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Preliminary detection of haemoplasma in Thai cat blood samples using universal primers: identifying '*Candidatus* Mycoplasma haemominutum' and closely related species

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Abstract

Objectives This study examined feline haemoplasmas (*Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum' [*C*Mhm] and '*Candidatus* Mycoplasma turicensis') infecting Thai domestic cats, using the 16S and 23S rRNA genes as genetic markers.

Methods Blood samples from 20 cats were obtained from a diagnostic laboratory and nucleic acids were extracted from each sample using a commercial kit. PCR targeting the 16S rRNA gene was used to screen haemoplasmas in the samples. Positive PCR samples were further sequenced using the 16S and 23S rRNA genes. The sequences from each genetic marker were analysed using Nucleotide BLAST, phylogeny and genetic network analyses.

Results Among the 20 samples, five were infected with haemoplasmas. In the 16S rRNA gene sequencing, four sequences were assigned to *C*Mhm and the remaining sequence was likely to be a closely related species of *C*Mhm. In the 23S rRNA gene sequencing, four sequences from the same samples used for 16S rRNA gene sequencing were identified as *C*Mhm and one sequence could be a putative novel haemoplasma species closely related to *C*Mhm.

Conclusions and relevance Only *C*Mhm and its closely related species were identified in this study. Although *C*Mhm has been recognised as a low-virulence parasite, cases of severe anaemia in cats infected with *C*Mhm have been found. Thus, such cases could be confirmed via the analysis of 16S and 23S rRNA genes. Furthermore, molecular detection and genetic analyses of feline haemoplasmas in additional cat blood samples should be conducted using PCR assay and DNA sequencing based on universal primers of 16S rRNA and 23S rRNA genes to enable more specific identification.

Plain language summary

Detection of blood parasites in Thai cats: finding '*Candidatus* Mycoplasma haemominutum' and similar species using universal primers

This study focused on identifying blood parasites called haemoplasmas that infect cats in Thailand. Specifically, it looked at three species: *Mycoplasma haemofelis, Candidatus* Mycoplasma haemominutum (*C*Mhm) and

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Candidatus Mycoplasma turicensis. Researchers tested blood from 20 cats using a PCR targeting the 16S rRNA gene to see if they were infected. They found that five of the cats were infected with haemoplasmas. Further analysis showed that four of the samples were infected with *C*Mhm, while one sample likely had a closely related species. This was confirmed by sequencing two genetic markers, the 16S and 23S rRNA genes. Even though *C*Mhm is usually considered a low-risk parasite, it has been linked to severe anaemia in some cats. This study highlights the importance of using PCR targeting the 16S rRNA and 23S rRNA genes to better understand and identify these infections. The researchers recommend further testing of more cat blood samples to help with more accurate identification of these parasites.

Keywords: *Candidatus* Mycoplasma haemominutum; genetic marker; identification; 16S rRNA gene; 23S rRNA gene

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Introduction

Haemotropic mycoplasmas (haemoplasmas) comprise a group of mollicutes bacteria that lack a cell wall, parasitise erythrocytes and induce haemolytic anaemia in mammals.[1,2] In domestic cats, three common haemoplasma species, *Mycoplasma haemofelis* (*Mhf*), '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' (CMt), have been described.[3,4] These species have been reported in domestic cats from several countries, including Brazil,[5] Canada,[6] Chile,[7] China,[8] India,[9] Iran,[10] Italy,[11] Japan,[12] Spain,[13] Switzerland,[14] Thailand,[15–17] the UK[18] and the USA.[19]

The 16S rRNA gene has been commonly used as a genetic marker to characterise haemoplasmas at the species level; however, some strains cannot be specified at the species level using this marker alone because of their close phylogenetic relationships.[20] Therefore, other genetic markers (eg, *dnaK*, *gryB*, *rpoB*, *rpoC* and 23S rRNA) should be considered to distinguish certain closely related haemoplasma species.[20–25] For example, four haemoplasma species were characterised in Thai pigs using the 16S rRNA gene;[26] however, *Mycoplasma suis* samples have been frequently studied with the 23S rRNA gene and have been divided into two closely related species.[27] In addition, haemoplasmas in blood-sucking flies from a buffalo farm in Thailand were studied by targeting the 16S rRNA gene and the results suggested the existence of two subgroups of *Mycoplasma wenyonii*.[28] Representative samples from these subgroups were further confirmed via 23S rRNA gene sequencing to be closely related but different species.[28] Thus, sequencing targeting the 16S and 23S rRNA genes would be more beneficial for distinguishing haemoplasmas at the species level than sequencing the 16S rRNA gene alone.

Although numerous studies have reported feline haemoplasmas, there has been no genetic characterisation using the 23S rRNA gene in field samples. Moreover, the previously submitted partial sequences of the 16S rRNA gene from Thai isolates of CMhm appeared to be divided into two subgroups based on re-analysis using phylogeny and genetic network analyses.[29] Therefore, in the present study, established protocols for haemoplasma detection via 16S and 23S rRNA genes were initially used to study feline haemoplasmas in blood samples from Thai cats.

Materials and methods

Ethics and biosafety

This study was approved by the Chulalongkorn University Animal Care and Use Committee (protocol number 2331079). The sampling and laboratory procedures were approved by the CUVET-Institutional Biosafety Committee (protocol number 2331027).

Samples, nucleic acid extraction and haemoplasma detection

A total of 20 remnant blood samples from client-owned cats were obtained in 2023 from the Chulalongkorn University Veterinary Diagnostic Laboratory. Unfortunately, general information and the health status of the cats were not available. Nucleic acid was extracted from each blood sample using the IndiSpin Pathogen Kit (INDICAL BIOSCIENCE) according to the manufacturer's instructions. All nucleic samples were stored at −40°C until PCR analysis.

The presence of haemoplasmas in all nucleic acid samples was examined by PCR targeting the 16S rRNA gene as previously described.[26] PCR products were electrophoresed in a 1.5% agarose gel (Bio Basic) containing RedSafe (iNtRON Biotechnology) for 35 mins at 110 V and 400 mA. An ultraviolet transilluminator was employed to visualise the expected band, approximately 1000 bp,[26] on the agarose gel.

DNA sequencing and genetic analyses

PCR products with the expected bands were purified from the agarose gel using a GenepHlow Gel/PCR Kit (Geneaid Biotech) and submitted for bidirectional sequencing at U2Bio DNA sequencing service in Thailand. Primer sequences were trimmed using MEGA X.[30] All sequences

were deposited in GenBank under the accession numbers PQ045759–PQ045763. Nucleotide BLAST (BLASTn) was used to determine the percent identity of sequences compared with those already deposited in GenBank.[31]

All sequences were aligned with those of other haemoplasma species using ClustalW multiple sequence alignment in MEGA X.[30,32] In addition, *Mycoplasma bovis* strain CQ-W70 (CP005933) was used as an outgroup, representing a taxon outside the interesting group of haemoplasmas. 'Find Best DNA/Protein Models (ML)' was used to determine the optimal model for phylogenetic tree construction. A phylogenetic tree based on the 16S rRNA gene was generated in MEGA X using the following settings: maximum likelihood method, Tamura 3-parameter (T92) model, a distinct Gamma distribution, all sites for gaps or missing data treatment and 1000 bootstraps.[30] Two genetic networks (host species and countries) based on the Templeton–Crandall–Sing (TCS) method of *C*Mhm 16S rRNA sequences were generated using PopART version 1.7.[33,34]

Genetic characterisation based on the 23S rRNA gene

Nucleic acid samples with positive PCR results were subjected to nested PCR targeting the 23S rRNA gene using primers from a previous study.[35] Two rounds of PCR were performed using GoTaq Green Master Mix (Promega) and the T100 Thermal Cycler (Bio-Rad). PCR products were separated by 1.5% gel electrophoresis, purified from the agarose gel, submitted for bidirectional sequencing and analysed as described for the 16S rRNA gene. All sequences were deposited in GenBank under the accession numbers PQ083076–PQ083080.

Results

PCR demonstrated that 5/20 (25%) samples were infected with haemoplasmas. The results of the 16S and 23S rRNA gene analysis via BLASTn are presented in Table 1. Phylogenetic analysis of the 16S rRNA gene revealed two groups of haemoplasmas (Haemominutum and Haemofelis groups) (Figure 1). *C*Mhm sequences were placed in the Haemominutum group. *C*Mhm sequences from this study and previously deposited sequences could be roughly divided into three subgroups: A, B and C. Four sequences (PQ045760–PQ045763) were placed in subgroup A with the Birmingham 1 strain (HE613254)[36] and California strain (U88564).[37–39] One sequence (PQ045759) was placed in subgroup C with other sequences, such as isolate Purdue (FJ004275),[40] clone A1 (MN543623)[41] and isolates from wild felids (DQ825456 and DQ825457);[42] however, none were placed in subgroup B. Moreover, the genetic networks of *C*Mhm showed that the sequences from GenBank were diverse with several nucleotide sequence types (ntSTs) and have been detected in several hosts and countries (Figure 2).

In the phylogenetic analysis of the 23S rRNA gene (Figure 3), *C*Mhm sequences were also placed into the Haemominutum group. Despite limited information on 23S rRNA sequences in the database, *C*Mhm sequences could still be divided into subgroups A and B. *C*Mhm subgroup A consisted of four sequences from this study (PQ083077–PQ083080) and the Birmingham 1 strain. Only one sequence (PQ083076) from this study was categorised into a distinct clade (subgroup B) closely related to *C*Mhm. In the genetic network of the 23S rRNA gene (Figure 4), PQ083076 was separated from the *C*Mhm cluster, suggesting that this sequence is another putative novel species closely related to *C*Mhm.

Discussion

This study supports the use of the 23S rRNA gene to distinguish haemoplasmas at the species level as previously described.[20] Phylogenetic analysis revealed the existence of a *C*Mhm complex consisting of *C*Mhm (subgroup A) and its closely related species based on the 16S rRNA gene. Sequences in subgroup A could be *C*Mhm as initially described[38] because of their grouping with the Birmingham 1[36] and California[37–39] strains. This was also confirmed via the analysis of 23S rRNA sequences. In subgroup C, only one sequence from this study was confirmed using the 23S rRNA gene. The results of the genetic analyses suggested that subgroup C could be a putative novel species closely related to *C*Mhm. Unfortunately, samples belonging to subgroup B were not found in this study. Thus, subgroup B could not be characterised as another closely related species of *C*Mhm. Other clinical information (health status,

Table 1 Nucleotide BLAST results of the partial 16S and 23S rRNA gene sequences from five samples of haemoplasma in Thai cats

Sample ID	16S rRNA gene				23S rRNA gene			
	Accession number	Closest sequence	Species	Identity (%)	Accession number	Closest sequence	Species	Identity (%)
F66	PQ045759	MN543623	*C*Mhm	99.90	PQ083076	HE613254	*C*Mhm	96.69
F73	PQ045760	KR905451	*C*Mhm	100	PQ083077	HE613254	*C*Mhm	100
F80	PQ045761	KR905451	*C*Mhm	100	PQ083078	HE613254	*C*Mhm	99.53
F92	PQ045762	KR905451	*C*Mhm	100	PQ083079	HE613254	*C*Mhm	99.53
F93	PQ045763	KR905451	*C*Mhm	100	PQ083080	HE613254	*C*Mhm	99.53

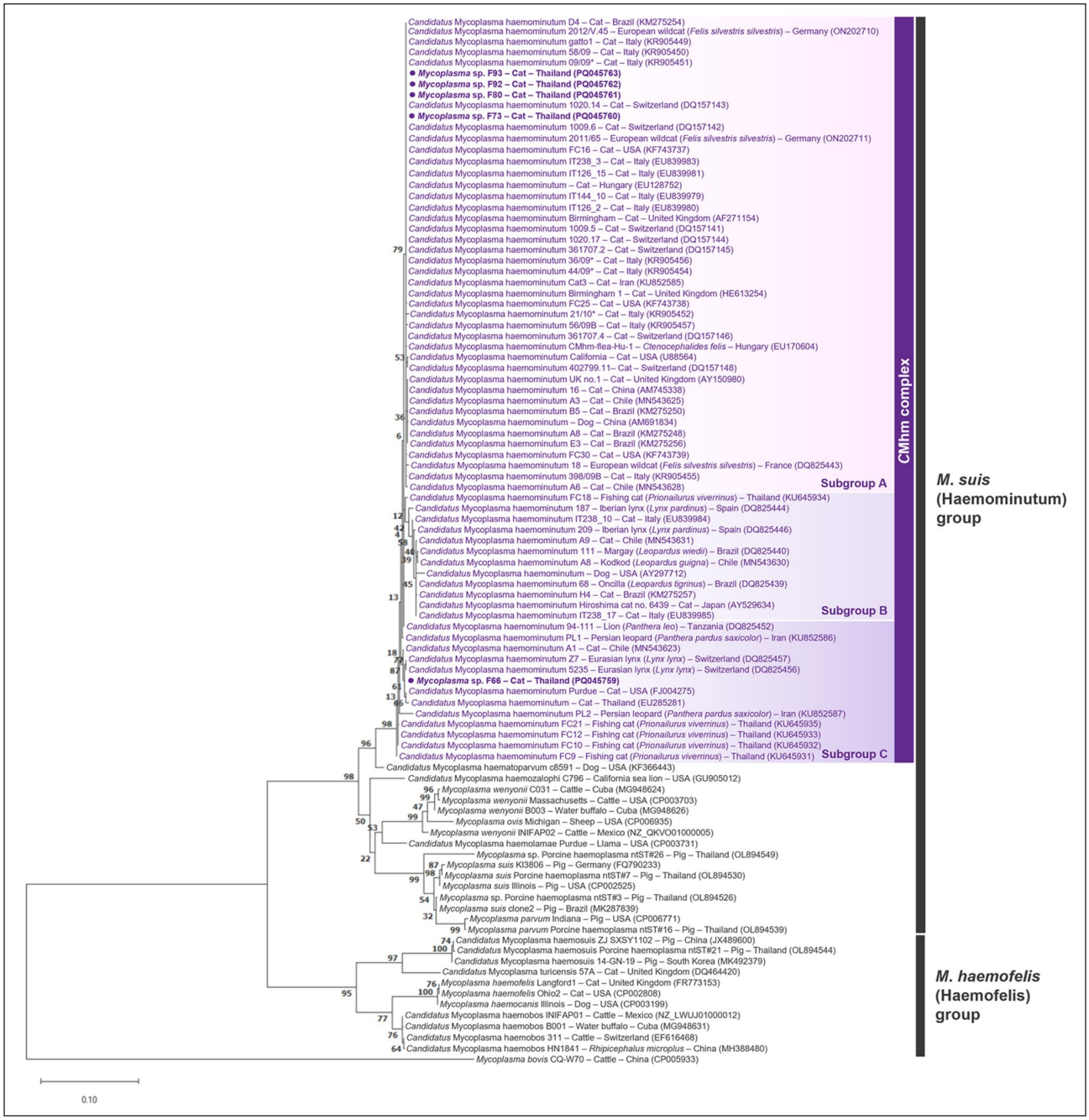

Figure 1 Phylogenetic relationships of the 16S rRNA sequences from *Candidatus* Mycoplasma haemominutum (*C*Mhm) obtained in this study, previously deposited *C*Mhm sequences and other haemoplasma species (approximately 960 bp). The tree was constructed in MEGA X based on the maximum likelihood method. The partial sequence of the 16S rRNA gene from *Mycoplasma bovis* strain CQ-W70 (accession number CP005933) was used as an outgroup

retroviral infection, anaemia and other concurrent diseases) for the cats in this study is unavailable. However, linking this putative novel species with clinical aspects would help practitioners better understand haemoplasma infection in cats. Thus, further studies should be conducted on the pathobiology of this novel species and its association with other diseases and infections.

In 2013, *M haemomuris* in rodents was classified into two clusters in 2013[43] using the 16S and 23S rRNA ITS genes. In 2015, these clusters were proposed to be named '*Candidatus Mycoplasma* haemomuris subsp musculi' and '*Candidatus Mycoplasma* haemomuris subsp ratti' using *rnpB* and *dnaK* genes.[44] In pigs, '*Candidatus Mycoplasma* haemosuis' was first identified in 2017 using the 16S rRNA gene and *rnpB*.[45] Moreover, several studies used the 23S rRNA gene to confirm novel haemoplasma species in wildlife, such as capybaras,[46] lowland tapirs,[47] porcupines,[25] coatis[48] and opossums.[49] Thus, molecular

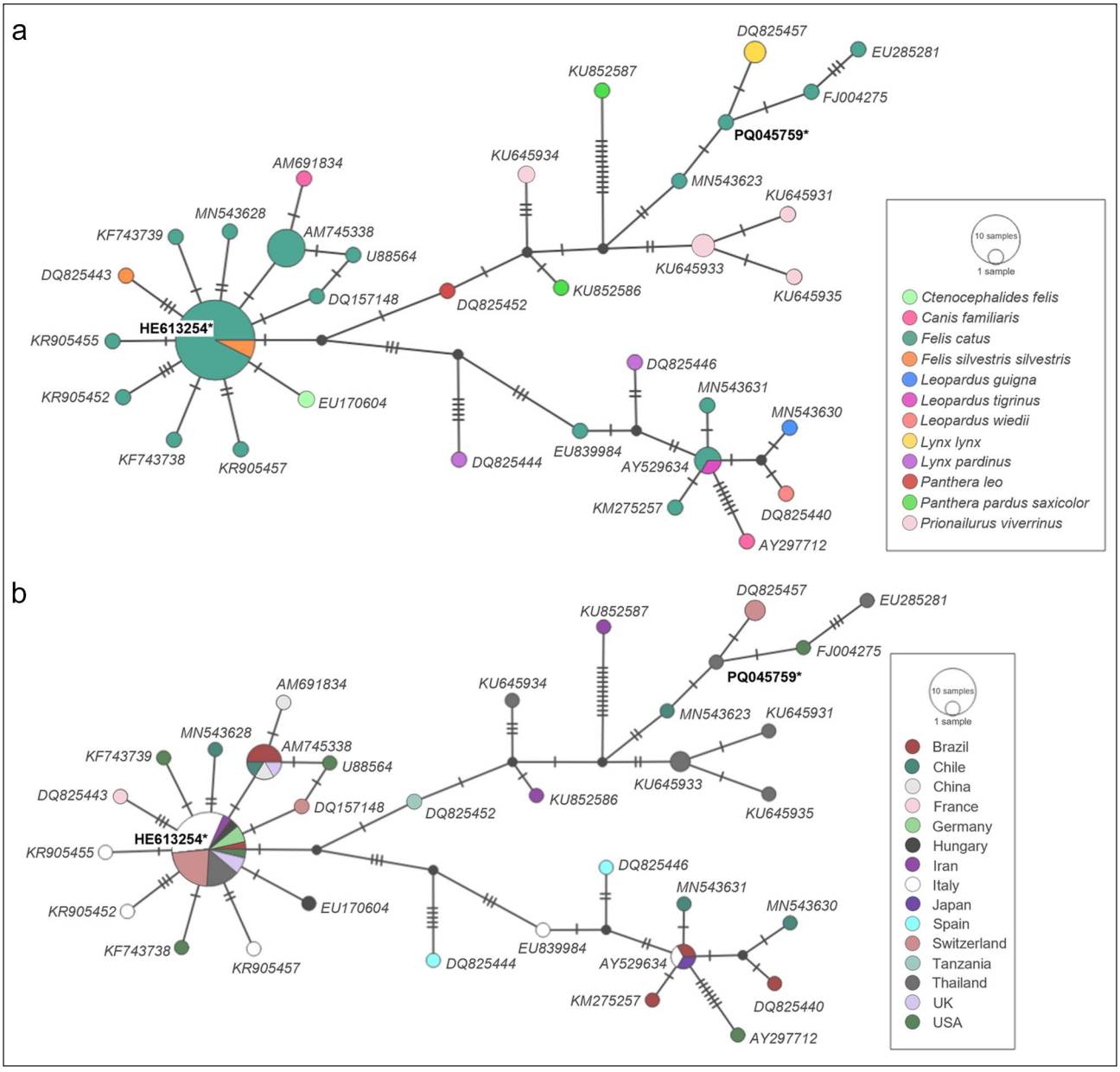

Figure 2 Genetic networks of the 16S rRNA sequences from *Candidatus* Mycoplasma haemominutum (approximately 960 bp) constructed via PopART 1.7 based on the Templeton–Crandall–Sing method. Each circle represents a different nucleotide sequence type (ntST). The size of the circle represents a number of sequences, whereas the colour within the circle represents the reported host (a) species and (b) countries. Bars between ntSTs indicate the number of nucleotide differences. Labels next to circles are the representative GenBank accession numbers of each ntST. The bold ntSTs with an asterisk (*) contain sequences from this study

detection and genetic analyses of feline haemoplasmas using the 16S rRNA gene together with other genetic markers (eg, *rnpB*, *dnaK* and the 23S rRNA gene) should be further investigated to confirm feline haemoplasmas at the species level.

Previously, some studies used the 16S rRNA gene to study feline haemoplasmas at the species level; for instance, three feline haemoplasma species were detected in client-owned cats,[16] semi-domesticated cats[15] and stray cats[17] in Thailand. Two species (*Mhf* and CMhm) have been reported in fishing cats[50] and in the flea pools of Thai cats.[51] Only CMhm was detected in fleas collected from cats in Indonesia and Vietnam.[52] Positive amplicons of haemoplasmas from cat fleas may represent *Spiroplasma* or other bacteria.[53] Similarly, the PCR primers used in this study could amplify other bacterial species in blood-sucking flies, such as *Arsenophonus nasoniae*, *Providencia* species and *Providencia stuartii*;[28] therefore, confirming positive amplicons using DNA sequencing is essential when working with fleas or other

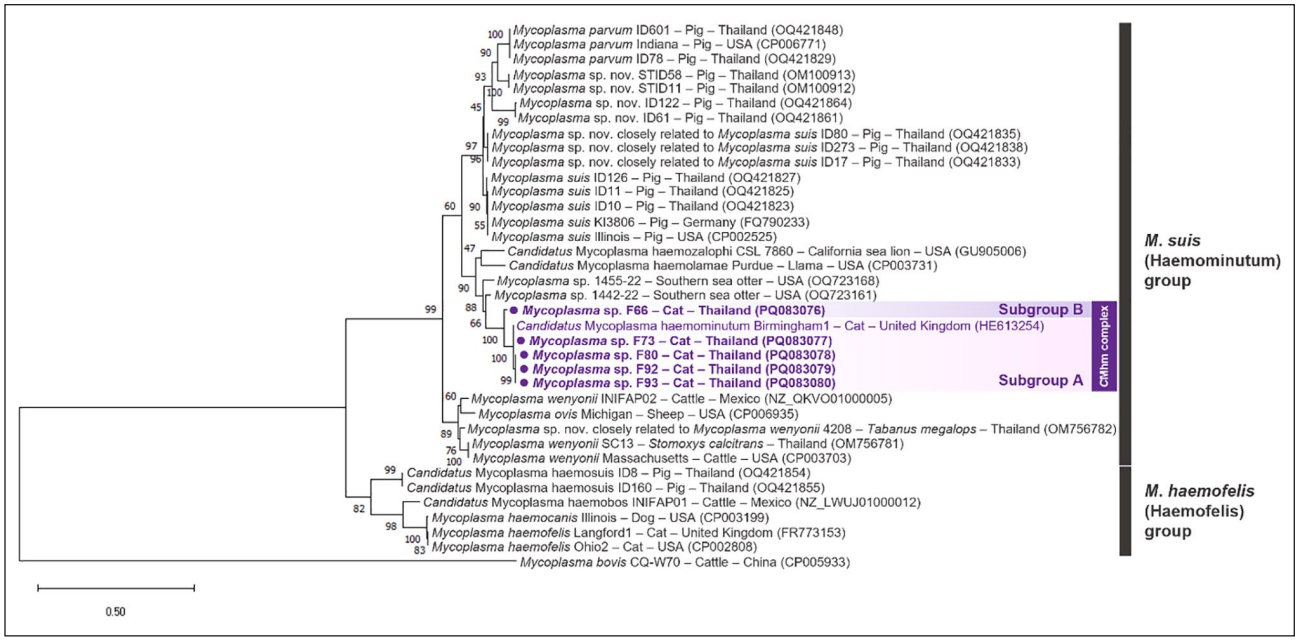

Figure 3 Phylogenetic relationships of the 23S rRNA sequences from *Candidatus Mycoplasma* haemominutum (*C*Mhm) obtained in this study, previously deposited *C*Mhm sequences and other haemoplasma species (approximately 1060 bp). The tree was constructed in MEGA X based on the maximum likelihood method. The partial sequence of the 16S rRNA gene from *Mycoplasma bovis* strain CQ-W70 (accession number CP005933) was used as an outgroup

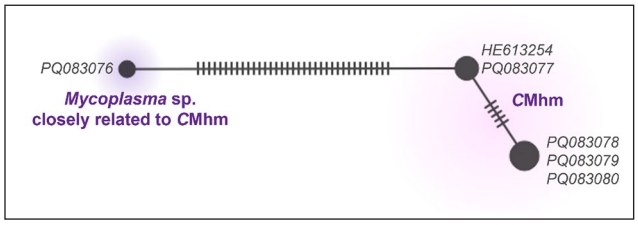

Figure 4 Genetic network of the 23S rRNA sequences from *Candidatus* Mycoplasma haemominutum (approximately 960 bp) constructed via PopART 1.7 based on the Templeton–Crandall–Sing (TCS) method. Each circle represents a different nucleotide sequence type (ntST). Bars between ntSTs indicate the number of nucleotide differences. Labels next to circles are the representative GenBank accession numbers of each ntST

insects.[28,53] CMhm has also been detected in European wildcats in Germany without evidence of other feline haemoplasma species.[54] Thus, because of hidden closely related species within the CMhm complex, the probability of detecting CMhm was likely higher than that of other species. Importantly, CMhm has been recognised as a low-virulence parasite of cats with minor or absent clinical diseases;[38] however, there is evidence that CMhm is a causative agent of anaemia in cats without concurrent diseases from Thailand[55,56] and Germany.[57] Notably, most CMhm-like sequences of the 16S rRNA gene in GenBank have been characterised as CMhm and so BLASTn results should be interpreted carefully with further investigation. We hypothesised that other virulent species might be grouped within the CMhm complex, emphasising that 16S rRNA gene sequences alone cannot differentiate some closely related haemoplasmas at the species level, especially CMhm and its closely related species.

The zoonotic potential and cross-species transmission of feline haemoplasmas should be considered. *Mhf*-like infection has been reported in a patient with HIV,[58] and CMhm has been detected in dogs from China,[59] Japan,[60] Thailand,[61] Türkiye[62] and the USA (AY297712). In addition, interspecies transmission from other mammals to cats and wild felids should be considered. For example, *Ca* M haematoparvum-like species have been detected in cats from Portugal[63] and the USA.[64] In European wildcats, one sample was infected with *Mycoplasma ovis* (ON202709);[54] therefore, using species-specific primers for haemoplasmas in domestic cats and wild felids may overlook uncommon species within this group. More reliable genetic markers, such as the 23S rRNA gene, would enhance our understanding of haemoplasma classification at the species level.

Conclusions

In the present study, CMhm and closely related species were detected in blood samples from Thai cats using the 16S and 23S rRNA genes. However, further molecular detection and genetic analysis of feline haemoplasmas using these markers should be conducted in larger sample

sizes, both in Thailand and other countries. In addition, research into the pathobiology and clinical outcomes of infection, such as signs of anaemia, associated with species closely related to CMhm is warranted.

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Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval The work described in this manuscript involved the use of non-experimental (owned or unowned) animals. Established internationally recognised high standards ('best practice') of veterinary clinical care for the individual patient were always followed and/or this work involved the use of cadavers. Ethical approval from a committee was therefore not specifically required for publication in JFMS. Although not required, where ethical approval was still obtained, it is stated in the manuscript.

Informed consent Informed consent (verbal or written) was obtained from the owner or legal custodian of all animal(s) described in this work (experimental or non-experimental animals, including cadavers, tissues and samples) for all procedure(s) undertaken (prospective or retrospective studies). For any animals or people individually identifiable within this publication, informed consent (verbal or written) for their use in the publication was obtained from the people involved.

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