

Common occurrence of *Enterocytozoon bieneusi* genotypes SHR1 and PL2 in farmed masked palm civet (*Paguma larvata*) in China

Xuhui Lin^a, Luyao Xin^{a,b}, Yangwenna Cao^b, Minyu Hou^b, Fei Qiao^b, Juan Li^a, Meng Qi^{b,*}, Mingfei Sun^{a,**}

^a Key Laboratory of Livestock Disease Prevention of Guangdong Province, Scientific Observation and Experiment Station of Veterinary Drugs and Diagnostic Techniques of Guangdong Province, Ministry of Agriculture, Institute of Animal Health, Guangdong Academy of Agricultural Sciences, Guangzhou, Guangdong Province, 510640, PR China

^b College of Animal Science, Tarim University, Alar, Xinjiang, 843300, PR China

ARTICLE INFO

Keywords:

Enterocytozoon bieneusi
Masked palm civet
Genotypes

ABSTRACT

Enterocytozoon bieneusi is a common pathogen in a broad range of vertebrate hosts. To assess the prevalence and genotypes of *E. bieneusi* in farmed masked palm civet (*Paguma larvata*), 537 fecal samples from seven provinces in China were tested by nested PCR of the polymorphic internal transcribed spacer (ITS) region. Among of all the samples, 60.0% (325/531) were positive for *E. bieneusi*, with the highest prevalence in Hebei province (85.0%). Sequence analysis revealed the presence of nine *E. bieneusi* genotypes, including four known genotypes (SHR1, PL2, PL4, CHG19) and five novel genotypes (PL12 to PL16). Genotypes SHR1 and PL2 were the most common genotypes in seven provinces. Phylogenetic analysis showed that three genotypes (CHG19, PL4 and PL16) were distributed to Group 1, and six genotypes (SHR1, PL2, PL12, PL13, PL14 and PL15) formed a novel clade, which was named group 12. Findings highlight the need to conduct additional research to elucidate the epidemiology of *E. bieneusi* in farmed masked palm civet.

1. Introduction

Enterocytozoon bieneusi is an important enteric pathogen in humans and animals (both wild and domestic) worldwide, causing chronic diarrhea and other gastrointestinal symptoms, especially in AIDS patients (Matos et al., 2012). To date, over 230 animal species have been reported to be hosts for *E. bieneusi* worldwide (Wang et al., 2018). Furthermore, it is believed that several animal species and environments with high levels of fecal pollutants are important sources for human *E. bieneusi* infections (Galvan-Diaz et al., 2014).

The most versatile tool used for the identification of *E. bieneusi* has been PCR (Santin and Fayer, 2009). The highly polymorphic internal transcribed spacer (ITS) region of the small-subunit ribosomal RNA (SSU rRNA) has been used as a genetic marker for genotyping and exploration of genetic diversity among *E. bieneusi* isolates (Santin and Fayer, 2011). More than 500 *E. bieneusi* genotypes have been identified in humans, livestock, wild animals, and the environment through ITS sequencing, which have been divided into 11 major phylogenetic groups (Li et al.,

2019c). Most of the genotypes classified in Group 1 are known to be zoonotic, whereas those belonging to other groups exhibited varying degrees of host specificity (Li et al., 2019a; Li and Xiao, 2019). Genotype determination and phylogenetic analysis of *E. bieneusi* isolates from various sources has deepened the current understanding of this pathogen.

The similar distribution of *E. bieneusi* genotypes between humans and animals indicates routine cross-species transmission (Li et al., 2019c). The main distribution of masked palm civet was in the southern regions of China, as well as Hubei, Shannxi, Shanxi, Sichuan and Xizang provinces. In China, masked palm civets (*Paguma larvata*) are farmed for their valuable fur and meat, the latter considered as a culinary delicacy (Zhang et al., 2016). The consumption of masked palm civet dates back to the 1950s and has increased since the 1990s (Jiang et al., 2003). The size of civet farming has gradually increased, and by 2003 there were about 660 farms with about 40 000 civets in China (Jiang et al., 2003). While after the COVID crisis, this activity has been banned. Limited information is available regarding the prevalence and genotypes of

* Corresponding author.

** Corresponding author.

E-mail addresses: 596229015@qq.com (X. Lin), 1321253371@qq.com (L. Xin), 1047681451@qq.com (Y. Cao), 1649635894@qq.com (M. Hou), 1969838166@qq.com (F. Qiao), lijuan413@126.com (J. Li), qimengdz@163.com (M. Qi), smf7810@126.com (M. Sun).

<https://doi.org/10.1016/j.ijppaw.2021.08.009>

Received 6 July 2021; Received in revised form 20 August 2021; Accepted 20 August 2021

Available online 21 August 2021

2213-2244/© 2021 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND

license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

E. bieneusi in farmed masked palm civet in China. This information is very important due to their potentially contaminated meat is frequently handled by people. The aim of this study was to improve the understanding of the prevalence and genetic diversity of *E. bieneusi* in farmed masked palm civet throughout China.

2. Materials and methods

2.1. Ethics statement

No experimental animals were involved in this study, and this research was not suitable for the approbation by the ethics committee. Permission was obtained from the farm managers prior to collection of fecal samples.

2.2. Collection of samples

From February 2018 to June 2019, a total of 531 fresh fecal samples were collected from masked palm civet in Chongqing Municipality, Guangdong Province, Hebei Province, Henan Province, Jiangxi Province, Sichuan Province, and Yunnan Province in China. There was no specific official report about the number and distribution of animals/farm in China, it is difficult to design a solution for sampling. The selection of sampling provinces was random, and the survey was mainly conducted in the provinces where masked palm civets were farmed. In fact, two farms were sampled in Guangdong province and Jiangxi province, and only one farm was sampled in most provinces, such as Chongqing province, Henan province, Sichuan province, Hebei province and Yunnan province. Masked palm civets were kept in groups with 2–5 animals per cage, and only one fecal sample was collected into individual plastic bags from each cage using sterile disposable latex gloves. All farmed masked palm civets have no direct contact with wildlife and no repeated collection of feces. All fecal samples were stored at 4 °C until the DNA was extracted from the samples.

2.3. DNA extraction and PCR amplification

About 200 mg of each fecal sample was used for extraction of genomic DNA using the E.Z.N.A. Stool DNA kit (Omega Biotek Inc., USA) according to the manufacturer's instructions. The extracted DNA was stored in a refrigerator at –20 °C until the time of PCR analysis. The primers and thermal cycling parameters used for nested PCR amplification have been previously reported (Sulaiman et al., 2003). The outer forward and reverse primers were 5'-GATGGTCATAGGGATGAAGAGCTT-3', and 5'-AATACAGGATCACTTGGATCCGT-3', respectively. The nested forward and reverse primers were 5'-AGGGATGAAGAGCTTCGGCTCTG-3', and 5'-AATATCCCTAATACAGGATCACT-3', respectively. The 2 × Easy-Taq PCR SuperMix (TransGene Biotech Co., Beijing, China) was used for PCR amplification. Each batch of PCR amplification included both a positive (DNA from dairy cattle-derived genotype I) and negative controls (distilled water). The secondary PCR amplicons were examined by electrophoresis on 1% agarose gels (w/v) using GelRed™ (Biotium Inc., Hayward, CA, USA) staining.

2.4. Nucleotide sequencing and data analysis

All the positive secondary PCR products of expected size were sequenced by GENEWIZ (Suzhou, China). All products were sequenced in both directions to ensure accurate sequencing results. Sequences were aligned with reference sequences obtained from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>) by BLAST analysis to determine genotypes. ClustalX 2.1 (<http://www.clustal.org/>) was used to compare all sequences with the reference sequences to illustrate the diversity and genetic relationships between novel and known genotypes. The sequences with a 100% similarity with deposited sequences in the 243 bp ITS region was determined as a known

Table 1

Providence and genotypes of *Enterocytozoon bieneusi* in farmed masked palm civets in China.

Province	No. of specimens	Positive (%)	Genotype (n)
Chongqing	43	19 (44.2)	SHR1 (14), PL2 (5)
Guangdong	219	156 (71.2)	SHR1 (119), PL2 (36), PL12 (1)
Hebei	20	17 (85.0)	SHR1 (9), PL2 (8)
Henan	14	11 (78.6)	SHR1 (1), PL2 (10)
Jiangxi	148	74 (50.0)	SHR1 (42), PL2 (27), PL4 (3), CHG19 (1), PL15 (1)
Sichuan	44	24 (54.5)	SHR1 (2), PL2 (21), PL13 (1)
Yunnan	49	24 (49.0)	SHR1 (18), PL2 (4), PL14 (1), PL16 (1)
Total	537	325 (60.0)	SHR1 (205), PL2 (111), PL4 (3), CHG19 (1), PL12 (1), PL13 (1), PL14 (1), PL15 (1), PL16 (1)

genotype, and the novel genotypes were confirmed as previously described (Santin and Fayer, 2009). The nucleotide sequences of the ITS in the present study were submitted to GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) under accession numbers MZ400631–MZ400639.

2.5. Phylogenetic analysis

A neighbor-joining phylogenetic tree of the ITS sequences was constructed using Mega 7.0 (<https://www.megasoftware.net/>) based on evolutionary distances calculated by a Kimura 2-parameter model. The reliability of branches in the tree was evaluated by bootstrap analysis with 1000 replicates. Bootstrap values above 70% were presented and considered in the analysis.

2.6. Statistical analysis

Differences in rates of infection between locations were compared using chi-square tests in the software SPSS version 22.0 (International Business Machines Corporation, New York, NY, USA) (<https://www.ibm.com/products/spss-statistics>). Differences were considered statistically at a *p*-values < 0.05.

3. Result and discussion

Of the 537 fecal samples collected from masked palm civet, 325 (60.0%) were positive for *E. bieneusi*. The highest prevalence of *E. bieneusi* was observed in Hebei Province (85.0%, 17/20), followed by Henan Province (78.6%, 11/14), Guangdong Province (71.2%, 156/219), Sichuan Province (54.5%, 24/44), Jiangxi Province (50.0%, 74/148), Yunnan Province (49.0%, 24/49) and Chongqing Municipality (44.2%, 19/43) (Table 1). The prevalence of *E. bieneusi* was higher than was reported previously in Guangdong (46.2%, 49/106) (*P* < 0.05) and Jiangxi Provinces (35.2%, 172/489) (*P* < 0.05), but was lower than previously reported in Chongqing Municipality (85.9%, 73/85) (*P* < 0.05) (Yu et al., 2020). The difference in prevalence of *E. bieneusi* in masked palm civet in difference areas may be related to the overall sample size and the health of the animals at the time of sampling. Drugs use in different farms, such as antibiotics and disinfectants, may also have impacted *E. bieneusi* infection rates. It's meaningful to explore the true sight that the effects of drug treatment on this parasite in wild animals, However, this requires detailed documentation and adequate data on their breeding process, which is difficult to do on most farms due to a lack of professional veterinarians or researchers.

Sequence analysis of the ITS PCR products showed nine *E. bieneusi* genotypes in this study, including four known genotypes (SHR1, PL2, PL4 and CHG19) and five novel genotypes (PL12–PL16) (Table 1). Genotypes SHR1 (63.1%, 205/325) and PL2 (34.2%, 111/325) were the most prevalent. Genotype PL1 was the dominant genotype in Chongqing

Table 2
Variation in the ITS sequences of *Enterocytozoon bieneusi* in farmed masked palm civets.

Genotypes	Location										
	86	89	94	106	107	126	127	176	205	223	234
J	G	G	G	T	G	T	G	G	A	A	A
PL12	A	G	T	C	A	T	G	G	G	A	G
PL13	A	A	G	C	A	T	G	G	G	G	G
PL14	A	G	G	C	A	–	–	G	G	A	G
PL15	A	G	G	C	A	T	G	T	G	A	G

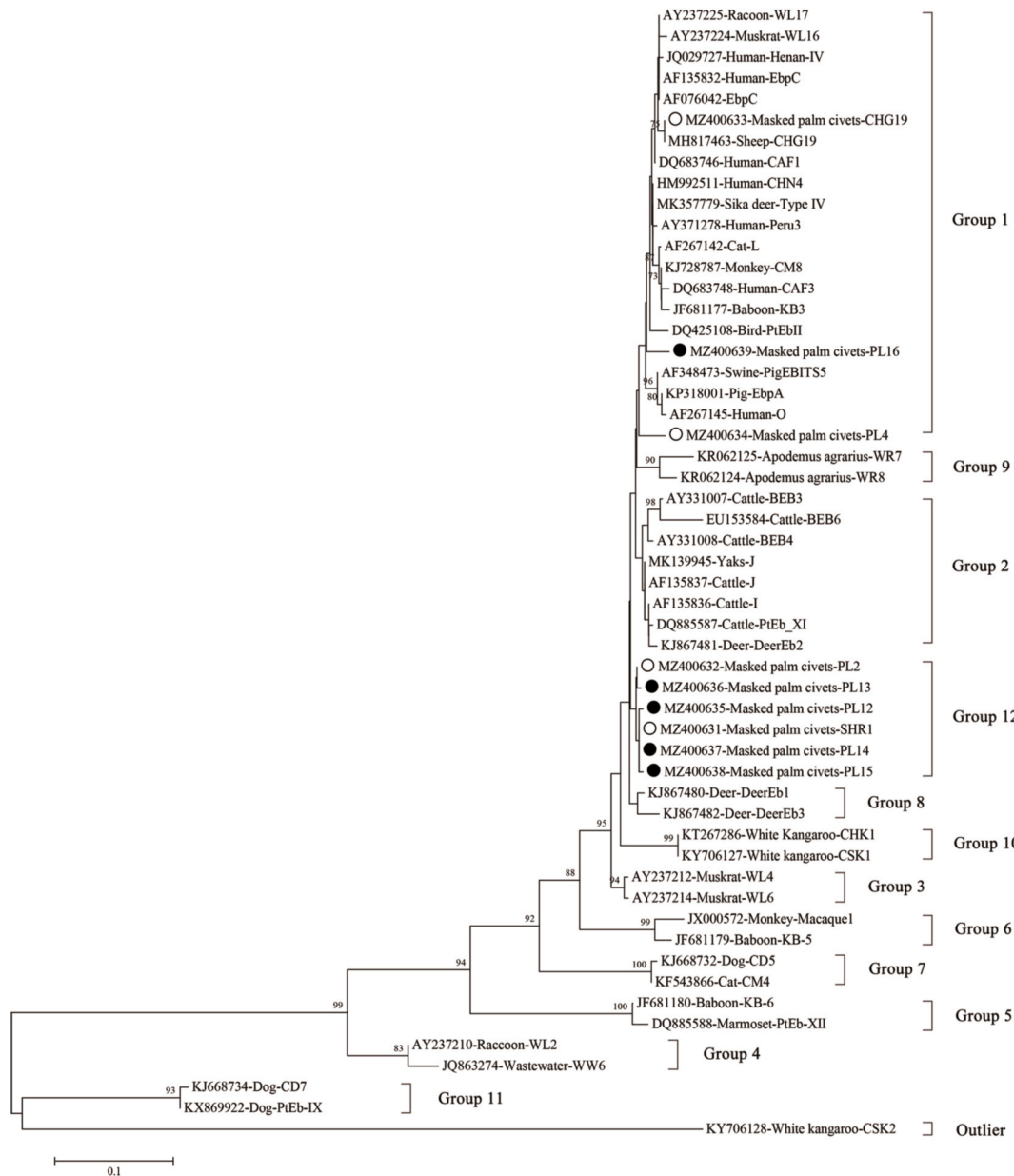


Fig. 1. Phylogenetic relationships of the *E. bieneusi* genotypes. The relationships were inferred using NJ analysis of the ITS rRNA gene and the values generated greater than 70% are shown beside the nodes. Genotypes with hollow circles and filled circles are known and novel genotypes identified in this study, respectively.

Municipality, and Guangdong, Jiangxi and Yunnan Provinces, which differed from a previous report out of Guangdong Province, where the dominant genotype was PL2 (Table 1) (Yu et al., 2020). However, PL2 was the most common genotype in Henan and Sichuan Provinces (Table 1). Genotype SHR1 has been identified in experimental rats and pet snakes in China. (Li et al., 2020a, 2020b). Genotype CHG19 has been

identified in sheep, wild and domestic pigs, and horses (Qi et al., 2016; Li et al., 2017, 2019b; Chang et al., 2020), which suggest that this genotype has a broad host-range. For the novel genotypes of *E. bieneusi* in this study, genotypes PL12 (No.109), PL13 (No.375), PL14 (No.487), PL15 (No. 487) had six, seven, seven and six single nucleotides polymorphisms (SNPs) relative to genotype J (GenBank Accession No.

MK139945) (Table 2), respectively. Genotype PL16 (No.471) had seven SNPs in comparison to genotype Type IV (GenBank Accession No. MK357779), with A→G in loci 107, 119, 192, 226 269, T→C in loci 140 and 157.

Phylogenetic analysis was conducted to reveal the relationship and genetic diversity between the genotypes identified in this study and other representative genotypes (Fig. 1). Genotypes CHG19, PL4 and PL16 belonged to group 1, which represents most human and zoonotic genotypes, including those of domesticated animal, wildlife, birds and companion animals, suggesting a high probability of cross-species transmission (Li et al., 2019d). Genotypes SHR1, PL2, PL12, PL13, PL14 and PL15 formed a clade named group 12. This finding was similar to a previous report which showed that genotypes PL1, PL2, PL3, PL6, PL7 and PL8 formed a unique clade. However, this clade was related to Group 2 and was thus named group 2-like (Yu et al., 2020). Group 2 was previously considered to include ruminant specific genotypes. However, some of the Group 2 genotypes, such as BEB4, BEB6, I, and J have been identified in some other hosts, suggesting a more diverse host range in this group than was initially believed (Li et al., 2019d). Genotypes in Group 12 may be adapted to masked palm civet. The host adaptation and specificity of these genotypes identified in masked palm civet will require further investigation by large scale samplings from various animal species and also humans in China.

This report of the prevalence and geographic distributions of *E. bieneusi* genotypes in farmed masked palm civets in China extends our knowledge of the genetic diversity and transmission potential of these pathogens in farmed wild animals. Further studies involving extensive sampling of farmed wildlife and other animal species around the farm as well as farmworkers are necessary to elucidate the roles of newly domesticated wildlife in the transmission of *E. bieneusi* between animals and/or humans.

Declaration of competing interest

All authors declare no conflicts of interest.

Acknowledgments

This work was supported in part by the Program for Young and Middle-Aged Leading Science, Technology, and Innovation of Xinjiang Production & Construction Corps (2018CB034), and Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams (2020KJ119), Special fund for scientific innovation strategy-construction of high level Academy of Agriculture Science (R2020PY-JG013).

References

- Chang, Y., Wang, Y., Wu, Y., Niu, Z., Li, J., Zhang, S., Wang, R., Jian, F., Ning, C., Zhang, L., 2020. Molecular characterization of *Giardia duodenalis* and *Enterocytozoon bieneusi* isolated from Tibetan Sheep and Tibetan Goats under natural grazing conditions in Tibet. *J. Eukaryot. Microbiol.* 67 (1), 100–106.
- Galvan-Diaz, A.L., Magnet, A., Fenoy, S., Henriques-Gil, N., Haro, M., Gordo, F.P., Millan, J., Miro, G., del Aguila, C., Izquierdo, F., 2014. Microsporidia detection and genotyping study of human pathogenic *E. bieneusi* in animals from Spain. *PLoS One* 9 (3), e92289.
- Jiang, Z.G., Li, C.W., Zeng, Y., 2003. Status of the research on masked palm civets. *Chin. J. Zool.* 38, 120–122.
- Li, D., Zhang, Y., Jiang, Y., Xing, J., Tao, D., Zhao, A., Cui, Z., Jing, B., Qi, M., Zhang, L., 2019a. Genotyping and zoonotic potential of *Enterocytozoon bieneusi* in pigs in Xinjiang, China. *Front. Microbiol.* 10, 2401.
- Li, D., Zheng, S., Zhou, C., Karim, M.R., Wang, L., Wang, H., Yu, F., Li, J., Wang, W., Wang, Y., Zhang, S., Jian, F., Wang, R., Ning, C., Zhang, L., 2019b. Multilocus typing of *Enterocytozoon bieneusi* in pig reveals the high prevalence, zoonotic potential, host adaptation and geographical segregation in China. *J. Eukaryot. Microbiol.* 66 (5), 707–718.
- Li, J., Jiang, Y., Wang, W., Chao, L., Jia, Y., Yuan, Y., Wang, J., Qiu, J., Qi, M., 2020a. Molecular identification and genotyping of *Enterocytozoon bieneusi* in experimental rats in China. *Exp. Parasitol.* 210, 107850.
- Li, J., Li, D., Zhang, H., Wang, R., Lin, Z.X., Zhang, L.W., Cao, Y., Qi, M., 2020b. Molecular characterization and novel genotypes of *Enterocytozoon bieneusi* in pet snakes in Beijing, China. *Int J Parasitol Parasites wildl* 12, 172–175.
- Li, W., Deng, L., Wu, K., Huang, X., Song, Y., Su, H., Hu, Y., Fu, H., Zhong, Z., Peng, G., 2017. Presence of zoonotic *Cryptosporidium scrofarum*, *Giardia duodenalis* assemblage A and *Enterocytozoon bieneusi* genotypes in captive Eurasian wild boars (*Sus scrofa*) in China: potential for zoonotic transmission. *Parasites Vectors* 10 (1), 10.
- Li, W., Feng, Y., Santin, M., 2019c. Host specificity of *Enterocytozoon bieneusi* and public health implications. *Trends Parasitol.* 35 (6), 436–451.
- Li, W., Feng, Y.Y., Zhang, L.X., Xiao, L.H., 2019d. Potential impacts of host specificity on zoonotic or interspecies transmission of *Enterocytozoon bieneusi*. *Infect. Genet. Evol.* 75, 104033.
- Li, W., Xiao, L.H., 2019. Multilocus sequence typing and population genetic analysis of *Enterocytozoon bieneusi*: host specificity and its impacts on public health. *Front. Genet.* 10, 307.
- Matos, O., Lobo, M.L., Xiao, L.H., 2012. Epidemiology of *Enterocytozoon bieneusi* infection in humans. *J Parasitol Res* 981424, 2012.
- Qi, M., Wang, R., Wang, H., Jian, F., Li, J., Zhao, J., Dong, H., Zhu, H., Ning, C., Zhang, L., 2016. *Enterocytozoon bieneusi* genotypes in grazing horses in China and their zoonotic transmission potential. *J. Eukaryot. Microbiol.* 63 (5), 591–597.
- Santin, M., Fayer, R., 2009. *Enterocytozoon bieneusi* genotype nomenclature based on the internal transcribed spacer sequence: a consensus. *J. Eukaryot. Microbiol.* 56 (1), 34–38.
- Santin, M., Fayer, R., 2011. Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. *Res. Vet. Sci.* 90 (3), 363–371.
- Sulaiman, I.M., Fayer, R., Lal, A.A., Trout, J.M., Schaefer, F.W., Xiao, L., 2003. Molecular characterization of microsporidia indicates that wild mammals harbor host-adapted *Enterocytozoon* spp. as well as human-pathogenic *Enterocytozoon bieneusi*. *Appl. Environ. Microbiol.* 69 (8), 4495–4501.
- Wang, S., Wang, R., Fan, X., Liu, T., Zhang, L., Zhao, G., 2018. Prevalence and genotypes of *Enterocytozoon bieneusi* in China. *Acta Trop.* 183, 142–152.
- Yu, Z., Wen, X., Huang, X., Yang, R., Guo, Y., Feng, Y., Xiao, L., Li, N., 2020. Molecular characterization and zoonotic potential of *Enterocytozoon bieneusi*, *Giardia duodenalis* and *Cryptosporidium* sp. in farmed masked palm civets (*Paguma larvata*) in southern China. *Parasites Vectors* 13 (1), 403.
- Zhang, D., Xu, L., Bu, H., Wang, D., Xu, C., Wang, R., 2016. The complete mitochondrial genome of the masked palm civet (*Paguma larvata*, *Mammalia*, *Carnivora*). *Mitochondrial DNA ADNA Mapp Seq Anal* 27 (5), 3764–3765.