Analysis of *Dipylidium caninum* tapeworms from dogs and cats, or their respective fleas

**Part 1. Molecular characterization of *Dipylidium caninum*: genetic analysis supporting two distinct species adapted to dogs and cats**

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Received 25 January 2018, Accepted 21 April 2018, Published online 28 May 2018

**Abstract** -- A 28S rDNA PCR detection assay was previously developed to identify *Dipylidium caninum* DNA inside single fleas collected from both cats and dogs. Sequence analysis of the 28S rDNA fragment indicated two genetically distinct variations of the target region. The two genotypes, so-called “*D. caninum* canine genotype” and “*D. caninum* feline genotype”, based on host origin, are further investigated and described in this paper. Restriction fragment length polymorphism (RFLP) analysis and hydrolysis probe-based genotyping assays were developed and validated for genotyping *D. caninum* DNA. The complete mitochondrial (mt) genome of the “feline genotype” was sequenced and compared to the *D. caninum* mt genome available in GenBank. The molecular characterization of *D. caninum* isolates collected from infected fleas, and also proglottids collected from dogs and cats, confirmed the existence of two distinct genotypes. These genotypes are related to host origin (dogs or cats), irrespective of their geographical origin, and they present a biological adaptation to their respective host, as confirmed by the comparison of biological development and host preference in another study. The genetic differences (Part 1, present paper) and biological observations (Part 2, in this journal) enabled us to suggest the existence of two distinct species within *D. caninum*, which will have to be clarified.

**Key words:** *Dipylidium caninum*, mitochondrial genome, *Ctenocephalides felis*, dogs, cats, genotypes

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**Introduction**

*Dipylidium caninum* (Linnaeus, 1758), a globally distributed cestode, infects domestic cats and dogs [8], wild canids and felids [7,10], and occasionally humans [15]. In 1758, Linnaeus recognized the parasite and named it *Taenia canina*. In 1863, Leuckart erected the genus *Dipylidium*, but it was not until 1893 that the internal anatomy and observations of the life history of *Dipylidium caninum* was described by Diamare (in [16]). The cat flea, *Ctenocephalides felis*, is considered the main intermediate host of *D. caninum* [5]. Furthermore, *C. felis* has the ability to infest both dogs and cats. The dog flea (*Ctenocephalides canis*) also acts as an intermediate host, but exceptionally infests cats. The life cycle of *D. caninum* can be summarized as follows: flea larvae ingest eggs of *D. caninum*, followed by development of the egg to the metacestode stage inside the flea. When a canine or feline host ingests adult fleas infected with suitably developed metacestodes, the parasite establishes in the small intestine of its definitive host.

Several species belonging to the genus *Dipylidium* have historically been suggested based on morphological observations [16]. However, significant overlap in morphological traits led to recognition of a single species, namely *D. caninum* [16,18]. As a result, the genus *Dipylidium* is currently considered monotypic. However, in the absence of distinguishing morphological characters or significant overlap thereof, modern molecular techniques often allow us to differentiate hidden genetic lineages and cryptic species [e.g. 1,6,12,17,19]. A molecular approach would potentially be highly beneficial in investigating and confirming species status within the genus *Dipylidium*.

In 2014, Beugnet et al. [3] investigated the prevalence of *D. caninum* in fleas from client-owned cats and dogs in Europe, using a new PCR detection assay targeting a region within the 28S rDNA. The results confirmed the spread of *D. caninum sensu lato* in fleas of dogs and cats throughout Europe. Preliminary analyses indicated genetic differences between *D. caninum* metacestodes in fleas collected from dogs and cats, respectively. These preliminary analyses are described in the present article as well as all further molecular assessments that were performed in order to confirm the existence of multiple *D. caninum* genotypes.

Original sequence analysis of PCR products from *Dipylidium* infected fleas collected in 2011 and 2012 (Beugnet et al., 2014 [3]) indicated two 28S rDNA sequence variants of the target region. This preliminary analysis suggested that a host-specific preference may be applicable, and hence the two distinct 28S rDNA sequence variants were defined as the so-called “*D. caninum* canine genotype” and the “*D. caninum* feline genotype” [previously unpublished, see Tables 1–Tables 1 to 3]. The *D. caninum* canine genotype was found in >95% cases in infected *C. felis* fleas collected on dogs and 100% of *C. canis* infected fleas, whereas the *D. caninum* feline genotype was identified in >95% of *C. felis* infected fleas collected on cats. It was thus decided to further investigate these differences, with specific reference to possible genotype-host associations.

The present paper reports the several steps of analysis since the original finding. Firstly, the development of restriction fragment length polymorphism (RFLP) analysis and hydrolysis probe-based genotyping assays, for genotyping *D. caninum* DNA. Secondly, a sensitive, non-invasive nucleic acid detection assay applied for the detection of *D. caninum* DNA in faeces. And finally, the complete sequencing of the mt genome from a feline host to compare the “*D. caninum* feline genotype” and the *D. caninum* mt genome available in GenBank.

The objectives of this study were thus to develop the necessary assays for genotyping *D. caninum* DNA to discriminate between the two *D. caninum* genotypes. These comparisons allowed the identification and breeding of the two genotypes, in order to further evaluate their biological differences (see Beugnet et al., 2018, part 2, [4]).

**Materials and Methods**

**Parasites and DNA extraction**

28S rDNA PCR detection of *D. caninum* was performed on 6116 crude flea (*C. felis, C. canis and Pulex irritans*) extracts as described by Beugnet et al [3]. A total of 192 *D. caninum*-positive DNA extracts were included in the present genotype analysis. A total of 57 *D. caninum*-positive samples were subjected to DNA sequencing. Subsequently, all positive samples (Table 1) were subjected to RFLP (restriction fragment length polymorphism) analysis.

In addition, a total of 55 fleas collected from cats were obtained from Mike Lappin (Colorado State University, USA) and 9 of the PCR positive samples were subjected to sequencing (Table 2). Adult fleas (n=100) were also obtained from New Zealand and 2 of the PCR positive samples were subjected to sequencing. All adult fleas were supplied in 70% (v/v) ethanol.

Five adult *D. caninum* tapeworms were also obtained from various sources, including 2 worms from Clinvet International (South Africa), 1 worm from École Nationale Vétérinaire de Maisons-Alfort (France), and 2 worms from Guangxi University Animal Hospital (China). All adult worms were supplied in 70% (v/v) ethanol. Genomic DNA was isolated from the adult worm proglottids using the GeneJet Genomic DNA isolation kit (Thermo Fisher Scientific), according to the manufacturer’s recommendation for tissue samples.

The original *D. caninum* strain maintained at Clinvet International research centre on cats and fleas was included. A new *Dipylidium* sp. strain originating from proglottids collected on dogs in the village near the research centre was also included, genotyped, and maintained on dogs. The two identified genotypes are thus maintained on dogs, cats, and their fleas, respectively, at Clinvet Research Centre, allowing further studies (Table 3).
Table 1. Details on two distinct 28S rDNA sequence variants (“canine” and “feline”) defined from fleas infected by Dipylidium caninum, obtained from PCR products collected by Beugnet et al. [3] from Europe and South Africa.

| Sample ID    | Source | Geographic origin | Host | RFLP Genotype |
|--------------|--------|-------------------|------|---------------|
| CVML12/008/006/024 | C. felis | Czech Republic    | Cat  | Canine        |
| CVML12/008/023/002 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/023/003 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/023/004 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/023/005 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/023/006 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/023/009 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/023/010 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/034/003 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/041/001 | C. felis | Slovenia          | Cat  | Feline        |
| CVML12/008/060/007 | C. felis | Portugal          | Cat  | Not determined|
| CVML12/008/105/002 | C. felis | France            | Cat  | Canine        |
| CVML12/008/118/002 | C. felis | France            | Cat  | Not determined|
| CVML12/008/193/001 | C. felis | France            | Dog  | Feline        |
| CVML12/008/198/006 | C. felis | France            | Dog  | Canine        |
| CVML12/008/198/007 | C. felis | France            | Dog  | Canine        |
| CVML12/008/231/007 | C. felis | France            | Dog  | Canine        |
| CVML12/008/231/008 | C. felis | France            | Dog  | Canine        |
| CVML12/008/246/002 | C. felis | France            | Dog  | Canine        |
| CVML12/008/248/025 | C. felis | France            | Dog  | Canine        |
| CVML12/008/248/036 | C. felis | France            | Dog  | Canine        |
| CVML12/008/265/001 | C. felis | Hungary           | Cat  | Feline        |
| CVML12/008/265/010 | C. felis | Hungary           | Cat  | Feline        |
| CVML12/008/277/006 | C. felis | Germany           | Cat  | Feline        |
| CVML12/008/277/013 | C. felis | Germany           | Cat  | Feline        |
| CVML12/008/279/003 | C. felis | Portugal          | Cat  | Feline        |
| CVML12/008/279/014 | C. felis | Portugal          | Cat  | Feline        |
| CVML12/008/364/001 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/364/004 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/366/001 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/373/002 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/397/004 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/474/013 | C. felis | France            | Dog  | Canine        |
| CVML12/008/523/005 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/530/003 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/539/002 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/550/001 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/598/002 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/601/003 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/601/004 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/603/003 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/632/002 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/642/004 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/651/002 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/651/003 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/653/002 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/654/004 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/664/005 | C. felis | Sicily            | Dog  | Canine        |
| CVML12/008/671/001 | C. felis | France            | Cat  | Feline        |
| CVML12/008/739/005 | C. felis | Romania           | Cat  | Feline        |
### Table 1. (continued).

| Sample ID | Source | Geographic origin | Host | RFLP Genotype |
|-----------|--------|-------------------|------|---------------|
| CVML12/008/739/007 | C. felis | Romania | Cat | Feline |
| CVML12/008/739/009 | C. felis | Romania | Cat | Feline |
| CVML12/008/754/002 | C. felis | Romania | Cat | Feline |
| CVML12/008/757/002 | C. felis | Romania | Cat | Not determined |
| CVML12/008/758/002 | C. felis | Romania | Cat | Feline |
| CVML12/008/758/003 | C. felis | Romania | Cat | Feline |
| CVML12/008/758/004 | C. felis | Romania | Cat | Feline |
| CVML12/008/791/001 | C. felis | Hungary | Cat | Feline |
| CVML12/008/800/002 | C. felis | Hungary | Cat | Feline |
| CVML12/008/800/009 | C. felis | Hungary | Cat | Feline |
| CVML12/008/800/011 | C. felis | Hungary | Cat | Feline |
| CVML12/008/803/001 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/001 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/002 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/003 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/004 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/005 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/006 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/007 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/008 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/009 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/010 | C. felis | Hungary | Cat | Feline |
| CVML12/008/807/011 | C. felis | Hungary | Cat | Feline |
| CVML12/008/812/005 | C. canis | Albania | Dog | Canine |
| CVML12/008/823/003 | C. canis | Albania | Dog | Canine |
| CVML12/008/830/004 | C. canis | Albania | Dog | Canine |
| CVML12/008/830/005 | C. canis | Albania | Dog | Feline |
| CVML12/008/832/001 | C. canis | Albania | Dog | Feline |
| CVML12/008/1067/002 | C. canis | Hungary | Dog | Feline |
| CVML12/008/934/001 | C. canis | Romania | Dog | Canine |
| CVML12/008/941/004 | C. canis | Romania | Dog | Canine |
| CVML12/008/1193/004 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/1196/001 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/1196/010 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/1196/014 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/1197/006 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/1198/010 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/997/002 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/997/005 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/998/003 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/998/006 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/001 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/002 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/003 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/005 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/008 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/022 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/026 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/027 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/028 | C. canis | Bulgaria | Dog | Canine |
| CVML12/008/999/029 | C. canis | Bulgaria | Dog | Canine |
| Sample ID   | Source   | Geographic origin | Host | RFLP Genotype |
|------------|----------|-------------------|------|---------------|
| CVML12/008/999/039 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1010/007 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1010/008 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1011/013 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1231/002 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1231/003 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1231/004 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1234/001 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1235/001 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1239/002 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1253/006 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1254/001 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1028/001 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1028/002 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1028/005 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1029/001 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1029/002 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1029/005 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1030/003 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1259/001 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1259/003 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1302/010 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1302/014 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1302/017 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/005 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/007 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/018 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/019 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/022 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/029 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/034 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/037 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/045 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1314/052 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1317/002 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1324/021 | C. canis | Albania           | Dog  | Canine        |
| CVML12/008/1328/001 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1333/001 | C. felis | France            | Dog  | Canine        |
| CVML12/008/1347/003 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1348/001 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1348/002 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1349/001 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1380/003 | C. canis | Romania           | Dog  | Canine        |
| CVML12/008/1383/003 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1383/005 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1383/007 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1383/010 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1383/015 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1383/018 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1383/020 | C. canis | Bulgaria          | Dog  | Canine        |
| CVML12/008/1384/002 | C. canis | Bulgaria          | Dog  | Canine        |
DNA sequencing, RFLP, and hydrolysis probe genotyping of *D. caninum*

Primer pairs DC28S-1F and DC28S-1R [3] were used to amplify part of the 28S rDNA region from the genome of *D. caninum*. PCR products were subjected to Sanger sequencing and sequence assembly of both strands were performed using Geneious assembler (Geneious 8.0.5). All PCR products obtained from Beugnet et al. [3] (Table 1) were subjected to RFLP analysis. PCR product (2 µl) was subjected to direct digestion using 10 units *Stu*-I restriction enzyme (New England Biolabs) in a final volume of 20 µl for 1 hour at 37°C. The complete digestion mixture was electrophoretically separated using a 2% (m/v) TAE agarose gel at 6 V/cm for 1 hour. All appropriate controls were included.

| Sample ID     | Source | Geographic origin | Host | RFLP Genotype |
|---------------|--------|-------------------|------|---------------|
| CVML12/008/1384/003 | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1385/001  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1385/003  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/003  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/011  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/015  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/017  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/020  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/022  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/026  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/027  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/032  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/040  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/045  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/048  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1386/050  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1394/014  | *C. canis* | Bulgaria         | Dog  | Canine        |
| CVML12/008/1466/001  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1541/007  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/001  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/003  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/006  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/011  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/012  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/013  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/014  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/015  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/019  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/024  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/028  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1542/029  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1544/009  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1544/025  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1544/036  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1545/006  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1546/001  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1549/001  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1551/001  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1556/002  | *P. irritans* | Europe    | Dog  | Canine        |
| CVML12/008/1556/005  | *P. irritans* | Europe    | Dog  | Canine        |

Canine = “*D. caninum* canine genotype”, Feline = “*D. caninum* feline genotype”
Table 2. Details on two distinct 28S rDNA sequence variants (“canine” and “feline”) defined from fleas infected by *Dipylidium caninum*, received from the United States of America.

| Sample ID          | Source | Geographic origin       | Host      | RFLP Genotype |
|--------------------|--------|-------------------------|-----------|---------------|
| CVML13/004/043     | C. felis | United States of America | Cat       | Feline        |
| CVML13/004/044     | C. felis | United States of America | Cat       | Not determined |
| CVML13/004/045     | C. felis | United States of America | Cat       | Feline        |
| CVML13/004/046     | C. felis | United States of America | Cat       | Feline        |
| CVML13/004/049     | C. felis | United States of America | Cat       | Not determined |
| CVML13/004/050     | C. felis | United States of America | Cat       | Feline        |
| CVML13/004/051     | C. felis | United States of America | Cat       | Feline        |
| CVML13/004/052     | C. felis | United States of America | Cat       | Feline        |
| CVML13/004/053     | C. felis | United States of America | Cat       | Feline        |

Canine = “D. caninum canine genotype”, Feline = “D. caninum feline genotype”

Table 3. Details on two distinct 28S rDNA sequence variants (“canine” and “feline”) defined from non-invasive anal swabs collected from cats and dogs infected by *Dipylidium caninum* in South Africa.

| Sample ID | Source | Geographic origin | Host      | “Genotype” |
|-----------|--------|-------------------|-----------|------------|
| CV1       | Swabs  | South Africa      | Cat       | Feline     |
| CV2       | Swabs  | South Africa      | Cat       | Feline     |
| CV3       | Swabs  | South Africa      | Cat       | Feline     |
| CV4       | Swabs  | South Africa      | Cat       | Feline     |
| CV5a      | Swabs  | South Africa      | Cat       | Feline     |
| CV5b      | Swabs  | South Africa      | Dog       | Canine     |
| CV7       | Swabs  | South Africa      | Dog       | Not determined |
| CV8       | Swabs  | South Africa      | Dog       | Canine     |
| CV9       | Swabs  | South Africa      | Dog       | Canine     |
| CV10      | Swabs  | South Africa      | Dog       | Canine and feline |
| CV11      | Swabs  | South Africa      | Dog       | Canine     |
| CV12      | Swabs  | South Africa      | Dog       | Canine     |
| CV13      | Swabs  | South Africa      | Dog       | Canine     |
| CV14      | Swabs  | South Africa      | Dog       | Canine     |
| CV16      | Swabs  | South Africa      | Dog       | Canine     |
| CV23      | Swabs  | South Africa      | Dog       | Canine     |
| CV24      | Swabs  | South Africa      | Dog       | Canine     |
| CV25      | Swabs  | South Africa      | Dog       | Canine     |
| CV26      | Swabs  | South Africa      | Dog       | Canine     |
| CV27      | Swabs  | South Africa      | Dog       | Canine     |
| CV28      | Swabs  | South Africa      | Dog       | Canine     |
| CV29      | Swabs  | South Africa      | Dog       | Canine     |
| CV30      | Swabs  | South Africa      | Dog       | Canine     |
| CV31      | Swabs  | South Africa      | Dog       | Feline*     |
| CV32      | Swabs  | South Africa      | Dog       | Feline*     |
| CV33      | Swabs  | South Africa      | Dog       | Not determined |
| CV34      | Swabs  | South Africa      | Dog       | Feline*     |
| CV35      | Swabs  | South Africa      | Dog       | Feline*     |
| CV36      | Swabs  | South Africa      | Dog       | Feline*     |
| CV37      | Swabs  | South Africa      | Dog       | Not determined |
| CV38      | Swabs  | South Africa      | Dog       | Feline*     |
| CV39      | Swabs  | South Africa      | Dog       | Feline*     |
| CV40      | Swabs  | South Africa      | Dog       | Feline*     |
| CV41      | Swabs  | South Africa      | Dog       | Not determined |
| CV42      | Swabs  | South Africa      | Dog       | Canine     |
| CV43      | Swabs  | South Africa      | Dog       | Canine     |
| CV44      | Swabs  | South Africa      | Dog       | Canine     |
| CV45      | Swabs  | South Africa      | Dog       | Canine     |
The hydrolysis probe qPCR assays to discriminate between the two genotypes were based on a qPCR assay with the following setup. Primers DC28S-1F and DC28S-1R [3] were added to a final concentration of 900 nM each. Probes D_caninum dog (FAM-GTGTGTGCACAGTC-NFQ-MGB) and D_caninum cat (VIC-CCTGTGTGTCAGTCG-NFQ-MGB) were added to a final concentration of 200 nm each. qPCR was performed using a QuantStudio 6 instrument fitted with a 384-well block in a final reaction volume of 10 μl using SsoAdvanced™ Universal Probes Supermix (Bio-Rad) under the following cycling conditions: initial denaturation of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Pre-and post-read stages at 25°C for 30 sec were included. Analysis was performed using the QuantStudio 6 Real-time PCR system Software. All appropriate controls were included.

Table 3. (continued).

| Sample ID | Source | Geographic origin | Host | “Genotype” |
|-----------|--------|-------------------|------|------------|
| CV57      | Swabs  | South Africa      | Dog  | Canine     |
| CV59      | Swabs  | South Africa      | Dog  | Canine     |
| CV60      | Swabs  | South Africa      | Dog  | Canine     |
| CV61      | Swabs  | South Africa      | Dog  | Canine     |
| CV64      | Swabs  | South Africa      | Dog  | Canine     |
| CV66      | Swabs  | South Africa      | Dog  | Canine     |
| CV67      | Swabs  | South Africa      | Dog  | Canine     |
| CV68      | Swabs  | South Africa      | Dog  | Canine     |
| CV70      | Swabs  | South Africa      | Dog  | Canine     |
| CV71      | Swabs  | South Africa      | Dog  | Canine     |
| CV72      | Swabs  | South Africa      | Dog  | Canine     |
| CV73      | Swabs  | South Africa      | Dog  | Canine     |
| CV74      | Swabs  | South Africa      | Dog  | Not determined |
| CV75      | Swabs  | South Africa      | Dog  | Canine     |
| CV77      | Swabs  | South Africa      | Dog  | Canine     |
| CV80      | Swabs  | South Africa      | Dog  | Canine     |
| CV81      | Swabs  | South Africa      | Dog  | Canine     |
| CV82      | Swabs  | South Africa      | Dog  | Canine     |
| CV83      | Swabs  | South Africa      | Dog  | Canine     |
| CV84      | Swabs  | South Africa      | Dog  | Canine     |
| CV85      | Swabs  | South Africa      | Dog  | Canine     |
| CV86      | Swabs  | South Africa      | Dog  | Not determined |
| CV87      | Swabs  | South Africa      | Dog  | Canine     |
| CV88      | Swabs  | South Africa      | Dog  | Canine     |
| CV89      | Swabs  | South Africa      | Dog  | Canine and feline |
| CV90      | Swabs  | South Africa      | Dog  | Canine     |
| CV91      | Swabs  | South Africa      | Dog  | Canine     |
| CV92      | Swabs  | South Africa      | Dog  | Canine     |
| CV93      | Swabs  | South Africa      | Dog  | Canine     |
| CV94      | Swabs  | South Africa      | Dog  | Canine     |
| CV95      | Swabs  | South Africa      | Dog  | Canine     |
| CV96      | Swabs  | South Africa      | Dog  | Canine     |
| CV97      | Swabs  | South Africa      | Dog  | Canine     |
| CV98      | Swabs  | South Africa      | Dog  | Canine     |
| CV99      | Swabs  | South Africa      | Dog  | Canine     |
| CV100     | Swabs  | South Africa      | Dog  | Canine     |
| CV102     | Swabs  | South Africa      | Dog  | Canine     |
| CV103     | Swabs  | South Africa      | Dog  | Canine     |
| CV104     | Swabs  | South Africa      | Dog  | Canine     |
| CV105     | Swabs  | South Africa      | Dog  | Canine     |

Canine = “D. caninum canine genotype”, Feline = “D. caninum feline genotype”

* Dogs participated in an experimental infection efficacy study, and were infected with the D. caninum feline genotype.
Non-invasive field sampling of animals: detection and genotyping of *D. caninum*

Ninety-nine dogs were sampled in villages under field conditions in the area surrounding Bloemfontein (Free State, Republic of South Africa). The sampling procedure entailed the gentle swabbing of the anal region (including surrounding hair) with a sterile cotton swab. Swabs were collected from dogs living with their owners and stored at room temperature until processing for DNA isolation using the GeneJet Genomic DNA isolation kit (Thermo Fisher Scientific), according to the manufacturer’s recommendation for tissue samples. PCR based detection of *D. caninum* DNA obtained from 99 anal swabs from dogs resulted in successful amplification of the target region.

During the study, the anal area and hair surrounding it, as well as the sleeping area of the dog were checked for signs of proglottids. Proglottids were expelled by 12 animals at the time of swabbing. The swabs were subjected to PCR analyses, and if positive for *D. caninum* DNA (Table 3), the dogs were individually placed in kennels at Clinvet and screened for signs of proglottids.

**Targeted nuclear DNA amplification and sequencing**

Primer pairs *WormA* (5'-GGGATGCTCATTAAATCCAG-3') and *WormB* (5'-CTTTGTCAGCTTTTACTTCC-3') [11] were used to amplify the 18S ribosomal DNA region from the proglottids of feline (R166 and #1431) and canine origin (CV_ref). The complete 18S region was sequenced using a primer walking strategy. Sequence alignments were performed using the MAFFT plugin in Geneious 8.0.5. Molecular phylogenetic analyses using Bayesian inference and Neighbour-Joining were performed according to Guo [9] using the sequence of Schistosoma solidus (Accession number: KF202097) (as published by East et al. [7]).

**Mitochondrial genome sequence analysis**

Illumina Miseq and Sanger sequencing data were assembled, after quality trimming, using the Geneious assembler (Geneious 8.0.5). Contigs were mapped to the only available *D. caninum* mt DNA genome (AB732959), using Geneious assembler. Annotation and similarity percentages of the *D. caninum* R166 mt genome was performed using the Geneious Live annotate and predict function, making use of Illumina’s Miseq instrument and 2×250 bp reads using the MiSeq Reagent Kit v2. Primers F_1DNA-F (TCTCTGAGTTTTGCTGCTGTTT) and F_1DNA-R (AACGAGCA-CATAGCTTACCTT) were used to amplify 1126 bp, including the DcMitoUni-INV-1F and DcMitoUni-INV-1R primer binding sites and the PCR product was subjected to Sanger sequencing.

**Sequencing of the mitochondrial DNA**

Primer pairs DeMitoUni-1F (5'-GGGATGCTCATTAAATCCAG-3') and DeMitoUni-1R (5'-CCTTTGTCAGCTTTTACTTCC-3') were based on the sequence of the *D. caninum* partial mt DNA sequence isolated from spotted hyena (Accession number: KP202097) (as published by East et al. [7]).

The 842 bp PCR product was amplified using total DNA isolated from R166 (obtained from the École Nationale Vétérinaire de Maisons-Alfort) and was sequenced using Sanger methodology. The above mentioned primer pairs were reverse complemented, resulting in primers pair DcMitoUni-INV-1F (5'-CTTAGTTT-TTTAACTAAATGTTGTGCGCAGAAGTG-3') and DcMitoUni-INV-1R (5'-CAATTATCTTTGCTAAACCCATTCAAACACGCC-3'). These primers were used at 400 nM final concentration to amplify the remainder of the mt DNA genome using LongAmp® Taq DNA Polymerase (NEB) using 50 ng total DNA as template. Thermal cycling entailed 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 59°C for 30 sec, 65°C for 12 min. The thermal cycling was concluded with a final extension of 10 min at 65°C. Purified PCR product of approx. 12500 bp was submitted to Inqaba Biotec (South Africa) for next generation sequencing making use of Illumina’s Miseq instrument and 2×250 bp reads using the MiSeq Reagent Kit v2. Primers F_1DNA-F (TCTCTGAGTTTTGCTGCTGTTT) and F_1DNA-R (AACGAGCA-CATAGCTTACCTT) were used to amplify 1126 bp, including the DcMitoUni-INV-1F and DcMitoUni-INV-1R primer binding sites and the PCR product was subjected to Sanger sequencing.

The 12S rDNA sequence from the newly generated *D. caninum* feline genotype mt sequence was compared to *D. caninum* 12S rDNA sequences recently deposited and analyzed by Low et al. [13] using the Mr Bayes (HKY85 substitution model; 1 000 000 chain length and 25% burn-in length) and Schistocephalus solidus as the outgroup.

The 12S rDNA sequence from the newly generated *D. caninum* feline genotype mt sequence was compared to *D. caninum* 12S rDNA sequences recently deposited and analyzed by Low et al. [13] using the Mr Bayes (HKY85 substitution model; 1 000 000 chain length and 25% burn-in length) and Schistocephalus solidus as the outgroup.
**Results**

**Sequencing of the 28S and 18S ribosomal DNA regions.**

DNA sequence analysis of a 655 bp region of the 28S ribosomal DNA region used in the *D. caninum* detection PCR (see Table 4) resulted in the identification of two unique groups when these DNA sequences were compared to the GenBank reference sequence (Table 5). One group exhibited 99.5% DNA sequence identity towards the published reference sequence (AF023120) and 100% DNA sequence identity towards the canine derived *D. caninum* isolated at Clinvet (MH040832), whereas the other group (i.e. “feline genotype”) exhibited a 93.5% sequence identity towards the published reference sequence. As described below in detail, the “100% identity group” DNA isolates were almost all of dog and dog flea origin, whereas the “93.5% identity group” DNA extracts almost all came from cats and fleas collected on cats.

**Table 4.** Percentage DNA sequence identity obtained from approximately 650 bp PCR product in *D. caninum* positive samples.

| Sample ID     | Source | Geographic origin | Host | Sequence target | GenBank accession number |
|---------------|--------|-------------------|------|----------------|--------------------------|
| CVML12_008_023| *C. felis* | Slovenia          | Cat  | 650 bp 28S rDNA | MH040824                |
| CVML12_008_034| *C. felis* | Slovenia          | Cat  | 650 bp 28S rDNA | MH040825                |
| CVML12_008_041| *C. felis* | Slovenia          | Cat  | 650 bp 28S rDNA | MH040826                |
| CVML12_008_118| *C. felis* | France            | Cat  | 650 bp 28S rDNA | MH040827                |
| CVML12_008_193| *C. felis* | France            | Dog  | 650 bp 28S rDNA | MH040828                |
| CVML12_008_265| *C. felis* | Hungary           | Cat  | 650 bp 28S rDNA | MH040829                |
| CVML12_008_277| *C. felis* | Germany           | Cat  | 650 bp 28S rDNA | MH040830                |
| CVML12_008_279| *C. felis* | Portugal          | Cat  | 650 bp 28S rDNA | MH040831                |
| CVML12_008_CV_dog| *C. felis* | Worm South Africa | Dog  | 650 bp 28S rDNA | MH040832                |
| CVML12_008_006| *C. felis* | Czech Republic    | Cat  | 650 bp 28S rDNA | MH040833                |
| CVML12_008_198| *C. felis* | France            | Dog  | 650 bp 28S rDNA | MH040834                |
| CVML12_008_231| *C. felis* | France            | Dog  | 650 bp 28S rDNA | MH040835                |
| CVML12_008_364| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040836                |
| CVML12_008_365| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040837                |
| CVML12_008_642| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040838                |
| CVML12_008_366| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040839                |
| CVML12_008_397| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040840                |
| CVML12_008_474| *C. felis* | France            | Dog  | 650 bp 28S rDNA | MH040841                |
| CVML12_008_523| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040842                |
| CVML12_008_530| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040843                |
| CVML12_008_601| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040844                |
| CVML12_008_373| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040845                |
| CVML12_008_539| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040846                |
| CVML12_008_550| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040847                |
| CVML12_008_598| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040848                |
| CVML12_008_603| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040849                |
| CVML12_008_632| *C. felis* | Sicily            | Dog  | 650 bp 28S rDNA | MH040850                |
| CVML12_008_246| *C. felis* | France            | Dog  | 650 bp 28S rDNA | MH040851                |
| CVML12_008_105| *C. felis* | France            | Cat  | 650 bp 28S rDNA | MH040852                |
| CVML13_004_043| *C. felis* | USA               | Cat  | 650 bp 28S rDNA | MH040853                |
| CVML13_004_044| *C. felis* | USA               | Cat  | 650 bp 28S rDNA | MH040854                |
| CVML13_004_045| *C. felis* | USA               | Cat  | 650 bp 28S rDNA | MH040855                |
| CVML13_004_046| *C. felis* | USA               | Cat  | 650 bp 28S rDNA | MH040856                |
No intra-group differences in DNA sequencing could be observed for any of the samples analyzed using the 655 bp PCR product. Sequence submission to GenBank was under accession numbers indicated in Table 5.

The new “93.5% identity group” corresponds to the feline associated D. caninum samples from the USA, New Zealand, Europe, China and South Africa [with no intrinsic variation related to geographical origin (see Discriminant RFLP analysis below)]. For the clarity of the analysis, we propose to name it “D. caninum feline genotype”.

The “100% identity group” exhibited no intrinsic variation related to geographical origin between all samples from Europe and South Africa. It corresponds to the DNA extracts from infected dogs or fleas collected on dogs (see discriminant RFLP analysis below); therefore we propose to name it “D. caninum canine genotype”.

Amplification and sequence analysis of approx. 2.4 kb 28S rDNA region were performed for two of the “D. caninum feline genotype” representatives and no sequence difference was detected with the 3 ambiguous nucleotides
identical between two feline genotypes. The *D. caninum* canine genotype yielded a sequence with 28 ambiguous nucleotides detected. In spite of the ambiguous nucleotides, a non-ambiguous DNA sequence comparison resulted in differences of 2% (with a 3.1% difference when ambiguous nucleotides were included) between the “*D. caninum* canine genotypes” (MH045482; MH045483) and the “*D. caninum* canine genotype” (MH045484) for the 2437 bp compared, with a 12 bp and a 4 bp insertion/deletion event detected.

Amplification of the 18S rDNA region resulted in the amplification of 2.4 kb DNA fragments. 18S rDNA sequence analysis performed on two “*D. caninum* canine genotype” (MG582181, MG582183) representatives revealed 2.7% differences with the canine genotype (MG582184) with 2 conserved 6 bp and one 8 bp insertions/deletions detected in the feline sequence when compared to the canine sequence (Figure 1). Pairwise sequence comparison also indicate that the *D. caninum* canine and feline genotypes share sequence identity to a level that can be observed for different species of cestodes when comparing 18S rDNA sequences (Table 6). Phylogenetic analysis based on the 18S rDNA sequences were conducted using sample sets from previous studies [14] and analysis of the sequence identity and patristic distance between the feline genotype, the canine genotype and other cestodes indicated that the sequence identity and patristic distance between the *D. caninum* canine genotypes and the *D. caninum* canine genotype was larger than the patristic distance observed between *Taenia serialis* and *Taenia modocae; Taenia saginata* and *Taenia asiatica; Taenia martis* and *Taenia tawschelli* as well as between *Echinococcus canadensis* and *Echinococcus ortleppi* (Table 6). This suggests that the two *D. caninum* genotypes are genetically (based on the 18S rDNA sequence data) more distant from a common ancestor than the different pairs of cestode species mentioned above.

**RFLP and hydrolysis probe genotyping assays.**

Sequence data from 28S rDNA region targeted for the PCR revealed the presence of conserved nucleotide differences when comparing the two genotypes. These conserved differences allowed the design of an RFLP assay using the restriction enzyme *SstI* to generate a product that would allow direct discrimination between the two groups. The *SstI* recognition occurs twice in the feline genotypes and will result in the fragmentation of the 653 bp feline PCR product into 404 bp, 223 bp and 26 bp, and occurs only once in the reference canine genotype, yielding 629 bp and 26 bp fragments from the 655 bp PCR canine product, thereby allowing a clear discrimination between the two identified groups. RFLP analysis was also performed on the 57 sequence verified samples to validate the RFLP assay for accuracy. All results were in concordance.

**Mitochondrial DNA amplification of sequence analysis.**

Total DNA extract from adult *D. caninum* R166 isolate (representing the feline genotype based on 18S and 28S sequence analysis and genotyping) was used to amplify the complete mt genome in two fragments. The partial feline genome 842 bp fragment revealed a 99% identity towards KF202097, the partial sequence obtained from the spotted hyena [7], and only an 89% identity towards the completed *D. caninum* mt genome from the reference group, i.e. NC_021145; “canine genotype”.

Identification and isolation of a *Dipylidium* canine genotype strain at ClinVet International

In order to collect a local canine genotype of *D. caninum* and to assess the possibility of using the PCR technique on anal swabs, 38 flea-infested dogs living in the village near ClinVet were assessed through a veterinary consultation (Table 3). Twelve dogs expelling *Dipylidium* proglottids were placed in kennels at ClinVet. Anal swabbing was conducted on all of these dogs and the proglottids were genotyped to confirm the canine genotype, and were then pooled to start breeding the canine genotype colony at ClinVet by passing through fleas and dogs.
The remainder of the mt genome was amplified by simply reverse complementing both primers used to amplify the 842 bp fragment. The approx. 13 kb fragment was sequenced using Illumina chemistry resulting in 215 263 reads with a mean read length of 196 bp resulting in >3000X coverage of the expected mt genome. Mapping the reads to the *D. caninum* mt genome (AB732959) resulted in assembly of the complete mt genome from *D. caninum* R166 (representing the “cat genotype”) with an mt genome size of 13,598 bp (Figure 2; GenBank accession number: MG587892). Nucleotide analysis revealed an overall base composition of 22.3% A, 8.8% C, 19.7% G and 49.1% T, resulting in a low GC content of 28.5%.

Direct DNA comparison of the complete mtDNA genomes of *D. caninum* R166 (“feline genotype” MG587892) and the reference *D. caninum* mtDNA genome (AB732959 and NC_021145; “canine genotype”) indicate only a 78.7% identity on the DNA level. The DNA sequences of AB732959 and NC_021145 are identical, but they are annotated differently. Analyses of the CDS, rRNA and tRNA regions (36 in total) with the specific associated identities between the two mt genomes are represented in Table 7. The ATP8 coding gene, present in mammalian mt genomes, could not be observed in the *D. caninum* mt genome, which is in agreement with published tapeworm mt genomes. No STOP codon could be detected for the COX1 protein-coding region from MG587892 and differently annotated COX1 encoding sequences are reported for AB732959 and NC_021145. Both COX1 annotations for the “canine genotype” mt DNA are under review from GenBank (GenBank email communication).

**Figure 2.** Graphical representation of the complete mitochondrial genome of *D. caninum* R166 (MG587892) including the organization and direction of 36 genes within the mitochondrial genome.

| Organisms compared | Accession numbers                  | Pairwise DNA sequence identity | Patristic distance |
|--------------------|-----------------------------------|--------------------------------|-------------------|
| *D. caninum* feline genotype: *D. caninum* canine genotype | MG582181: MG582184 | 97.3                     | 0.024             |
| *T. serialis*: *T. modoquae* | AB731620: AB731623 | 97.6                     | 0.021             |
| *T. saginata*: *T. asiatica* | AB731616: AB731617 | 97.8                     | 0.011             |
| *T. martis*: *T. twichelli* | AB731625: AB731626 | 96.6                     | 0.021             |
| *E. canadensis*: *E. ortleppi* | AB731642: AB731641 | 99.0                     | 0.013             |

**Table 6.** 18S rDNA sequence identity and patristic distance observed between the *D. caninum* feline and canine genotypes and different cestodes.
Mitochondrial phylogenetic analysis.

Mitochondrial genomes used by Guo [9], including any updated and additional genomic DNA sequences, were downloaded from GenBank and used in all subsequent analyses. Concatenation of the 12 protein-coding genes from *D. caninum* feline genotype (R166 adult tapeworm isolate), canine genotype, and those of 52 other tapeworms were subjected to multiple alignment followed by maximum likelihood and Bayesian inference analysis (average standard deviation of split frequencies was below 0.005) using *Schistosoma japonicum* as the outgroup. Both analysis methods exhibited the same topology and confidence and the tree obtained from the Bayesian inference is shown in Figure 3.

Protein identity analysis (based on the concatenated mt protein sequences) between the “canine genotype” and the “feline genotype” of *D. caninum* mt genomes revealed only an 81.8% identity between the two genotypes, which is 17.2% lower than the average protein identity calculated for the other tapeworm genotypes available from GenBank. The patristic distance of 0.33 between the two genotypes is more than 7-fold the average patristic distance of the other sequence genotypes used in the

Table 7. Results following analysis of the CDS, rRNA and tRNA regions (36 in total) with the specific associated identities between the two mitochondrial genomes.

| Name     | Type     | Start (codon) | Stop (codon) | Length | DNA Identity |
|----------|----------|---------------|--------------|--------|--------------|
| atp6     | CDS      | 3658 (ATG)    | 4173 (TAA)   | 516    | 76.94%       |
| Cob      | CDS      | 899 (ATG)     | 1993 (TAA)   | 1095   | 81.70%       |
| cox1     | CDS      | 6926 (ATG)    | > 8527 (†)   | > 1602 | 88.30%       |
| cox2     | CDS      | 10357 (ATG)   | 19932 (TAA)  | 576    | 88.19%       |
| cox3     | CDS      | 182 (ATG)     | 828 (TAA)    | 657    | 80.53%       |
| nad1     | CDS      | 5267 (ATG)    | 6160 (TAA)   | 894    | 84.79%       |
| nad2     | CDS      | 4183 (ATG)    | 5054 (TAA)   | 872    | 84.52%       |
| nad3     | CDS      | 6445 (ATG)    | 6789 (TAA)   | 345    | 81.40%       |
| nad4     | CDS      | 2220 (ATG)    | 3467 (TAA)   | 1248   | 78.30%       |
| nad4L    | CDS      | 1993 (ATG)    | 2253 (TAA)   | 261    | 84.29%       |
| nad5     | CDS      | 12921 (ATG)   | 15586 (TAA)  | 1566   | 76.40%       |
| nad6     | CDS      | 11010 (ATG)   | 11465 (TAA)  | 456    | 79.17%       |
| rrnL     | rRNA     | 8563          | 9559         | 997    | 87.80%       |
| rrnS     | rRNA     | 9702          | 10343        | 642    | 87.30%       |
| trnA     | tRNA     | 5123          | 5193         | 71     | 81.69%       |
| trnC     | tRNA     | 9560          | 9623         | 64     | 75.00%       |
| trnD     | tRNA     | 5200          | 5265         | 66     | 87.88%       |
| trnE     | tRNA     | 10938         | 11004        | 67     | 75.00%       |
| trnF     | tRNA     | 3529          | 3589         | 61     | 86.15%       |
| trnG     | tRNA     | 117           | 179          | 63     | 87.69%       |
| trnH     | tRNA     | 829           | 894          | 66     | 89.71%       |
| trnL     | tRNA     | 6300          | 6362         | 63     | 92.19%       |
| trnK     | tRNA     | 6376          | 6440         | 65     | 84.62%       |
| trnL (CUN) | tRNA    | 11804         | 11865        | 62     | 84.62%       |
| trnL (UUR) | tRNA    | 11874         | 11937        | 64     | 83.08%       |
| trnM     | tRNA     | 3586          | 3653         | 68     | 90.00%       |
| trnN     | tRNA     | 6169          | 6232         | 64     | 91.04%       |
| trnP     | tRNA     | 6239          | 6300         | 62     | 89.23%       |
| trnQ     | tRNA     | 3470          | 3538         | 69     | 93.65%       |
| trnR     | tRNA     | 11956         | 12015        | 60     | 80.00%       |
| trnS (AGN) | tRNA   | 6798          | 6856         | 59     | 81.67%       |
| trnS (UCN) | tRNA   | 11740         | 11799        | 60     | 90.32%       |
| trnT     | tRNA     | 8528          | 8589         | 62     | 87.30%       |
| trnV     | tRNA     | 5055          | 5117         | 63     | 92.42%       |
| trnW     | tRNA     | 6860          | 6920         | 61     | 87.30%       |
| trnY     | tRNA     | 11473         | 11538        | 66     | 89.39%       |

* stop codon not determined.
The molecular characterization of *D. caninum* isolates from dogs, cats, and in infected fleas collected either from dogs or cats allowed the identification of two distinct genotypes that clearly differ from each other.

East et al., 2013, collected *D. caninum* proglottids from six spotted hyena [7]. Initial PCR amplification and sequencing of the 314 bp fragment indicated identical sequence data for the partial 12S mt rDNA region from all six proglottids. Comparison of 314 bp sequence data with two published *D. caninum* sequences revealed a high (99%) similarity to one sequence from Europe (accession number L49460.1) but a considerably lower similarity (89%) to one sequence from Asia (accession number AB031362.1). They selected one of the six samples and PCR amplified and sequenced 1176 bp of the 12S mt rDNA (accession number KF202097). Comparison of this sequence to a similar fragment from *D. caninum*, again revealed a relative low similarity (89%). Pairwise sequence comparison between the sequences of East et al., 2013 and our complete mt sequence of the *D. caninum* feline genotype (MG567892), revealed a 99.1% identity between the *D. caninum* isolated from the hyena (KF202097) and the *D. caninum* feline genotype (MG567892) isolated from a cat. When comparing these sequences to the mt genome of the *D. caninum* dog genotype, there is only an 88.5% and an 88.8% identity, respectively. This confirms that the *Dipylidium* isolate from hyena belongs to the "feline genotype".

More recently, Low et al., 2017, collected ectoparasites on dogs and cats in Malaysia [13]. In this study, *C. felis* (92 specimens) and *Felicola subrostratus* (30 specimens) were collected from 20 cats, whereas *C. orientis* (26 specimens) and *Rhipicephalus sanguineus sensu lato* (120 specimens) were collected from 29 dogs. PCR utilizing the primers we published in 2014 [3] was performed to amplify the partial 28S rDNA gene region of *D. caninum*. They found 2% of cat fleas and 10% of cat lice infected by *D. caninum*. They indicated that the representative 28S rRNA sequence isolated from their flea and louse specimens (accession no. KY751956) demonstrated 95% sequence similarity with that of *D. caninum* (accession no. AF023120), and they suggested the existence of a second distinct species from the one available in GenBank. This 5% divergence of the approx. 650 bp region of the 28S rDNA is consistent with data reported in this study. PCR amplification and sequencing of the partial 12S rDNA gene region indicated that the 12S rDNA sequences (accession no. KY751955) were clustered together with those adult specimens isolated from red fox (accession no. L49460) and spotted hyena (accession no. KF202097). They found a high level of genetic distance (9.59%) and concluded on the
being infested by the same fleas (i.e. C. felis), may explain the infection of cats and dogs by both genotypes, but the different observed prevalences suggest biological adaptation. On the other hand, in C. canis and P. irritans fleas, being more specific to dogs, 100% of the infected fleas were found to harbour the canine genotype of D. caninum (Table 1).

A comparison of biological development and host preference should confirm the genetic observations (Beugnet et al., 2018, [4]). The genetic differences observed in this analysis, which show a greater distance to what is known between different species of Taenia or Echinococcus, make it possible to suggest the existence of two distinct Dipylidium species, which will have to be confirmed or disproved.

**Acknowledgements.** The authors would like to thank the research personnel from both Clinomics and Clinvet International (Pty) Ltd for their assistance in conduct of the research. The study was funded by Merial SAS, France. There were no conflicts of interest.

Special thanks to Prof. M. Lappin, North Carolina State University, USA; Prof. Piyanan Taweethavonsawat, Chulalongkorn University, Bangkok, Thailand; Prof. Elias Papadopoulos, Thessaloniki Veterinary Faculty, Greece; Prof Smaro Sotiraki, Hellenic Agricultural Organisation, Greece; and Dr Maureen Forsyth, Auckland, New Zealand, for the collection and shipment of fleas and Dipylidium sp. tapeworms.

**Conflicts of interest**

The authors declare that they have no conflicts of interest in relation to this article.

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Cite this article as: Labuschagne M, Beugnet F, Rehbein S, Guillot J, Fourie J, Crafford D. 2018. Analysis of Dipylidium caninum tapeworms from dogs and cats, or their respective fleas. Parasite 25, 3017

Parasite (open-access) continues Parasite (print and online editions, 1994-2012) and Annales de Parasitologie Humaine et Comparée (1923-1993) and is the official journal of the Société Française de Parasitologie.

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