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Short Communication

Molecular identification of hemoplasma and piroplasma species from *Rattus edwardsi* based on sequences analysis of ribosomal DNA, China

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Abstract. The present study provides the first report on the molecular epidemiological data regarding infection by hemoplasma and piroplasma species in wild *Rattus edwardsi*, from China. In the current study, blood samples were investigated from 32 wild *Rattus edwardsi* from Hunan (23) and Guangxi (9) provinces, China. The prevalence of hemoplasma and piroplasma was 65.63% (21/32) and 6.25% (2/32), respectively. Phylogenetic analyses indicated that hemoplasmas (HQ183731, HQ183732) derived from wild *Rattus edwardsi* in China, can be grouped into a solitary clade closely related to *H. muris* (HMU82963) and *M. haemomuris* (AB758435). In addition, it was shown that piroplasmas from this study have very close genetic distance to other unidentified piroplasma species isolated from China (AB242140) and Japan (AB188086). The results suggested that hemoplasmas isolated in this study should be represented as a new genotype. Piroplasmas on the other hand needs more sequenced samples in its life-cycle and evidence to check its taxonomic status. These data may have important implications for researching on the epidemiology and population biology as well as for studying the taxonomy status of hemoplasmas and piroplasmids of wild rodents.

The *Hemotropic mycoplasma* (hemoplasma) is a group of hemotropic bacterial species, which inhabit red blood cells of humans, cattle, sheep, goats, pigs, cats, dogs, etc., (He *et al*., 2011; Neimark *et al*., 2001; Neimark *et al*., 2002a; Willi *et al*., 2005; Will *et al*., 2007b), causing anemia, retarded growth, slow onset, chronic dry cough and even sudden death (Rikihisa *et al*., 1997; Zhang & Rikihisa, 2002; Zhuang *et al*., 2010). Based on the morphology and host species, many previous haemotrophic species cannot be clearly distinguished and have been classified as the genus *Haemobartonella* or *Eperythrozoon*. Subsequently, the genus *Haemobartonella* or *Eperythrozoon* were more explicitly reclassified into the genus *Mycoplasma* according to the biological data, such as *H. felis*, *H. muris*, *E. suis* and *E. wenyonii* (Neimark *et al*., 2001; Neimark *et al*., 2002b). Due to its small, pleomorphic and non-cultivable pathogens *in vitro* (Johansson *et al*., 1999; Meli *et al*., 2010), the specific identifications are indistinguishable from mycoplasmas and have to depend mainly on molecular techniques (Barker *et*

al., 2011; Zhuang *et al.*, 2010). To date, several hemotropic bacterial species are reported and classified within the genus *Mycoplasma* by sequence-based analysis of 16S ribosomal DNA (rDNA) from different countries or regions, including USA, South Africa, Australia, Switzerland, Germany, Brazil, Italy, Southern Europe and Japan (Bauer *et al.*, 2008; Criado-Fornelio *et al.*, 2003a; Criado-Fornelio *et al.*, 2003b; Gentilini *et al.*, 2009; Kewish *et al.*, 2004; Maia *et al.*, 2013; Vieira *et al.*, 2009).

Piroplasmids are tick-borne parasitic protozoa which are differentiated into the genus *Theileria* and *Babesia*. "Classical" diagnosis of babesiosis relies mainly on microscopic identification of piroplasms in red blood cells (Foreyt, 1989), but it is an insensitive procedure, particularly for animals in the carrier state. To date, several piroplasmids were reported and classified by sequence-based analysis of 18S ribosomal DNA (rDNA) in different countries form different livestock such as horse, cattle, sheep, goats, dogs, cat, etc., (Carli *et al.*, 2009; Conrad *et al.*, Criado-Fornelio *et al.*, 2003b, Pietrobelli *et al.*, 2007, Schnittger *et al.*, 2012; Schnittger *et al.*, 2003). But there have few information from the wild animals, especially there isn't data from *Rattus edwardsi*.

The aims of the present study were to sequence and analyze the 16S rDNA or 18 rDNA of the hemotropic bacterial and piroplasmids isolated from wild *Rattus edwardsi* from Hunan and Guangxi provinces, China, and compare them with other genealogical *Mycoplasma* species or piroplasmids to infer phylogenetic relationship.

Thirty-two EDTA blood samples were collected from wild *Rattus edwardsi* of Yongzhou in Hunan province and Yulin in Guangxi province, China. Sources of samples and related species sequences were available in GenBank™ with hosts, geographical origins and GenBank™ accession numbers listed in Table 1 and Table 2. Firstly, all blood samples were examined by microscopy with Giemsa stain. Total DNA of each sample was extracted from 200 *µl* blood by using the Genome DNA Extraction Kit (Sangon Ltd, Shanghai), according to the manufacturer's recommendations, and each DNA sample was then stored at –20°C for further use.

Table 1. Hemoplasma samples used in the present study

Species/Sample code	Host	Geographical origin	GenBank™ accession number
Mycoplasma haemomuris Rattus edwardsi strain/MH1	*Rattus edwardsi*	Yongzhou, Hunan, China	HQ183731
Mycoplasma haemomuris Rattus edwardsi strain/MH2	*Rattus edwardsi*	Zhuzhou, Hunan, China	HQ183732
Haemobartonella muris	wild mice	Japan	HMU82963
Mycoplasma haemomuris	*Rattus rattus*	Japan	AB758435
Mycoplasma coccoides	mouse	United Kingdom	AY171918
Mycoplasma sp.	*Rattus norvegicus*	Japan	AB752303
Candidatus Mycoplasma turicensis	cat	South Africa	DQ464422
Mycoplasma haemofelis	cat	UK	AY150985
Mycoplasma erythrodidelphis	*Didelphis marsupialis*	America	AF178676
Mycoplasma ovis	sheep	America	AF338268
Mycoplasma wenyonii	cattle	Japan	EU367963
Mycoplasma suis	suis	China	AY492086
Mycoplasma haemolama	alpaca	America	AF306346
Candidatus Mycoplasma kahanei	*Saimiri sciureus*	–	AF338269
Mycoplasma haemocanis	Dog	Germany	AY150973
Candidatus Mycoplasma haemoparvum	Dog	French	AY532390
Mycoplasma pneumoniae	–	–	M29061

Table 2. Piroplasmida samples used in the present study

Species/Sample code	Host	Geographical origin	GenBank™ accession number
Piroplasmida sp.	Rattus edwardsi	China	HQ183733
Piroplasmida sp.	Niviventer confucianus	China	AB242140
Piroplasmida sp.	Apodemus speciosus	Japan	AB188086
Babesia caballi	Equine	South Africa	Z15104
Babesia bigemina	Bovine	China	HQ840960
Babesia sp. *Coco*	Canine	America	EU109716
Babesia bovis	Bovine	Portugal	AY150059
Babesia gibsoni	Canine	Europe	EU583386
Babesia canis rossi	Canine	/	L19079
Babesia canis canis	Canine	Europe	AY072926
Babesia canis vogeli	Canine	Europe	AY072925
Babesia odocoilei	Odocoileus virginianus	America	U16369
Babesia poelea	Sula leucogaster	Johnston atoll	DQ200887
Babesia sp.	Capybara	Brazil	EF222255
Babesia bicornis	Black Rhinoceros	South Africa	AF419313
Cytauxzoon felis	/	/	L19080
Cytauxzoon manul	Otocolobus manul	Mongolia	AY485690
Theileria sinensis	Yak	China	EU277003
Theileria sergenti	Bovine	Japan	AB016074
Theileria ovis	Sheep	Turkey	AY260172
Theileria annulata	Bovine	China	EU083799
Theileria parva	/	/	L02366
Theileria orientalis	Bovine	Australia	AB520953
Theileria orientalis	/	China	HM538266
Theileria mutans	Bovine	Kenya	AF078815
Theileria youngi	/	/	AF245279
Theileria equi	Equine	/	Z15105
Theileria equi	Equine	South Africa	EU642507
Babesia sp.	Human	America	AY027816
Babesia conradae	Canine	America	AF158702
Babesia sp.	Human	America	AY027815
Babesia lengau	Acinonyx jubatus	South Africa	GQ411417
Babesia microti-like sp.	Hamster	America	AF158700
Babesia microti-like sp.	Canine	Spain	AF188001
Babesia microti	Clethrionomys glareolus	Germany	AB085191
Neospora sp.	Bovine	/	U17345
Plasmodium falciparum	/	/	M19172

Primer for amplification of the 16S rDNA for *Mycoplasma haemomuris* was fHf1 (forward, 5'-ACGCGTCGACAGAGTTTGAT CCTGGCT-3') and rHf2 (reverse, 5'-CGC GGATCCGCTACCTTGTTACGACTT-3') (Messick *et al*., 1998). As for piroplasmids, the primer was designed for amplification of 18S rDNA: 5-22F (5'-GTTGATCCTGCC AGTAGT-3') and 1661R (5'-AACCTTGTTA CGACTTCTC-3') (Birkenheuer *et al*., 2003). PCR reactions were performed in a 50*μl* reaction system containing 25*μl* of PCR Premix Ex *Taq* (TaKaRa Ex *Taq* 1.25 U, 2 × Ex *Taq* Buffer, 4 mmol/*l* Mg^{2+}), 1*μl* of each primer (25 mmol/*l*), 1*μl* of DNA template under the following conditions: denaturation at 95ºC for 5 min followed by 32 cycles of 95ºC for 45 sec, 50ºC for 2 min, 72ºC for 1 min, and final annealing at 72ºC for 5 min. Recombinant plasmids of 18S rDNA from rat *Piroplasm* (Laboratory saved) and without gDNA samples were subjected to each amplification as positive and negative controls, respectively.

Sequences obtained from this study and those of other *Mycoplasma* spp. and piroplasmids in GenBank were analyzed using the online program Phylogeny.fr

(Dereeper *et al*., 2008) with the 'A la Carte' mode. And the pipeline was set up to run and connected well-recognized programs: MUSCLE (Edgar, 2004) for multiple alignment, Gblocks (Gastresana, 2000) for automatic alignment curation, PhyML 3.0 (Guindon *et al*., 2010) a nd for tree building. Finally, the result from the upstream was imported to MEGA 6.0 (Tamura *et al*., 2013) for tree drawing.

21 out of 32 blood samples collected from Hunan (23) and Guangxi (9) provinces were found to positive by PCR detection, with 91.3% (21/23) infection rate for *M. haemomuris* among isolates from Hunan province. All samples from Guangxi province showed negative results. Two representative amplicons from endemic regions (Yongzhou) in Hunan province were sequenced, and recorded as MH1 and MH2, respectively. Sequences of 1342 bp (MH1) and 1360 bp (MH2) in length (Fig. 1) were obtained and deposited in GenBank™ under accession number HQ183731 (MH1) and HQ183732 (MH2), representing a nearly complete 16S rDNA sequence. The A+T contents of 16S rDNA sequences were 54.55% and 54.63%, respectively. Pairwise comparison showed that the intra-specific sequence variations of *M. haemomuris* samples from two different regions in Hunan province were 1.43% (Table 3). The inter-specific sequence differences observed between *M. haemomuris* and other common corresponding sequences available in the GenBank™ revealed that the differences of the two *M. haemomuris* isolates in China (MH1, MH2) to the sequences in the GenBank™ database (HMU82963, AB758435) were both more than 4.7% and 3.2%, respectively (Table 3), which suggested that *M. haemomuris* isolates in China should be a new genotype of *M. haemomuris*. Phylogenetic analysis (Fig. 2) showed that the two nearly entire 16S rDNA sequences of the newly derived Chinese haemoplasma isolates formed a solitary clade, which consistently grouped in sister with other isolates including *H. muris* (HMU82963) from wild mice and *M. haemomuris* (AB758435) from *Rattus rattus* with high bootstrap values (>70%). These results further confirmed that the newly derived *Mycoplasma* isolates was most closely to the rodent infected mycoplasmas *M. hamomuris* (AB758435) and *H. muris* (HMU82963).

2 out of 32 blood samples collected from Hunan (23) and Guangxi (9) provinces were proved as positive by PCR detection, with 8.70% (2/23) infection rate from Hunan province. One representative obtained and the length was 1658 bp (Fig. 3) and deposited

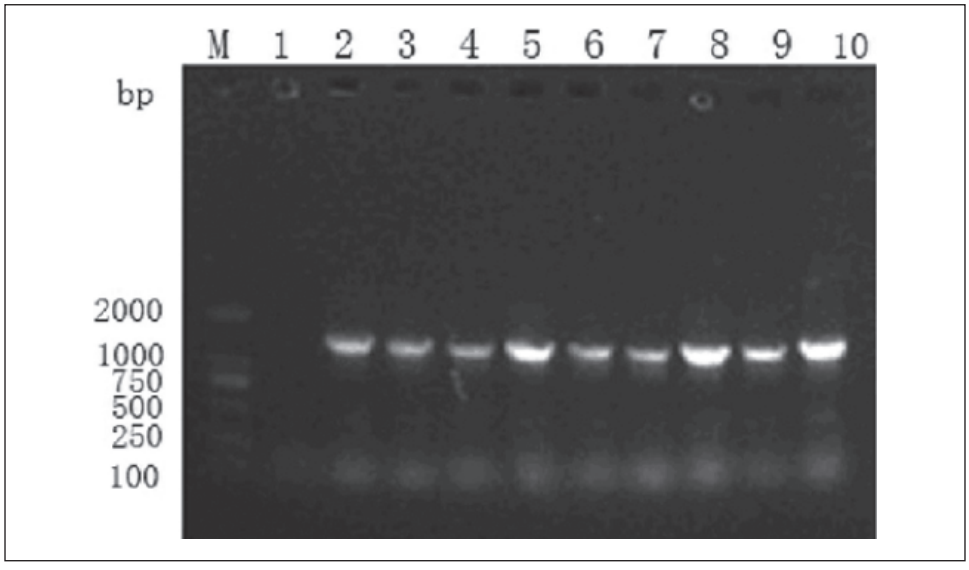

Figure 1. Results of PCR amplification of hemoplasma samples.
M: DL 2000 marker; 1: Negative control; 2~9: Samples from *Rattus edwardsi*; 10: Positive control.

Table 3. Estimates of Evolutionary Divergence between Sequences

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	MH1																
2	MH2	1.43%															
3	HMU82963	4.87%	3.42%														
4	AB758435	4.71%	3.26%	0.95%													
5	AY171918	14.04%	12.51%	13.17%	12.63%												
6	AB752303	12.97%	11.37%	11.27%	11.02%	12.65%											
7	DQ464422	12.80%	11.28%	11.46%	11.18%	9.82%	13.66%										
8	AY150985	16.13%	14.62%	15.02%	14.81%	12.17%	15.04%	13.56%									
9	AF178676	26.83%	24.89%	24.62%	24.38%	22.29%	26.51%	24.81%	22.98%								
10	AF338268	24.29%	22.43%	22.82%	22.79%	21.10%	24.50%	23.21%	22.68%	9.66%							
11	EU367963	24.00%	22.02%	21.93%	21.62%	19.81%	23.72%	21.54%	21.93%	10.07%	4.20%						
12	AY492086	25.75%	23.85%	24.81%	24.57%	22.27%	25.83%	23.63%	23.14%	11.71%	12.42%	11.42%					
13	AF306346	22.80%	20.99%	21.07%	20.98%	20.66%	22.65%	21.42%	21.62%	10.51%	8.64%	7.89%	10.99%				
14	AF338269	23.61%	21.77%	21.92%	21.91%	21.43%	24.83%	22.75%	22.40%	10.11%	11.25%	11.31%	11.72%	9.41%			
15	AY150973	15.81%	14.22%	14.58%	14.29%	11.62%	14.43%	13.79%	0.22%	22.32%	22.32%	21.65%	22.49%	21.17%	21.65%		
16	AY532390	24.03%	22.18%	22.66%	22.22%	20.45%	25.32%	22.72%	24.11%	10.42%	9.46%	9.29%	11.68%	8.42%	9.49%	23.44%	
17	M29061	26.97%	25.36%	27.41%	26.72%	25.05%	26.15%	26.70%	26.17%	31.76%	32.32%	31.06%	32.07%	30.99%	29.15%	25.65%	31.30%

The analysis involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1277 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

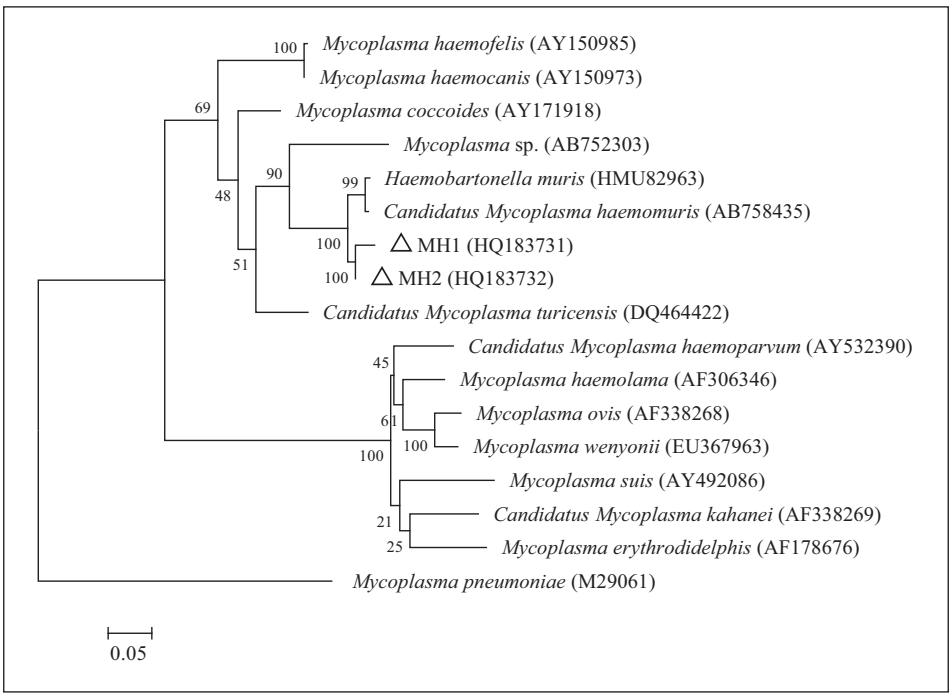

Figure 2. Maximum-likelihood trees obtained with partial 16S rDNA for the species reported in the present work and those present in GenBank database. Numbers in nodes correspond to the bootstrap support obtained in 100 replications with the PhyML program. Scale bar indicates an evolutionary distance of 0.05 nucleotides per position in the sequence. New sequences obtained are marked by triangle. *Mycoplasma pneumoniae* (M29061) was used as outgroup.

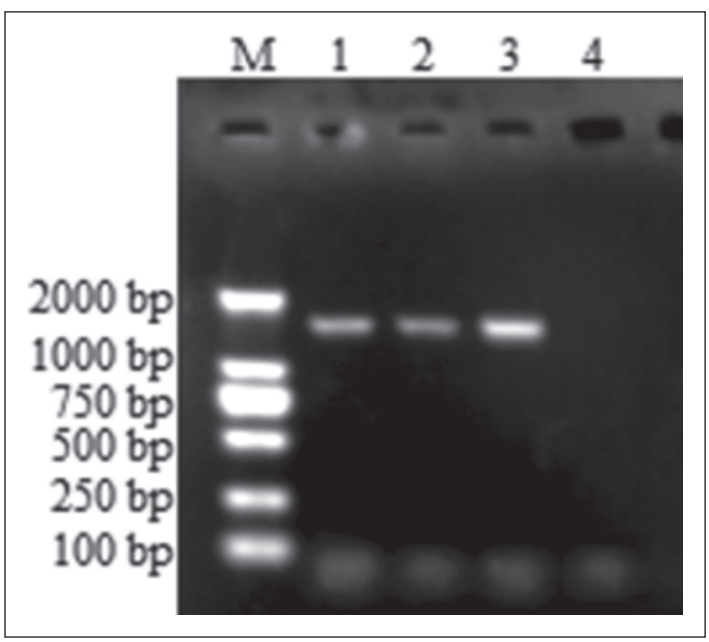

Figure 3. Results of PCR amplification of piroplasmids.
M: DL 2000 DNA Marker; 1: piroplasmids-HN sample 1; 2: piroplasmids-HN sample 2; 3: Positive control; 4: Negative control.

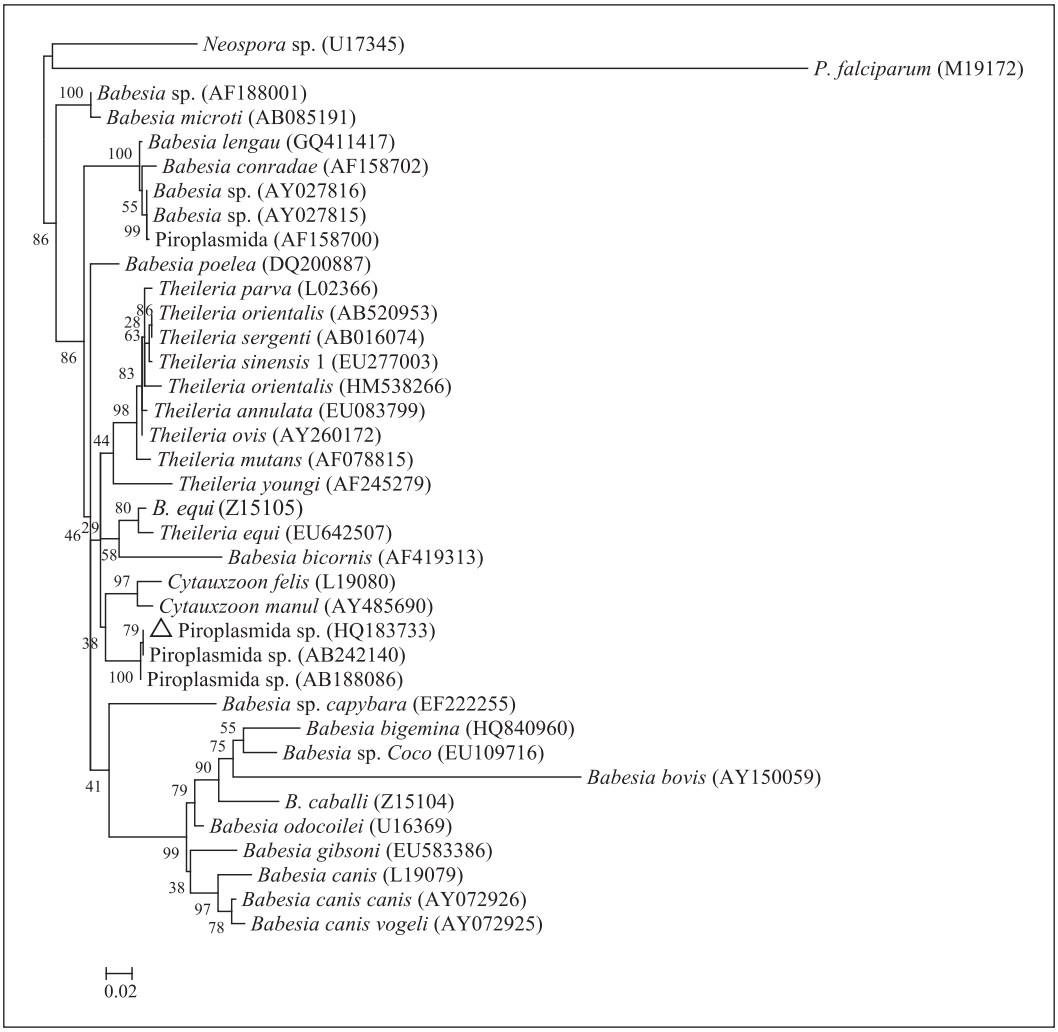

Figure 4. Maximum-likelihood trees obtained with partial 18S rDNA for the species reported in the present work and those present in GenBank database. Numbers in nodes correspond to the bootstrap support obtained in 100 replications with the PhyML program. Scale bar indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. New sequences obtained are marked by triangle. Plasmodium falciparum (M19172) and Neospora sp. (U17345) were used as outgroup.

in GenBank™ under accession number HQ183731. The A+T contents of 18S rDNA sequence was 54.16%. It is show that HQ183731 has nearest distance to an unidentified Piroplasmida (Accession Number: AB242140, Fig. 4) which isolated from *Niviventer confucianus* in China. In addition, it can cluster with *Cytauxzoon* spp. but show low bootstrap value (<50%).

Mycoplasma spp. and piroplasmids are vector-transmitted blood-borne pathogens of mammals such as mice, cattle, sheep and dogs. Clinical symptoms produced by *Mycoplasma* spp. and piroplasmids in mammals are sometimes similar. Diagnosis of these pathogens is difficult by microscopic procedures and molecular methods have been used as an alternative. In present study, the infection rate of *hemotropic mycoplasma* bacteria isolated from 32 wild *Rattus edwardsi* in two endemic southern provinces in China was 65.63% (21/32), especially in Hunan province with prevalence of 91.30% (21/23), but only two *Rattus edwardsi* are

infected by piroplasmids which affirmed that wild *Rattus edwardsi* has a common infection of blood hemoplasmas.

Generally, host species was considered as a useful character for speciation since most organisms were deemed to inhabit a single host. There are some genetic data on *Mycoplasma* spp. and piroplasmids detected in rodent from Genbank (Table 1, Table 2), but there no clear information on infected wild *Rattus edwardsi*. The present study is the first to report on the classification of wild *Rattus edwardsi* infected with *Mycoplasma* spp. and piroplasmids based on sequences of their 16S or 18S rDNA.

Various researchers have confirmed that the two hemotropic mycoplasma species, *M. haemomuris* and *M. coccoides*, could infect rodents (Harasawa *et al.*, Hayashi *et al.*, 2006). Analyses of genetic variations showed inter-specific differences between *M. haemomuris* isolates in China and related *Mycoplasma* species were bigger than that between two synonymic species with 0.8% (*H. muris* and *M. haemomuris*). Phylogenetic analysis (Fig. 1) showed that the two nearly entire 16S rDNA sequences of the newly derived Chinese haemoplasma isolates formed a solitary clade, which consistently grouped in sister with other isolates including *H. muris* (HMU82963) from wild mice and *M. haemomuris* (AB758435) from *Rattus rattus* with high bootstrap values (>70%). Therefore, we suggested that *M. haemomuris* derived from wild *Rattus edwardsi* in China should be a new genotype of *M. haemomuris*.

Piroplasmids are considered should differentiated into the genera *Babesia*, *Theileria* and *Cytauxzoon*, recently (Schreeg *et al.*, 2016). In addition, for *Cytauxzoon* spp. exists of schizonts, the piroplasmids which can grouped with *Cytauxzoon* spp. are sometimes referred to as *Theileria* s.l. (Schnittger *et al.*, 2012). The established phylogenetic tree shows that three sequences of piroplasmids derived from this study (HQ183733) and other work (AB188086, AB242140) formed a solitary clade, which consistently grouped in sister with *Cytauxzoon* spp. (L19080, AY485690) and *Theileria bicornis* (AF499604) with the low bootstrap values (<50%). The poorly supported phylogeny tree structure and complex composed this clade indicted that it is need more investigation to certify species in this group can represent at least one genus or not distinct from *Babesia* and *Theileria*.

In summary, due to deviation in selection of single rDNA fragment to affect phylogenetic analysis, the other rDNA sequences and complete mitochondrial genomic sequences will be sequenced and used to further ascertain taxonomic position of *hemoplasma* and *piroplasm* species from *Rattus edwardsi* in mainland China for future works.

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Conflict of interest

The authors declare that they have no conflict of interest.

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