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Original Article

Molecular identity of cat fleas (*Ctenocephalides felis*) from cats in Georgia, USA carrying *Bartonella clarridgeiae*, *Bartonella henselae* and *Rickettsia* sp. RF2125

Štěpán Šlapeta, Jan Šlapeta *

School of Life and Environmental Sciences, Faculty of Veterinary Science, McMaster Building B14, The University of Sydney, New South Wales 2006, Australia

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ABSTRACT

The cat flea (*Ctenocephalides felis*) is the most common ectoparasite of dogs and cats. Close association of humans with cats and dogs enables flea-borne disease transmission either directly, via flea bites, or indirectly, via pathogens excreted in flea faeces. The aim of this study was to evaluate molecular identity of *C. felis* from cats in Georgia, USA based on a molecular barcode marker gene (*cox1*) and the frequency at which the fleas were carriers of the vector-borne bacteria, *Bartonella* and *Rickettsia*. The multiplexed *Bartonella* and *Rickettsia* real-time PCR assay (targeting *ssrA* and *gltA*, respectively) adopted in this study, together with sequencing of the *ssrA* enabled rapid identification of *Bartonella* spp. in cat fleas. Eighteen out of 20 fleas examined were positive for *Bartonella* spp. and all fleas were positive for *Rickettsia* spp. DNA sequencing of the *ssrA* confirmed presence *B. clarridgeiae* and *B. henselae*. Amplification and DNA sequencing of *gltA* and *ompA* confirmed presence of *Rickettsia* sp. RF2125 (*Rickettsia felis*-like organism). All fleas from 21 cats in Georgia, USA were morphologically identical with *C. felis*. Sequencing of the flea *cox1* revealed identity with *C. felis* from Seychelles, Africa recognised as a subspecies *C. felis strongylus*, the African cat flea. Analysis of the *cox1* sequences of fleas improves understanding of global distribution of cat flea *cox1* clades (*C. felis*) when compared with sequences from *Ctenocephalides* spp. from Asia, Africa, Europe, Asia and Australia. Coupling flea barcoding approach with the multiplexed real-time PCR assay followed by *Bartonella* sequencing represents a rational approach for screening and elucidation of fleas' capacity to vector infectious agents.

1. Introduction

The cat flea (*Ctenocephalides felis*) is the most common ectoparasite of dogs and cats in tropical, subtropical, as well as temperate climates. The cat flea is a generalist parasite and potentially feeds on any mammal, including humans particularly when exposed to highly infested environments (Dryden and Rust, 1994; Rust, 2005, 2016). Most commonly, however, cat fleas are found on domestic and feral or community cats and dogs. Close association of humans with cats and dogs enables flea borne disease transmission either directly, via flea bites, or indirectly, via pathogens excreted in flea faeces (Boulouis et al., 2005; Chomel et al., 2006).

Recently, molecular tools were applied to characterise cat fleas and closely related species in the genus *Ctenocephalides* (Vobis et al., 2004; Šlapeta et al., 2011; Marrugal et al., 2013; Lawrence et al., 2014; Hii et al., 2015; Lawrence et al., 2015). A previously assumed subspecies *C. felis orientis*, Oriental cat flea, was confirmed to represent a full species *Ctenocephalides orientis* which is more closely related to the dog flea, *Ctenocephalides canis* than to *C. felis* (Hopkins and Rothschild, 1953; Menier and Beaucournu, 1998; Lawrence et al., 2015). The use of cytochrome c oxidase subunit I (*cox1*) marker to 'barcode' fleas has demonstrated that the cat flea is not as homogeneous as previously thought (Vobis et al., 2004; Marrugal et al., 2013), consisting of a number of defined clades whose global distribution is yet to be fully understood (Lawrence et al., 2014; Hii et al., 2015; Lawrence et al., 2015). The capacity of different *Ctenocephalides* species, subspecies or even *cox1* clades to vector infectious agents has been suggested on a small scale sample and requires further scrutiny (Hii et al., 2015).

The aim of this study was to evaluate the molecular identity of *Ctenocephalides felis* from cats in Georgia, USA based on a molecular barcoding of *cox1* marker gene sequence and the frequency at which the fleas were carriers of the vector-borne bacteria, *Bartonella* and *Rickettsia*. The multiplexed *Bartonella* and *Rickettsia* real-time PCR assay adopted in this study, together with sequencing of the *ssrA*, a gene that codes transfer-mRNA (tmRNA), enables rapid identification of *Bartonella* spp. in cat fleas. Analysis of the *cox1* sequences of fleas improved our understanding of global distribution of cat flea clades (*C. felis*) when compared with sequences from *Ctenocephalides* spp. from Asia, Africa, Europe, Asia and Australia.

* Corresponding author: McMaster Building B14, Faculty of Veterinary Science, The University of Sydney, New South Wales 2006, Australia.
E-mail address: jan.slapeta@sydney.edu.au (J. Šlapeta).

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2. Materials and methods

2.1. Flea collection, identification and extraction of the total DNA

Fleas were collected from cats in Georgia, USA (Fig. 1) between October and November 2015. Fleas (n = 74) were collected opportunistically from 20 cats during visits to a desexing veterinary clinic (neutered cats in a trap-neuter-return program), and one cat directly from a household (for details on locality see Table 1). Fleas were collected using flea comb and stored in 80% ethanol. Fleas were morphologically identified based on published keys (Hopkins and Rothschild, 1953). Total DNA was extracted whilst retaining flea exoskeletons using Isolate II Genomic DNA kit (BioLine, Australia) as previously described (Whiting et al., 2008; Lawrence et al., 2014). DNA was eluted into 100 µl of Tris buffer (pH = 8.5) and stored at −20 °C. Previously isolated DNA from *Ctenocephalides felis strongylus* (n = 5) collected on dogs from Seychelles was included in this study (Lawrence et al., 2014).

2.2. Amplification of the mitochondrial encoded cytochrome c oxidase subunit I and flea phylogeny

A 5′ fragment of the cytochrome c oxidase subunit I (*cox1*) coding for COX1 protein was PCR amplified using primers: LCO1490, 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and Cff-R (S0368) 5′-GAA GGG TCA AAG AAT GAT GT-3′ (Lawrence et al., 2014). Reactions of 30 µl contained MyTaq Red Mix (BioLine, Australia) with approximately 10–50 ng of genomic DNA template (2 µl) in a Veriti Thermal Cycler (Life Sciences, Australia). The cycling conditions were as follows: denaturing at 95 °C for 1 min followed by 35 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 10 s, and a final elongation for 5 min at 72 °C. Sterile PCR-grade water was used as negative control, and a positive control consisting of flea DNA known to amplify at these conditions was also run alongside. PCR products that yielded an unambiguous single band product (~600 bp) were directly and bidirectionally sequenced at Macrogen Inc. (Seoul, Korea). All sequences were assembled, aligned with related sequences and analysed using CLC Main Workbench 6.9.1 (CLC bio, Denmark) and deposited in GenBank (National Center for Biotechnology Information, NCBI) under the Accession Numbers: KX021755-KX021774.

Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Multiple sequence alignment was constructed with all available *Ctenocephalides* sequences belonging to defined clades (Lawrence et al., 2014; Hii et al., 2015; Lawrence et al., 2015). Sequence divergences were calculated using the Kimura 2 parameter (K2P) distance model, and phylogenetic tree was inferred using minimum evolution and the bootstrap support inferred from 500 replicates.

2.3. Diagnostic Rickettsia and Bartonella real-time PCR

A diagnostic TaqMan probe real-time PCR assay targeting the *glt*A (citrate synthase) gene of *Rickettsia* spp. was applied for the detection of *Rickettsia* (Stenos et al., 2005). A diagnostic TaqMan probe real-time PCR assay targeting *ssrA* gene was applied to specifically detect *Bartonella* (Diaz et al., 2012). The assay was multiplexed using *Rickettsia* oligonucleotide probe labelled with HEX and *Bartonella* oligonucleotide probe labelled with FAM. *Bartonella* assay used *ssrA*-F (S0508), 5′-GCT ATG GTA ATA AAT GGA CAA TGA AAT AA-3′, *ssrA*-R (S0509), 5′-GCT TCT GTT GCC AGG TG-3′ and a probe S0510, 5′-FAM-ACC CCG CTT AAA CCT GCG ACG-3′-BHQ1 (Diaz et al., 2012). CS-F (S0576), 5′-TCG CAA ATG TTC ACG GTA CTT T-3′, CS-R (S0577) 5′-TCG TGC ATT TCT

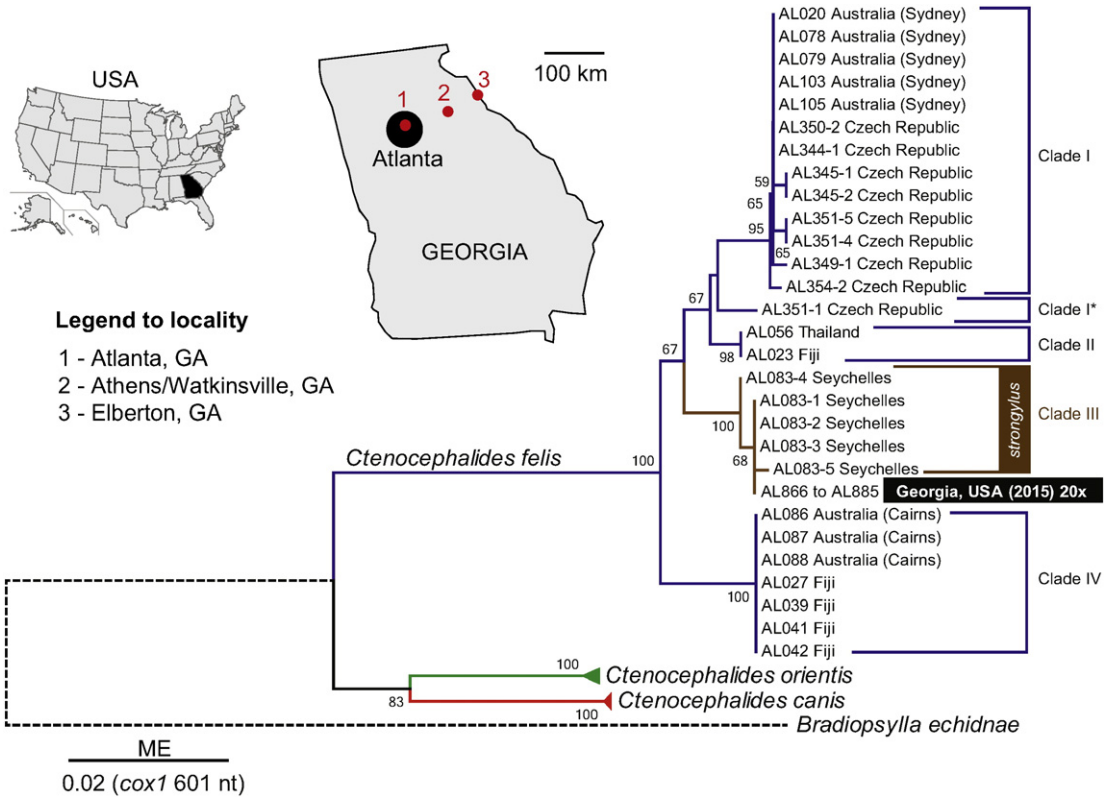

Fig. 1. Phylogenetic analysis of the genus *Ctenocephalides*. The evolutionary history of the *cox1* was inferred using the Minimum Evolution (ME) method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (>50%). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 41 nucleotide sequences of *cox1* mtDNA. All fleas (*C. felis*, n = 20) from cats in Georgia, USA (inset) has identical *cox1* and they are represented by a single sequence. There were a total of 601 nucleotide positions in the final alignment. Evolutionary analyses were conducted in MEGA6. *Bradiopsylla echidnae* served as an outgroup. Clade I-IV according to Lawrence et al. (2014) and Clade I* was identified in Lawrence et al. (2015).