P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2024.278

Molecular Identification of the Fleas *Ctenocephalides felis* and Tapeworm *Dipilydium caninum* Isolated from Fleas in Companion Dogs and Cats around Hanoi, Vietnam

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Article History: 24-704 Received: 10-Nov-24 Revised: 20-Dec-24 Accepted: 24-Dec-24 Online First: 07-Jan-25

ABSTRACT

This study aims to molecularly identify *Ctenocephalides* species and detect *Dipylidium caninum* from fleas parasitizing dogs and cats in Vietnam. The study samples comprise twenty pooled flea samples collected from dogs and cats. Methodologically, DNA extracted from fleas was utilized for PCR amplification of a 1200bp region of the flea 18S rDNA gene and a 653bp fragment of *D. caninum* 28S rDNA gene. Subsequently, two flea-positive PCR products and two *D. caninum*-positive PCR products (one each from dog and cat) were selected for phylogenetic tree analysis. The results indicated that all 20 samples were positive in the first PCR, exhibiting a 1200bp band, which corresponds to the estimated size of the flea 18S rDNA gene. Furthermore, 4 of 20 samples in the second PCR displayed a band of approximately 653bp, which is consistent with the expected size of the *D. caninum* 28S rDNA gene. Phylogenetic analysis further revealed that the isolated fleas to be *Ctenocephalides felis*. The percentage identity between the two *D. caninum* isolates in this study was 94.1%, indicating that these two isolates belong to two distinct genotypes (*D. caninum* feline and canine genotype). This study represents the first report of *D. caninum* tapeworm detection from dog and cat fleas in Vietnam. Additionally, this research provides a cautionary note to dog and cat owners regarding the importance of flea elimination from their companion animals to prevent *Dipylidium* infection.

Key words: *Ctenocephalides* sp., *Dipylidium caninum*, Dog, Cat, Vietnam

INTRODUCTION

Fleas (Siphonaptera) are the most significant external parasites in dogs and cats. Among them, the cat flea-*Ctenocephalides* genus is the most prevalent ectoparasite distributed worldwide, particularly for companion animals such as dogs and cats (Harman et al. 1987; Koutinas et al. 1995; Rust and Dryden 1997; Araujo et al. 1998; Visser et al. 2001; Akucewich et al. 2002; Bond et al. 2007; Traversa 2013; Ali et al. 2020). Two species were identified in this genus including *C. felis* and *C. canis*, with *C. felis* being more predominant compared to *C. canis* in most regions (Beck et al. 2006; Abdullah et al. 2019; Huynh et al. 2023). Animals infested with fleas frequently exhibit skin disorders. These parasites cause severe irritation in animals and are responsible for the development of allergic dermatitis (Traversa 2013). Furthermore, due to their blood-feeding behaviors in both sexes, they can serve as vectors for a range of pathogens such as *Rickettsia* sp., *Bartonella* sp., *Borrelia* sp., many of which are zoonotic pathogens (Horta et al. 2007; Bitam et al. 2010; Silaghi et al. 2012; Cicuttin et al. 2014; Driscoll et al. 2020; Eremeeva et al. 2020; Ferreira et al. 2020; Manvell et al. 2022). Additionally, they serve as the primary intermediate host for the tapeworm, *Dipylidium caninum*, in dogs and cats (Drummond et al. 2018; Scaramozzino et al. 2018). Recently, numerous human cases have been reported to be infected with *D. caninum* due to their exposure to fleas that inhabit their companion animals (Jiang et al. 2017; Chong et al. 2020; Meena et al. 2020; Gutema et al. 2021). In Vietnam, several studies have investigated fleas parasitizing dogs, cats, and other animals. These studies have focused on flea species identification and detection of pathogens such as bacteria, *Rickettsia*, and nematodes from fleas (Do et al., 2021; Huynh et al., 2023). Despite extensive research on flea-borne diseases, limited information is available regarding the prevalence of *D. caninum* in fleas in Vietnam. This study aims to address this knowledge gap by applying molecular techniques to identify *Ctenocephalides* species in dogs and cats and to detect *D. caninum* in these fleas in Vietnam for the first time. The results of this study provide information for

Cite This Article as: Nguyen YTH, Tran GTH and Vu TTT, 2025. Molecular identification of the fleas *Ctenocephalides felis* and tapeworm *Dipilydium caninum* isolated from fleas in companion dogs and cats around Hanoi, Vietnam. International Journal of Veterinary Science 14(3): 487-491. https://doi.org/10.47278/journal.ijvs/2024.278

owners of cats and dogs regarding the importance of eliminating fleas to prevent *Dipylidium* tapeworm infection in their companion animals as well as themselves.

MATERIALS AND METHODS

Flea sample collection

Ninety adult fleas were collected from 3 dogs and 15 cats. These dogs and cats were brought to animal hospitals around Ha Noi for grooming and health checks. To collect the fleas, the dogs and cats stood on a white top table and combed with a dampened flea comb, focusing on the lower back, tail head, and posterior and inner thighs (Abdullah et al. 2019). Finally, the fleas collected from each host were numbered, placed in a 15mL tube and covered. Fleas in each host were considered pooled samples, except for one flea from the dog and one flea from the cat, which were separated individually. Thus, 20 samples were analyzed.

DNA extraction and amplification

Flea samples were sectioned using a sterile surgical blade and placed into individual 1.5mL Eppendorf tubes. Flea DNA was subsequently extracted using the Genomic DNA Pre Kit (BioFact, Korea) with a minor modification. To ensure complete dissolution of flea tissue, the initial incubation step was extended to an overnight period at 56°C, deviating from the standard 10-minute protocol. Subsequent steps adhered to the manufacturer's instructions. The resulting 50µL DNA from each sample was stored at -20°C until further use.

Following DNA extraction and storage, amplification was performed using conventional PCR to amplify a 1200bp fragment of the flea 18S rDNA gene utilizing the following primer pair: 18S-F: GATCGTACCCACATTACTTG, and 18S-R: AAAGAGCTCTCAATCTGTCA (Kaewmongkol et al., 2011). The PCR reaction mixture was prepared in a 0.2mL tube with a total volume of 30µL, comprising 15µL of mastermix_2X (Phusa Biochem Ltd., Co., Vietnam), 0.4 mM of each primer, and 1µL DNA template. The mixture underwent amplification under the following conditions: initial denaturation at 95°C for 2min, followed by 40 cycles of 95°C for 20s, 56°C for 20s, 72°C for 90s; and a final extension step at 72°C for 5min in a DNA amplifier (TC-96/G/H (b) C, Bioer Technology, Hangzhou, China).

To detect the tapeworm *D. caninum* within fleas, an additional conventional PCR was conducted. A 653bp fragment of the 28S rDNA was amplified using the following primer set: F: GCATGCAAGTCAAAGGGTCCTACG, and R: CACATTCAACGCCCGACTCCTGTAG (Beugnet et al. 2014). The PCR mixture was prepared similarly to that of the flea, except that 2µL DNA was used as the template. Initial denaturation was performed at 95°C for 2min, followed by 40 cycles of 95°C for 20s, 66°C for 5s, 72°C for 30s; and a final extension step at 72°C for 10min in the aforementioned thermal cycler. Products from both PCR reactions were subjected to electrophoresis in 1.2% agarose gel pre-stained with GelRed and visualized under ultraviolet light.

Sequencing and phylogenetic tree analysis

The PCR products of two fleas and two tapeworms exhibiting strong positive bands (each from the canine and feline subjects) were selected for sequencing in both forward and reverse strands utilizing the same primers employed in the PCRs. This procedure was conducted by 1st BASE company (Selangon, MY). Nucleotide sequences were aligned using BioEdit 6.0. The obtained sequences were compared with those deposited in the GenBank database using BLAST analysis. Phylogenetic reconstructions were performed using the Maximum likelihood algorithm with the optimal substitution model in MEGA 6.

RESULTS

Molecular identification of fleas and phylogenetic tree analysis

In the initial PCR, all specimens exhibited robust positive bands, approximately 1200bp in length. Sequence alignment revealed 1088bp sequences in both feline and canine fleas (Fig. 1). When compared to GenBank entries, the isolates from this investigation were classified within the *Ctenocephalides* genus, sharing 99.08 - 100% identity with reference strains (PP455365, AF423914, ON745530, KC177274, JN008927, KM891264) (Fig. 2). The two *Ctenocephalides* spp. strains isolated in this study demonstrated 100% sequence identity with each other.

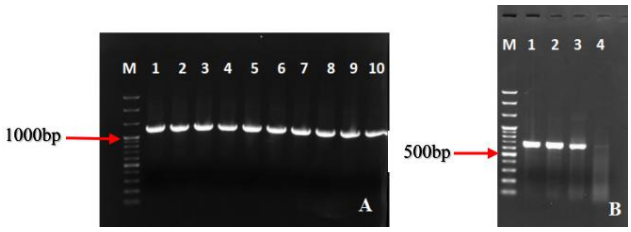

Fig. 1: A. The result of amplification of 1200bp bands from 18S rDNA gene of the fleas. (B) The result of amplification of 650bp bands from 28S rDNA gene of *D. caninum*. M: 100bp ladder; lane 1-10 (A), 1-4 (B): test samples.

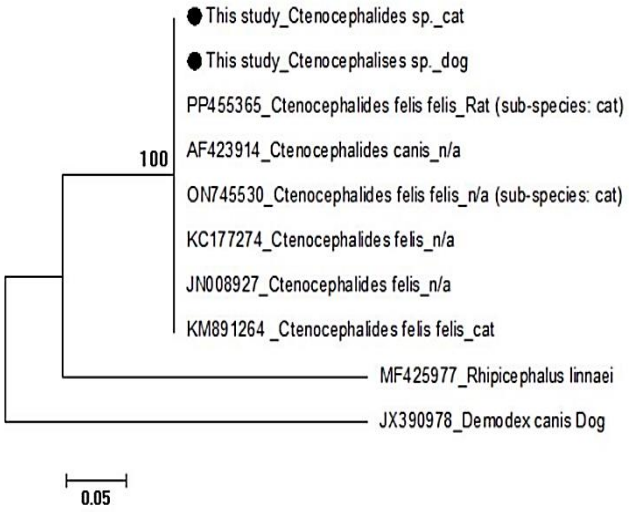

Fig. 2: The molecular phylogenetic relationship between *Ctenocephalides* genus in this study and related sequences obtained from GenBank (NCBI) was constructed based on the 18S rDNA gene. "n/a" indicated no information provided for host species.

Detection of *Dipyllidium caninum* within fleas and the phylogenetic analysis of *D. caninum*

The flea samples that yielded positive results in the initial PCR were subsequently examined for the presence of *Dipylidium* tapeworm. The analysis revealed that 4 out of 20 flea samples tested positive for *Dipylidium* sp. In the follow-up PCR, a distinct band approximately 650bp in size was observed. This band was detected in one pooled flea sample from dogs and three pooled samples from cats. The individual flea samples did not produce any positive results in the second PCR. Two *Dipylidium* sp. positive PCR products, one each from dog and cat flea origins, were randomly selected for sequencing. The sequencing and alignment results identified two sequences of the 28S rDNA gene of *D. caninum*, measuring 623 and 627bp in length, which were found in the cat and dog fleas, respectively. The *D. caninum* isolated from cats in this study showed a close relationship to *D. caninum* found in cat fleas (*C. felis*) and some cat worm reference sequences. Similarly, the *D. caninum* from dog fleas in this study was closely related to *D. caninum* from dog fleas or dog worms reported in earlier studies. The reference strains used in this research had a global distribution (Fig. 3).

In this study, the sequences were compared to previously published reference sequences (AF023120 and MH040832). The sequence derived from dog fleas (provisionally labeled "*D. caninum* canine genotype") demonstrated 99.7% and 100.0% similarity to the sequences with accession numbers AF02312 and MH040832 (obtained from proglottids collected from dogs), respectively (Abdullah et al. 2019). In contrast, the sequence isolated from cat fleas (provisionally labeled "*D. caninum* feline genotype") showed 93.4 and 94.4% similarity to the aforementioned published sequences, respectively. Notably, the percentage identity between the two isolates in this study reached only 94.1% (Table 1).

DISCUSSION

Ctenocephalides felis (Siphonaptera: Pulicidae) (Bouché, 1835), commonly known as the cat flea, is the most prevalent ectoparasite found on domestic cats and dogs globally (Labuschagne et al. 2018). This highly polyxenous ectoparasite infests a diverse array of hosts, including cattle, koalas and rodents (Šlapeta et al. 2011; Linardi and Costa Santos 2012). While cats serve as the primary host for only *C. felis*, dogs are the main hosts for multiple flea species, including *C. felis*, *C. canis*, *P. irritans*, and *P. simulans* (Guzman 1984; Huynh et al. 2023). Numerous studies have indicated that *C. canis* fleas occur more frequently than *C. felis* on dogs (Guzman 1984; Huynh et al. 2023). However, *C. felis felis* was identified as the most common species on household dogs in the UK (Beresford-Jones 1981; Chesney 1995) and Australia (Šlapeta et al. 2011). For molecular identification of fleas on domestic dogs and cats in this research, two isolates were randomly selected (one from each host) for sequencing analysis. The flea species in this study showed 100% identity with the published reference strain (PP455365), which was identified as the cat sub-species, *C. felis felis*. Additional research is necessary to determine the predominant flea species on dogs and cats within the scope of this study.

Table 1: The comparison of percentage identity of DNA sequences obtained from this study to the published reference sequences from GenBank

	D. *caninum* (AF023120) (%)	D. *caninum* (MH040832) (%)	*D. caninum* canine genotype (%)	*D. caninum* feline genotype (%)
D. caninum (AF023120) (%)			99.7	93.4
D. caninum (MH040832) (%)			100.0	94.4
D. caninum canine genotype (%)	99.7	100.0		94.1
D. caninum feline genotype (%)	93.4	94.4	94.1	

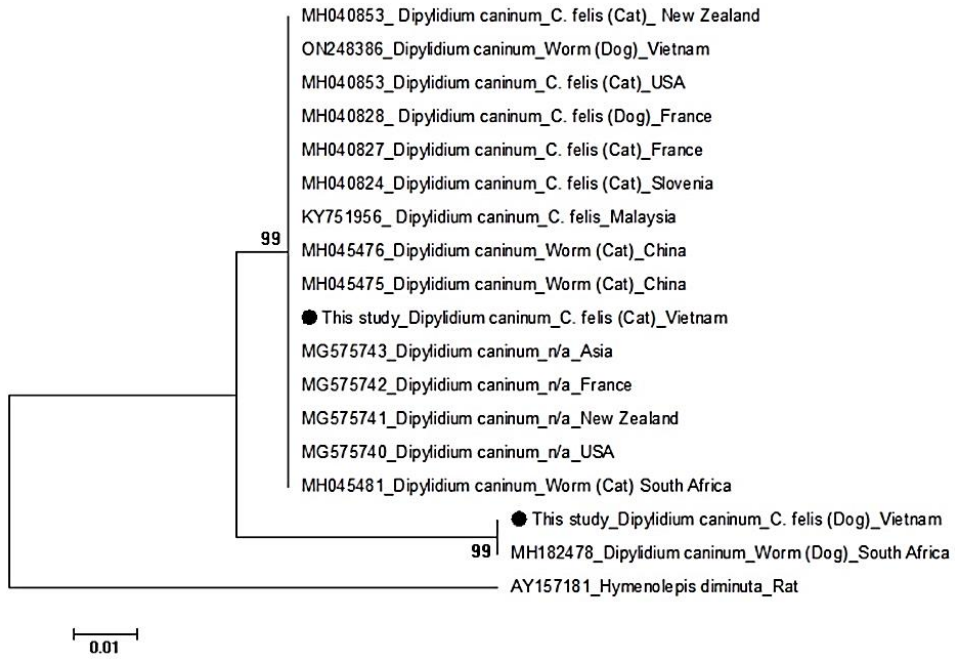

Fig. 3: The molecular phylogenetic relationship between *Dipylidium caninum* in this study and related sequences obtained from GenBank (NCBI) was constructed based on the 28S rDNA gene. "n/a" indicated no information provided for host species.

Fleas not only extract blood and cause severe clinical infestations on host skin but also serve as competent vectors for numerous pathogens, including *Dipylidium caninum* - a double-pored tapeworm that is a cosmopolitan cestode in the intestines of dogs and cats. In the *D. caninum* life cycle, the flea functions as the intermediate host in which the onchosphere larvae migrate to the hemocoel of the flea and develop into infective cysticercoid larvae. The detection rate of *D. caninum* in fleas using PCR assay was previously reported to range from 1.26 to 16.67% in cats, and from 0.0 to 14.39% in dogs (Beugnet et al. 2014; Labuschagne et al. 2018). In this study, we identified 4 of 20 flea samples (accounting for 20.0%) to be positive for *D. caninum*, of which three samples exhibited strong bands in PCR assay. The rate of *D. caninum* infection in dogs was reported to be 17.7% in Phu Tho, Vietnam (Nguyen et al. 2022). However, no official data on the rate of *D. caninum* infection in cats in Vietnam has been recorded, although we occasionally observe cases of cats infected with *D. caninum* by detecting proglottids excreted in cat feces. Furthermore, we observed a high level of genetic distance (5.9%) between two isolates in this study, between "*D. caninum* feline genotype" and the reference strains AF 023120 (6.6%), and MH040832 (5.6%). This finding indicated the existence of two distinct genotypes of *D. caninum* in the dog and the cat in this study. Some studies have reported that a small proportion of *D. caninum* DNA derived from cats or cat fleas or dogs or dog fleas belongs to the other genotype. Additionally, these two genotypes are not associated with geographical distribution (East et al., 2013; Low et al. 2017; Beugnet et al. 2018; Labuschagne et al. 2018).

Conclusion

This study molecularly identified the fleas parasitizing domestic dogs and cats as *Ctenocephalides felis*. Furthermore, this research presents the first report of *D. caninum* tapeworm detection from dog and cat fleas in Vietnam. The findings from this investigation provide valuable information for dog and cat owners regarding the importance of flea elimination from their companion animals to enhance both animal and human health. Nevertheless, further studies are warranted to confirm the predominant subspecies of *Ctenocephalides* flea as well as *D. caninum* in dogs and cats.

Authors' Contributions: Yen Thi Hoang Nguyen: Conceptualization, Writing - Original Draft, Review & Editing. Giang Thi Huong Tran: Methodology, Writing - Review; Thi Thu Tra Vu: Data Analysis, Writing - Review.

Funding: The authors express their gratitude to the Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Vietnam National University of Agriculture for providing financial support for this study.

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