiological investigation and population genetic analysis of *Eimeria* sp. of captive forest musk deer in 7 regions.

Methods: 328 faecal samples of forest musk deer were collected from 7 farms. The DNA of *Eimeria* sp. in the positive samples was extracted and used as template for nested PCR amplification. The 18S rRNA gene fragment was connected with the plasmid vector, and the products were introduced into *Escherichia coli* (DH5α). The culture bacterial solution was used as a PCR reaction template for identification.

Results: In total, we collected 328 faecal samples from forest musk deer in Lixian (*n*=54), Maoxian (*n*=52), Ma'erkang (*n*=49), Dujiangyan (*n*=55), Hanyuan (*n*=41), Luding (*n*=36) and Weinan (*n*=41). 198 (60.37%) faecal samples were positive for *Eimeria* sp. We analyzed the 18S rRNA gene sequence of *Eimeria* sp, and determined 34 types with similarity of 90.5% ~ 100%. A phylogenetic tree constructed based on the 18S rRNA gene sequence of *Eimeria* sp., it was confirmed that *Eimeria* sp. parasitized in the intestinal tract of forest musk deer was closely related to *Eimeria alabamensis* from *Bos taurus*, *Eimeria faurei* from *Ovis aries* and *Eimeria ahsata* from *Ovis aries*.

Conclusions: To our knowledge, this was the first molecular identification of *Eimeria* sp. in the intestinal tract of forest musk deer. The study suggested that *Eimeria* sp. parasitizes in the intestinal tract of forest musk deer, which is their carrier.

Background

Forest musk deer (*Moschus berezovskii*) is a medium-sized mammal that inhabits alpine forests [1, 2]. The forest musk (*Moschus* spp.) is an endangered species in China and is currently listed as a class I protected species at the national level [3, 4, 5]. Musk is secreted by the musk gland in the groin of adult male forest musk deer, it has high economic and medicinal value [6, 7]. In recent years, due to habitat destruction and other reasons, the population of wild forest musk deer has decreased sharply [4]. Therefore, China began to carry out research on artificial breeding forest musk deer in Sichuan, Shaanxi and other regions [8, 9]. However, the requirements of forest musk deer on breeding conditions are strict [10, 11], and the prevention and control technology for herd diseases of captive musk deer is weak. The actual number of captive forest musk deer is still very small, and large-scale breeding of ruminants similar to cattle and sheep has not been successfully realized [12].

Coccidia have been described in a diverse range of vertebrate host groups [13, 14]. Several species cause significant mortality or morbidity in some hosts, which has attracted special attention in animal

production industries [15, 16, 17]. In ruminants the genus *Eimeria* sp. is commonly referred to by the term coccidia. *Eimeria* sp. species are generally gastrointestinal parasites, and most species of *Eimeria* sp. are exclusively located in the intestine [18]. Coccidiosis is widespread among ruminants [19], the intestinal coccidiosis of captive forest musk deer is one of the important mass diseases, especially endangering young animals, which causes the decrease of feed utilization rate, growth rate, production performance, and musk production capacity [20]. The forest musk deer that are parasitized by *Eimeria* sp. are emaciated, anaemic and have intestinal inflammation [18, 21, 22]. Sha et al. [23] found two kinds of forest musk deer coccidia, and named them *Eimeriamoschus* and *Eimeriajinfengshanenisis.* Afterwards, they were detected in captive forest musk deer farms in Sichuan and Shaanxi, with a high positive rate of infection [24, 25]. Zhao et al. [25] detected 7 species of *Eimeria* sp. from 50 fresh feces of captive forest musk deer collected from the musk deer farm of Chongqing Institute of Drug Cultivation. The species of coccidia eggs were identified as *Eimeria stiedai*, *Eimeria perforans*, *Eimeria magna, Eimeria media*, *Eimeria irresidua, Eimeria piriformis* and *Eimeria coecicola.* Lu et al. [26] conducted an investigation on intestinal parasite infection of wild musk deer feces collected in Qinghai Province, of which the infection rate of *Eimeria* sp. coccidia was 43.66% (31/71). So far, there was no report on the molecular identification of *Eimeria* sp. parasitized on forest musk deer, this study aimed to provide scientific basic experimental data for the molecular epidemiological investigation and population genetic analysis of *Eimeria* sp. of captive forest musk deer in 7 regions.

Methods

Fecal sample collection

In the spring and autumn of 2018, 328 faecal samples of forest musk deer with clinical symptoms of emaciation, diarrhea, lumpy or unformed stool, listlessness and yellowish fur were collected from 7 forest musk deer farms in Lixian, Maoxian, Ma'erkang, Dujiangyan, Hanyuan, Luding and Weinan of China. The specific sampling information about faecal samples of captive forest musk deer was as follows (Table 1). At 7: 30 in the morning, fresh faecal samples were collected using sterile disposable PE gloves, put into sterile individual plastic bags and marked with numbers, and recorded the sampling place, time, longitude, latitude and altitude. Finally, the collected samples were placed on ice bag in containers, and then directly brought back to the Animal Quarantine Laboratory of Sichuan Agricultural University for inspection.

Table 1
Sampling information

					NO. of sample	
Province	Location	Longitude/E (°)	Latitude/N (°)	Altitude	Spring	Autumn
Sichuan	Hanyuan	102.5800	29.3103	1068 ± 7	12	29
	Luding	102.2269	29.7800	1179 ± 9	27	9
	Maoxian	103.6794	31.7413	2114 ± 11	25	27
	Lixian	103.2336	31.4103	2570 ± 10	13	41
	Dujiangyan	103.6039	31.0092	810 ± 5	30	25
	Ma'erkang	102.1211	31.8992	2601 ± 3	23	26
Shaanxi	Weinan	109.7125	34.3950	792 ± 3	21	20

Parasitological Examination

All faecal samples of captive forest musk deer were tested for parasite eggs or oocysts by saturated saline floating method, and the eggs or oocysts found were judged to be positive. The mean eggs per gram (EPG) or oocysts per gram (OPG) of parasites in fecal samples were counted using the McMaster technique [27]. The eggs and oocysts were examined and photographed under a microscope at 400×, and recorded the number of observed coccidian eggs or oocysts [21].

Dna Extraction

Faecal samples were washed with double steaming water and vortexing for 2 min at 12000 rpm. This process was repeated three times until the supernatant was clear. Genomic DNA was then extracted from approximately 200 mg of each semi-purified product, using the stool DNA Kit (TD601; Tianmo, Beijing, China). DNA samples were stored in 200 µL of the kit Solution Buffer at -20 °C until use.

Nested Pcr Amplification And Amplicon Sequencing

The extracted DNA sample was used as template, a region of the 18S rRNA gene (1500 bp) was amplified with the forward primer EF1 5'-GAAACTGCGAATGGCTCATT-3' and the reverse primer ER1 5'-CTTGCGCCTACTAGGCATTC-3' [28]. PCR was performed in a 20 µL volume containing 10 µL 2 × *Taq* PCR Master Mix (Qingke, Beijing, China), 6 µL deionized water (Qingke), 2 µL DNA, and 1 µL each of forward and reverse primers. Reaction cycles consisted of an initial denaturation step at 95℃ for 5 min, 36 cycles of denaturation at 95℃ for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. The second PCR was conducted with the first PCR amplification mixture as the template. The target gene (830 bp) was amplified with the forward primer EF2 5'-TTTGATGGTCATTTTTAC-3' and the reverse primer ER2 5'-AATCCTTCTTATGTCTGG-3'. Second reaction system was the same as the first, thermocycling for target gene was done with an initial denaturation

step at 95℃ for 5 min, 32 cycles of denaturation at 95℃ for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. PCR products were subjected to 1% agarose gel electrophoresis [11]. The 18S rRNA target gene fragment was purified and placed in a T100 thermal cycler Bio-Rad together with plasmid pMD19-T vector (Katara, Beijing, China) for overnight connection at 16℃. The products were introduced into *Escherichia coli* (DH5α) (Tiangen, Beijing, China). Positive colonies were selected and inoculated with the Amp-containing liquid LB medium. The culture bacterial solution was used as a PCR reaction template for identification (Youkang, Hangzhou, China).

Data Analysis

The content of each base (A, T, G, and C) in the sequence was calculated by MEGA 6.0 software. The 18S rRNA sequences of different types of *Eimeria* sp. were compared with 18S rRNA sequence information of other protozoa retrieved (Table 2) from GenBank in NCBI (https://www.ncbi.nlm.nih.gov/guide/), and DNAMAN 6.0 software was used for sequence alignment analysis, moreover mutation sites in gene sequences were counted. Sequence similarity analysis was performed using DNAMAN 6.0 and Megalign in DNASTAR.Lasergene.v7.1. The 18S rRNA gene sequence of *Isospora ohioensis* was taken as the outer group of the phylogenetic tree. The software MEGA6.0 neighbor-joining method was used to construct the phylogenetic tree, and tree reliability was determined using bootstrap analyses of 1000 replicate.

Table 2
Eimeria 18S rRNA gene sequence information

Parasite type	Region	Host	GenBank accession number
Eimeria sp.	Colombia	Antillean Manatee	MG652359
Eimeria faurei	Turkey	*Ovis aries*	AF345998
Eimeria ahsata	Canada	*Ovis aries*	KT184334
Eimeria bovis	Canada	*Bos taurus*	KT184336
Eimeria auburnensis	Turkey	*Bos taurus*	KU052235
Eimeria alabamensis	Canada	*Bos taurus*	KT184335
Eimeria cylindrica	Japan	*Bos taurus*	AB769618
Isospora ohioensis	Canada	Collie	AF029303

Results

328 faecal samples were screened by the method of saturated salt water floatation, and *Eimeria* sp. coccidia oocysts were identified in 198 samples (Fig. 1). The positive rate of infection was 60.37%, and the OPG value ranged from 200 to 98,600. The positive rates of intestinal parasites detected in the feces

of forest musk deer in 7 forest musk deer farms in Lixian, Maoxian, Ma'erkang, Dujiangyan, Hanyuan and Luding and Shaanxi were quite different, with the infection rates of 91.84% (45/49) in Ma'erkang, 63.89% (23/36) in Luding, 57.69% (30/52) in Maoxian, 57.14% (31/54) in Lixian, 54.55% (30/55) in Dujiangyan, 48.78% (20/41) in Weinan and 46.34% (19/41) in Hanyuan, respectively.

The rate of intestinal *Eimeria* sp. positivity varies widely at different altitudes. The 7 forest musk deer farms are distributed at an altitude of 500 ~ 3000 m, of which Weinan and Dujiangyan are at an altitude of 0 ~ 1000 m, Hanyuan and Luding are at an altitude of 1000 ~ 2000 m, and Maoxian, Lixian and Ma'erkang are at an altitude of 2000 ~ 3000 m. The positive rates of *Eimeria* sp. were 52.08% (50/96), 54.55% (42/77) and 68.38% (106/155) at altitudes of 0 ~ 1000 m, 1000 ~ 2000 m and 2000 ~ 3000 m, respectively. The OPG values were 100 ~ 32,600, 200 ~ 57,200 and 800 ~ 98,600, respectively. The positive rate of intestinal *Eimeria* sp. infection (68.38%) in captive forest musk deer at 2000 ~ 3000 m above sea level was significantly higher than that at 0 ~ 1000 m (52.08%) and 1000 ~ 2000 m (54.55%) above sea level.

In this study, the positive rates of fecal *Eimeria* sp. detection in the spring and autumn of captive forest musk deer in 7 regions were quite different, and the intestinal *Eimeria* sp. infection was more serious in spring. The positive rates in spring and autumn were 66.23% (100/328) and 55.37% (98/328), respectively. The OPG values were 200 ~ 98,600 and 100 ~ 12,600, respectively.

Nested Pcr Amplification Of 18s Rrna

All *Eimeria* sp. positive samples were able to amplify 18S rRNA target gene fragment by nested PCR, the fragments from approximately to 830 bp (Fig. 2).

Pcr Identification Of 18s Rrna Gene Clone

The template of *Eimeria* sp. 18S rRNA gene clone was identified by routine PCR. The results of PCR products detected by agarose electrophoresis are as follows (Fig. 3).

Molecular Identification Results

After comparing and editing the sequencing results of *Eimeria* sp., the 18S rRNA gene sequence results of some samples were identical. The 132 18S rRNA gene sequences obtained by sequencing were divided into 34 species, including Maoxian (MX1-7), Dujiangyan (DJY1-5), Ma'erkang (MEK1-8), Lixian (LX1-3), Luding (LD1-2), Hanyuan (HY1-3) and Weinan (SX1-6), the GenBank accession numbers were shown in Table 3. The 18S rRNA gene sequences of 34 types of *Eimeria* were compared and analyzed in this experiment. The results showed that the intraspecific similarity was very high (Fig. 4). The sequence similarity of 18S rRNA genes of 34 types was 90.5% ~ 100%. The similarity of Maoxian (MX1-7) was 96.8% ~ 99.0%, Dujiangyan (DJY1-5) 95.0% ~ 96.8%, Ma'erkang (MEK1-8) 96.1% ~ 99.5%, Lixian (LX1-3) 95.2% ~ 97.0%, Luding (LD1-2) 92.6%, Hanyuan (HY1-3) 95.1% ~ 100%, and Weinan (SX1-6) 98.8%□99.0%.

The 18S rRNA gene sequence of *Eimeria* sp. determined in this study was located in two branch systems respectively, and it was under the same branch with *Eimeria alabamensis* from *Bos taurus* and *Eimeria faurei* from *Ovis aries*, which indicated that they were closely related. DJY3 and *Eimeria ahsata* were in the same branch (Fig. 5). The 18S rRNA gene sequences of 34 types of *Eimeria* sp. were 91.0% ~ 98.8% similar to those of *Eimeria cylindrica* from Japan *Bos taurus*, the similarity with *Eimeria faurei* from Turkey *Ovis aries* was 91.3% ~ 99.3%, the similarity with *Eimeria ahsata* from Canada *Ovis aries* was 91% ~ 99.3%, and the similarity with *Eimeria bovis* and *Eimeria alabamensis* of Canada *Bos taurus* was 90.6% ~ 99.0% and 91.6% ~ 98.0%, respectively. The similarity with *Eimeriidae auburnensis* from Turkey *Bos taurus* was 91.0% ~ 99.6%, and the similarity with *Isospora ohioensis* was 80.8% ~ 87.3%.

The 18S rRNA gene sequences of 34 types of *Eimeria* sp. obtained in this study had base variations (Table 3). There was only one type of *Eimeria* sp. 18S rRNA gene sequence base variation in sequence base positions 67, 166, 203, 363, 554, 668 and 695, and the bases at the variation positions were (A-G), (A-G), (A-G), (T-C), (C-T), (T-G), and (A-G), respectively. Only the 18S rRNA gene sequence of *Eimeria* sp. in Weinan had no variation at base positions 57, 411, 431, 456, 507 and 583. Both Dujiangyan and Ma'erkang (DJY1, MEK2, MRK7) changed from base C to T at base positions 427 and 568. The difference between Ma'erkang and Weinan was smaller than that in other regions.

Table 3 34 nucleotide mutation sites of *Eimeria* sp. 18S rRNA gene sequence **-Additional file 1**

Discussion

Compared with other countries, China's forest musk deer breeding is obviously more successful [29]. However, the incidence of intestinal diseases and mortality rate of forest musk deer are high in captivity [8], and *Eimeria* sp. infection may be one of the reasons. In general, more pathogenic species tend to inhabit the posterior part of the intestines [30]. Coccidia can invade and destroy intestinal epithelial cells of the host, resulting in digestive dysfunction, acute or chronic damage, and induce secondary damage, the above symptoms are more obvious in young animals [31, 32]. These are extremely unfavorable to the development of forest musk deer breeding industry.

In China, molecular data on *Eimeria* sp. in forest musk deer were not available. In our study, the infection level of intestinal parasites in captive forest musk deer was relatively high, which may be due to the single captive breeding mode in 7 regions. Because the captive mode of forest musk deer mainly consists of several individual brick cells and an outdoor yard (activity site), generally 2–6 single brick cells supported a large enclosure (activity site). Transmission is most efficient on farms with high stocking densities [22]. Parasites seemed to spread in forest musk deer in the same outdoor yard, which may become chronic carriers. Infected animals without clinical symptoms remain infected throughout the year and contaminate the environment continuously with oocysts [17]. Therefore, the infected forest musk deer are the source of reinfection and new infection in other animals. Oocysts can also be introduced into a susceptible herd by the fecal-oral route, contaminated clothing, boots, or cleaning tools [33, 34]. At the same time, the artificial enclosure can't completely simulate the field environment, habitat changes make

the animal in the state of stress for a long time, which will weaken their immunity [35], and then increase the parasitic infection rate.

The positive rates of *Eimeria* sp. infection was different in each areas, that may be related to the environmental climate, altitude, breeding and management mode of farms, and the control measures of parasites in various regions. Ma'erkang had the highest infection rate, which may be related to its large number of breeding and relatively old houses. The dissemination of coccidia is facilitated by high population density feeding [22]. The lowest infection rate was found in Hanyuan and Weinan, which might be the dosage and frequency of drugs used in Hanyuan and the management of the breeding farms in Weinan.

Regional factors seem to have an impact on the epidemiology of parasitic diseases, which can be attributed to geographical location and climatic conditions [36, 37]. The altitude of Maoxian, Lixian and Ma'erkang was higher than that of other areas. The conditions of moderate heat and moisture in these areas are very favorable for the development, survival and transmission of *Eimeria* sp. [38]. Because for the reason that the oocysts can survive for weeks or months in such an environment [33], the positive rate in these areas is higher.

In addition, it is worth noting that the feeds provided to forest musk deer in different regions are not identical, which may make the composition of the intestinal microbial community of animals very different, thereby affecting the parasitism of coccidia in the intestine [39, 40].

There is an increasing recognition that *Eimeria* sp. prevalence and intensity has a relevant relationship with seasonality [41]. Temperature, humidity, and rainfall are the main environmental factors affecting the survival and transmission of gastrointestinal parasites [34]. *Eimeria* sp. can remain viable and infectious for at least 1 year in vitro and able to withstand many adverse environmental influences because of their thick oocyst wall [42]. However, direct exposure to ultraviolet light for a few hours or extremely dry is detrimental to the oocysts [43], none of the 7 areas we sampled had such climatic conditions. Sichuan and Shaanxi are similar in geographical location and climate. The annual average temperature is above 10 ℃, and the temperature fluctuates greatly in different seasons. The precipitation in spring is several times more than that in winter. The total sunshine hours of the whole year are between 1500 ~ 2800 h [44, 45]. In spring, oocysts tend to sporulate faster and disperse better with increasing precipitation [46], whereas cold and dry weather during winter inhibits development and transmission [34]. Therefore, the intestinal *Eimeria* sp. infection was more serious in spring. This situation should lead to the importance of farming work and strengthen the seasonal prevention and control of coccidiosis.

In this study, the 18S RNA gene sequence of different species of *Eimeria* sp. was highly similar. These results indicated that the 18S rRNA gene sequences of *Eimeria* sp. parasitized in captive forest musk deer in this study were homologous between species.

The 18S rRNA gene sequences of 34 types of *Eimeria* sp. determined in 7 regions had some base variations, and the similarity between sequences was 90.5% ~ 100%, which may be due to the fact that

ribosomal rRNA gene was a highly repetitive tandem sequence unit in eukaryotes and its 18S rRNA gene sequence was highly conservative in the evolution process of biological genetic population [28, 47, 48]. *Eimeria* sp. has a wide variety of species and can be parasitic on many animals, such as chickens, rabbits, cattle, sheep, goats, and deer, etc. *Eimeria* sp. is considered to have high host specificity and has strict selectivity to parasitic host animals and parts [18]. Therefore, each host animal has its own *Eimeria* origin in the classification of *Eimeria* sp. and cannot cross-infect each other [19, 49, 50].

According to the phylogenetic tree, DJY3 and *Eimeria ahsata* were in the same branch. This may be related to the fact that sheep, cattle and musk deer were ruminants. Combining with the phylogenetic tree, it showed that the closer the relationship of host animals was, the closer the relationship of *Eimeria* sp. parasitized on host animals was [51, 52].

Conclusions

This experiment confirmed the prevalence of intestinal coccidiosis in captive forest musk deer in China. As far as we know, this is the first molecular identification of *Eimeria* sp. in the intestinal tract of forest musk deer. This study showed that there were significant differences in the prevalence of coccidiosis in different regions, altitudes and seasons. Based on the sequence identification of 18S rRNA gene, it was confirmed that *Eimeria* sp. parasitized in the intestinal tract of forest musk deer was closely related to *Eimeria alabamensis* from *Bos taurus*, *Eimeria faurei* from *Ovis aries* and *Eimeria ahsata* from *Ovis aries*. At present, there is no known effective drug to treat the *Eimeria* sp. infection. Therefore, measures should be taken to prevent forest musk deer from being infected.

Declarations

Ethics approval and consent to participate

In this study, the process of specimens collected was accorded with animal protection law of the People's Republic of China. And all institutional and national guidelines for the care and use of laboratory animals were followed and animal experiments were approved by the National Institute of Animal Health Care and Use Committee at Sichuan Agricultural University (approval number SYXK2019-187).

Consent for publication

Not applicable.

Availability of data and materials

All data involved and arising from the study are included in this published article.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This work was funded by the Science & Technology Achievements Transfer Project in 2020 from Scientific Research Institutions of Science & Technology Department of Sichuan Province, Sichuan, China (No. 2020JDZH0024).

Authors' contributions

ZWR and DY carried out the final analysis and drafted the manuscript. JGC, YW, ZXY, XPY assisted in the analysis. WZ and YL participated in the design of the study and helped to draft the manuscript. WY, XW and YML participated in the sample collection and DNA extraction. All authors read and approved the final manuscript.

Acknowledgments

We are grateful to the 7 forest musk farms for their support of this experiment, and thank the Animal Quarantine Laboratory of the College of Veterinary Medicine of Sichuan Agricultural University (Wenjiang, China) for providing experimental conditions. We also thank Hangzhou Youkang Biotechnology Co. for providing help for the experiment. We extend our thanks to the hard work of the experimental contributors.

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Figures

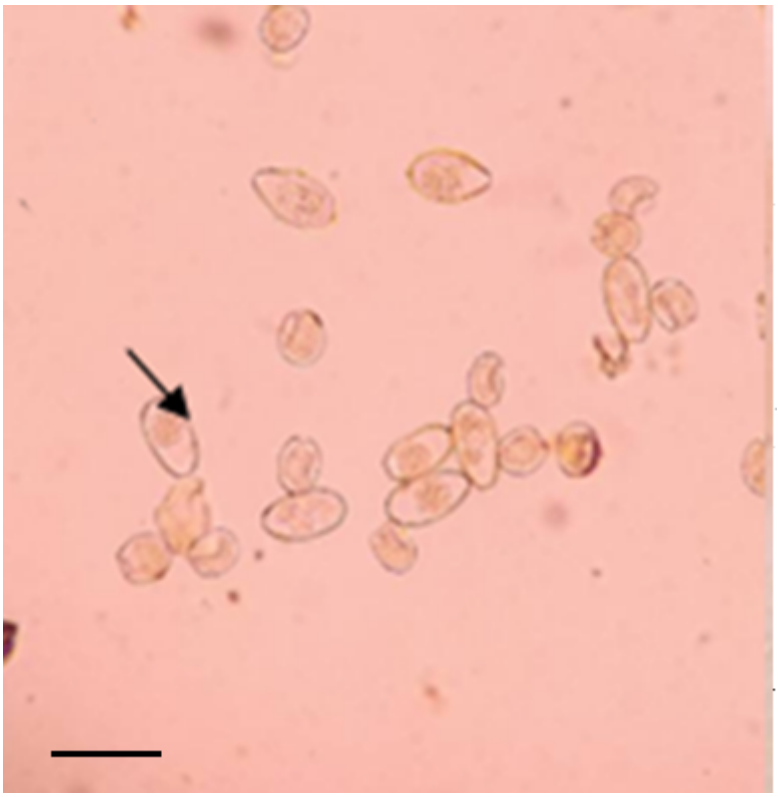

Figure 1

Intestinal Eimeria sp. coccidia oocysts in captive forest musk deer carp (400×) (scale bar=10 μm)

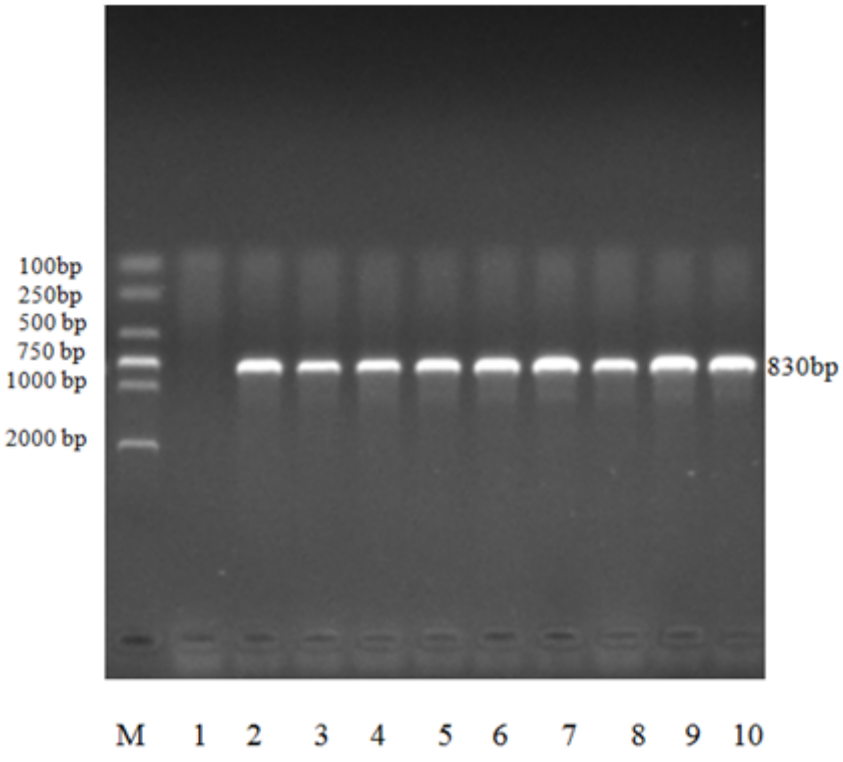

Figure 2

Electrophoresis map of nested PCR products of 18S rRNA gene of Eimeria sp. in forest musk deer

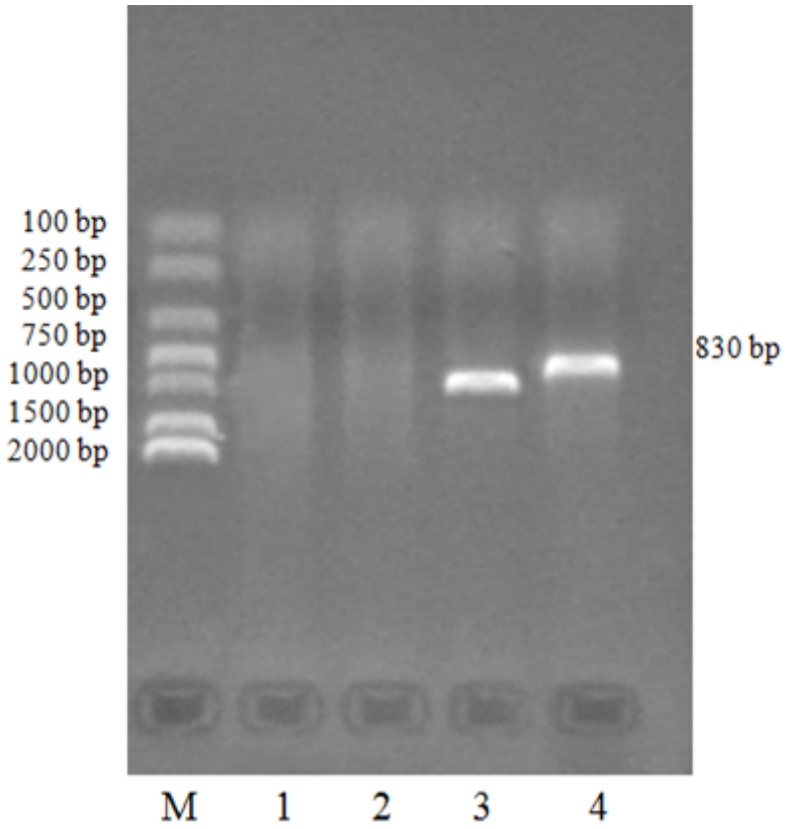

Figure 3

PCR identification of pMD19-T / 18S rRNA recombinant cloning vector. Note: M: DNA Marker DL2000, 1:18S rRNA negative control; 2: M13 negative control; 3: M13 positive control; 4: 18S rRNA gene positive clone.

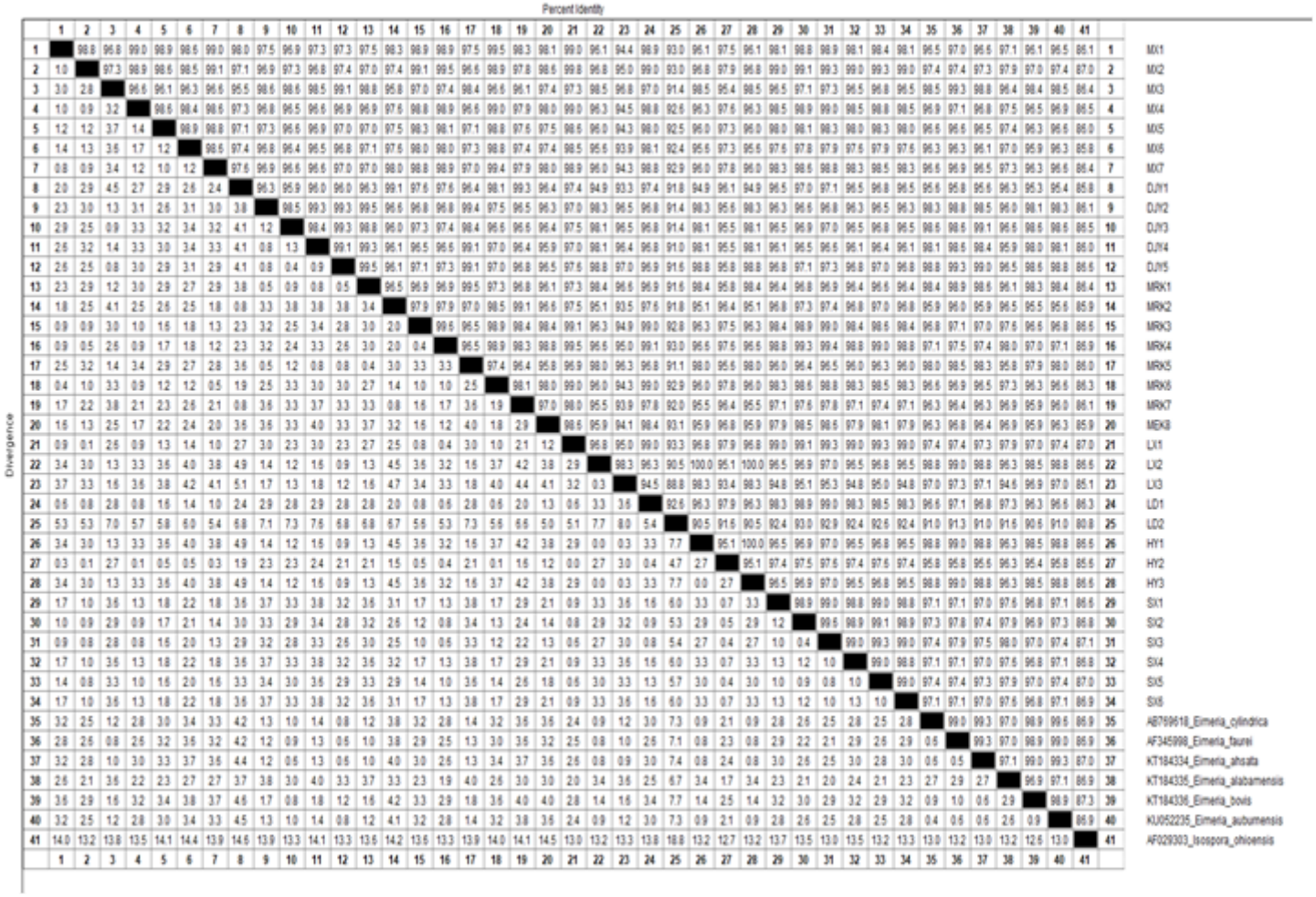

Figure 4

Sequence similarity analysis of Eimeria sp. 18S rRNA gene.

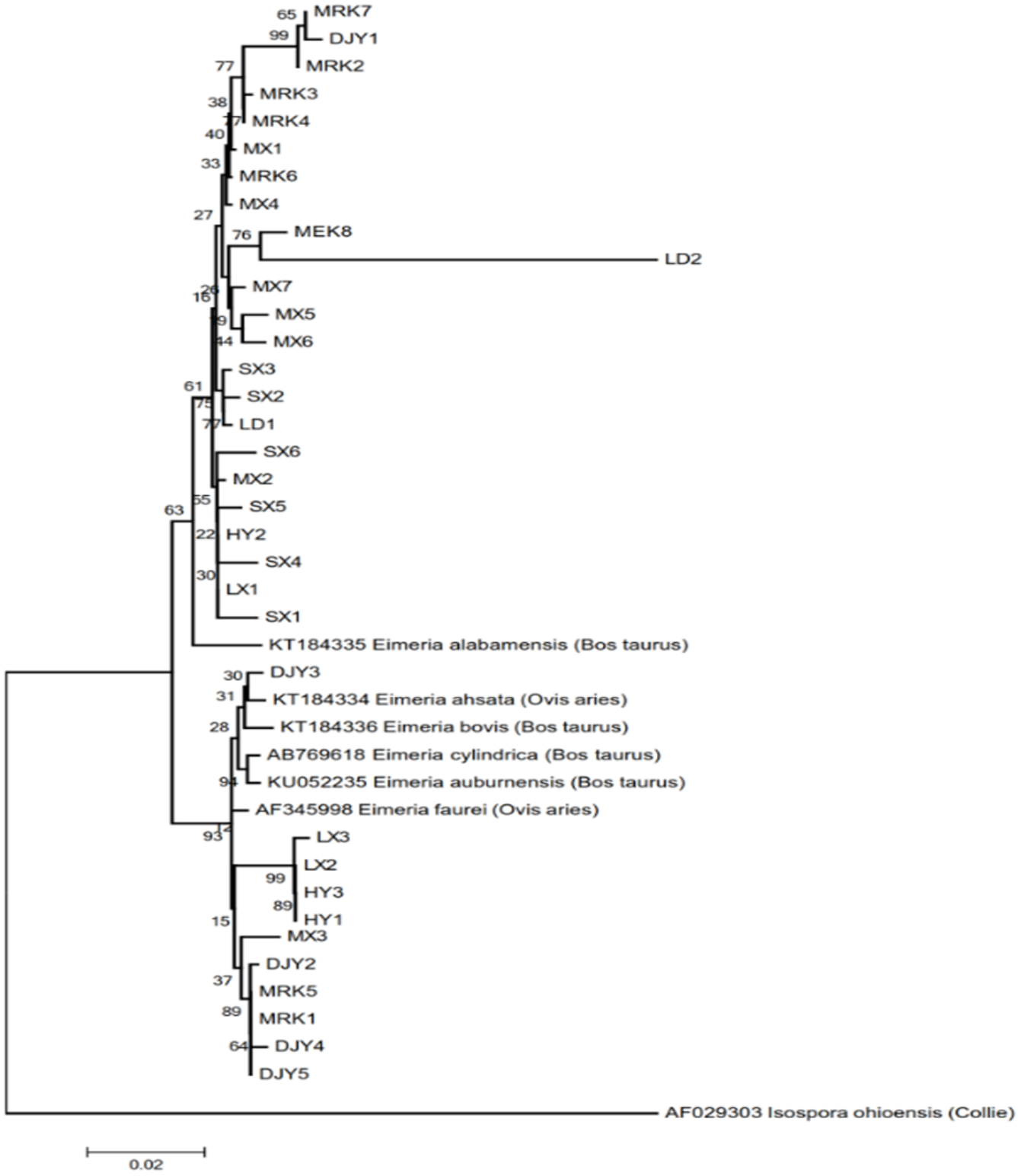

Figure 5

Phylogenetic tree of Eimeria sp. based on the 18S rRNA gene sequence. Note: MX1-7, DJY1-5, MEK1-8, LX1-3, LD1-2, HY1-3, and SX1-6 are the 18S rRNA gene sequences determined by this experiment, and Isospora ohioensis is the phylogenetic tree outgroup.

Supplementary Files

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- Additionalfile1.xlsx