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## Molecular biological investigations of Brazilian *Tunga* sp. isolates from man, dogs, cats, pigs and rats

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**Abstract** Twenty-four isolates of *Tunga* were collected from afflicted humans, dogs, cats, pigs and rats in Brazil. To investigate genetic diversity, a hypervariable section of mitochondrial 16S rDNA was amplified using PCR and subsequently sequenced. In order to compare results with another species of the genus *Tunga*, three isolates of the recently described *Tunga trimamillata* were also checked. Whereas eleven isolates (five from cats, three from dogs and three from humans) were of identical sequence, thirteen isolates collected from dogs, humans, pigs and rats showed differences in sequence up to 49%, so that the existence of one or more new species of *Tunga* may be presumed.

### Introduction

*Tunga penetrans* is a hematophagous insect parasitizing man and various animals. Pregnant females of *T. penetrans* penetrate the skin of its host, leading to the clinical picture of tungiasis, whereas males and non-fertilized

females live as ectoparasites without penetrating permanently. The flea is of about 1 mm in length (Hopkins and Rothschild 1953) and is not only parasitizing man, but also uses a wide range of domestic animals as reservoirs (Heukelbach et al. 2004). Once penetrated, the flea is looking for nourishment by puncturing the blood vessels. Its tail is oriented towards the surface, keeping contact to the outside with a sore, which provides air for breathing and a passage for both excretion and eggs. The sore is an entry point for pathogenic microorganisms (Feldmeier et al. 2002) and is also associated with the risk of tetanus complications (Greco et al. 2001; Brothers and Heckmann 1980). Usually the flea infests the area of the toes, heels and soles, but can also be found on nearly every part of the body (Heukelbach et al. 2002). Of the ten current known species of the genus *Tunga*, only two are supposed to be parasites of man (Fioravanti et al. 2003). The host spectrum of the other eight species is supposed to be restricted to a few genera of wild mammals (e.g. rodents), which are an important reservoir for the parasite (Heukelbach et al. 2004). Tungiasis is mainly a problem of populations living in poverty (Heukelbach et al. 2001). In resource-poor communities in the endemic area, severe infestation with more than a 100 parasites occurs, and high morbidity is common (Feldmeier et al. 2003). Until now, ten different species of the genus *Tunga* have been described, but only two (*T. penetrans* and *T. trimamillata*) parasitizing man. The genus and species determination is generally based on a variety of morphological criteria like shape of abdomen, presence or absence of eyes or even size of the flea. In an approach to study the genetic diversity within the genus *Tunga*, we amplified and sequenced a partial sequence of mitochondrial 16S rDNA of 24 isolates of *Tunga*. To compare results with another species of *Tunga*, we also sequenced 16S rDNA from three isolates of the recently described *T. trimamillata* (Pampiglione et al. 2002). The amplified region of rDNA contains a hypervariable segment of approximately 200 bp, in which sequence comparison is useful (Parker and Kornfield 1996).

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## Materials and methods

### Parasites

All parasite material was collected in poor urban and rural communities in the State Ceará (northeast Brazil), where tungiasis is hyperendemic (Table 1). *T. trimamillata* specimens from Ecuador were provided by Prof. Dr. Pampiglione, Prof. Dr. Trentini and colleagues (Bologna).

### Preparation of genomic DNA

Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). One flea individual was frozen in liquid nitrogen and pulverized in a steel mortar. The powder was then mixed with lysis buffer containing Proteinase K and incubated for 4 h. Genomic DNA preparation from the resulting lysate was then performed according to the protocol for animal tissue provided by the manufacturer.

### Primers

Sen-mt16S TAC ATA ACA CGA GAA GAC C  
Rev-mt16S GTG ATT GCG CTG TTA TCC

Primers were used as previously described (Parker and Kornfield 1996).

### DNA amplification

Amplification of DNA of a single flea individual was performed in 100- $\mu$ l reaction tubes in a total volume of 50  $\mu$ l using the HotStar Master Mix Kit according to the manufacturer's instructions (Qiagen). Each reaction mixture contained 0.4 mM of each primer, 2.5 U of *Taq* polymerase, 200  $\mu$ M of each dNTP and 2 mM MgCl<sub>2</sub>. Each of the 30 cycles started with denaturation for 45 s at 94°C, followed by primer annealing at 40°C for 45 s and elongation at 72°C for 45 s.

### DNA purification and sequencing

DNA fragments were purified from agarose gels using QIAquick Gel Extraction Kit according to the protocol by the manufacturer (Qiagen). DNA was eluted in 30  $\mu$ l

H<sub>2</sub>O and sequenced by SEQLAB Sequence Laboratories GmbH (Göttingen, Germany).

### Phylogenetic analysis

The multiple sequence alignment program Clustal X version 1.8 was used to obtain a nucleotide sequence alignment file (Thompson et al. 1997). The phylogenetic tree was constructed by neighbour joining method using Kimura 2 parameter distances in MEGA 2.1 software (Kumar et al. 2001). Bootstrap analysis was employed to test the reliability of the phylogenetic tree (1,000 replications).

### Light microscopy

The specimens originating from humans (see Fig. 3) were photographed by light microscopy prior to the DNA analysis.

## Results

With one exception, all *Tunga* isolates had a hyper-variable section of mitochondrial 16S rDNA of approximately 200 bp. Isolate 23 (collected from an afflicted dog) differed in size with approximately 170 bp (Fig. 1, lane 7).

The PCR products were purified from agarose gel and subsequently sequenced. Aligned sequence data is shown in Fig. 2.

Data from Fig. 2 reveals, that the sequence is congruent for all isolates in a smaller region of 6 bp from nucleotide 154–159, and in a larger region of 12 bp from nucleotide 163–174. All isolates show further congruence in seven single positions at nucleotides 116, 122, 129, 134, 137, 150 and 177. In the first 115 bp, not even one position is identical for all isolates.

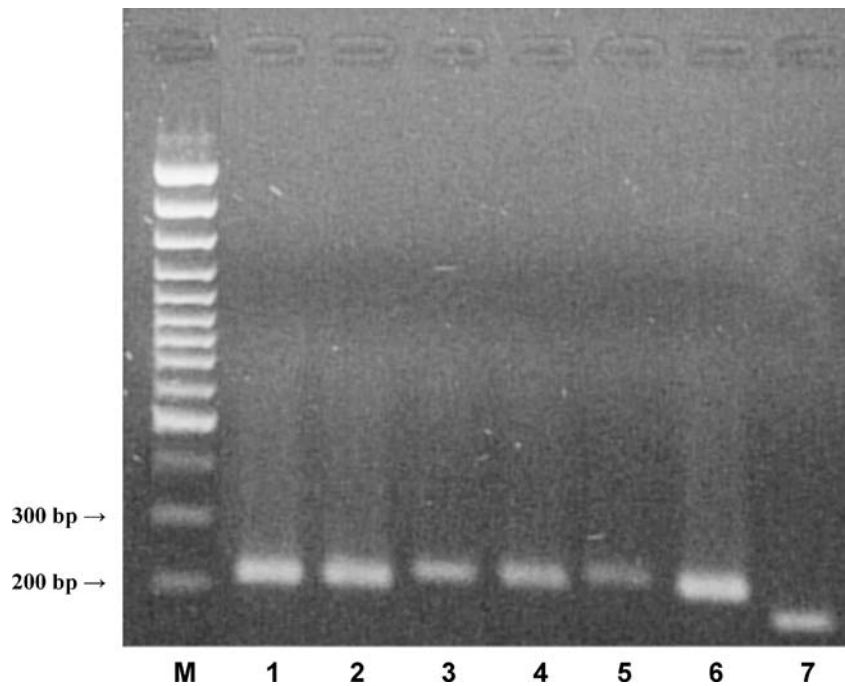
Sequences from Fig. 1 were used to generate a phylogenetic tree, based on neighbour joining method shown in Fig. 3.

When studying the light micrographs of the fleas obtained from humans, it was seen that three specimens differed from the others. In three specimens (No. 10, 11 and 12) the dorsal side of the head was more rounded (Fig. 4), while in the others the front of the head was somewhat depressed (Fig. 5) giving rise to a curved as-

**Table 1** List of isolates examined in this study

No.	Isolate
1–5	<i>Tunga</i> sp.—captured in the wild, Fortaleza, northeast Brazil. Five isolates collected from afflicted cats
6–12	<i>Tunga</i> sp.—captured in the wild, Fortaleza, northeast Brazil. Seven isolates collected from afflicted humans
13–15	<i>Tunga trimamillata</i> —captured in the wild, Catacocha, Ecuador. Three isolates collected from afflicted pigs
16–17	<i>Tunga</i> sp.—captured in the wild, Fortaleza, northeast Brazil. Two isolates collected from afflicted rats
18–19	<i>Tunga</i> sp.—captured in the wild, Cascavel, northeast Brazil. Two isolates collected from afflicted pigs
20–27	<i>Tunga</i> sp.—captured in the wild, Fortaleza, northeast Brazil. Eight isolates collected from afflicted dogs

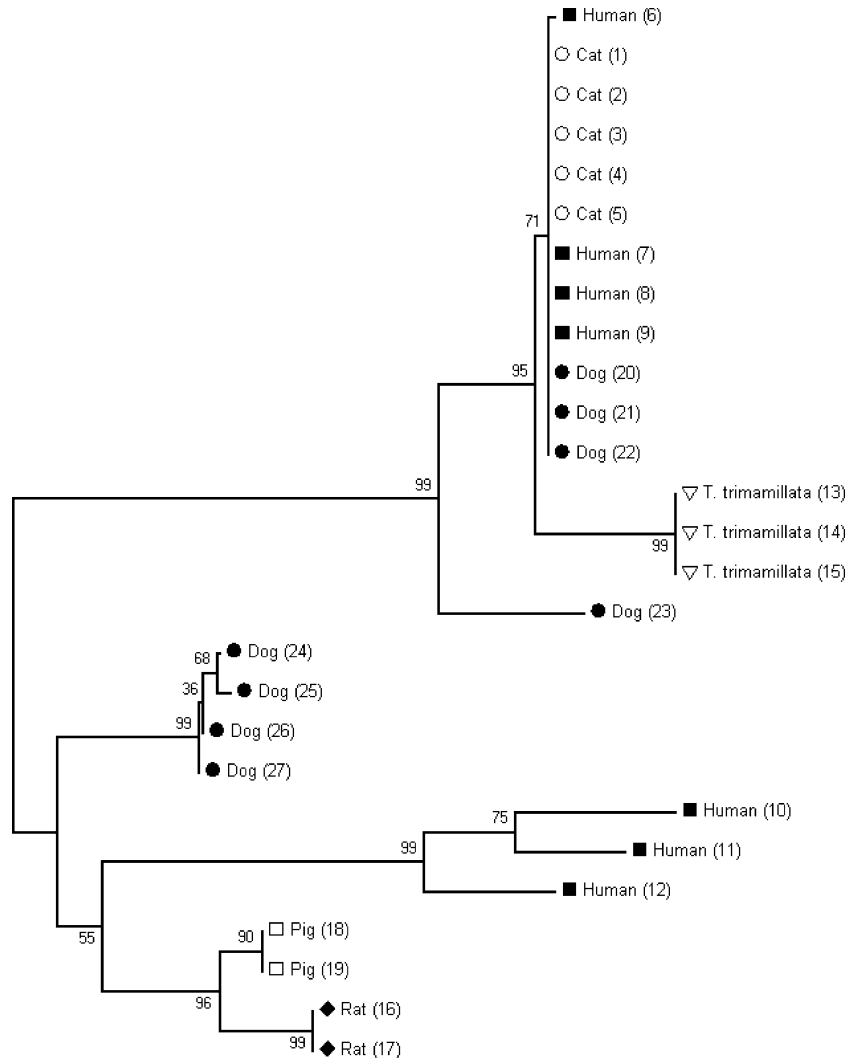
**Fig. 1** Amplification products of the hypervariable section of mitochondrial 16S-rDNA region of *Tunga* isolates. Isolates collected from the same host species showed the same size of amplification product, so that only one is shown representative. 1 isolates 1–5 (collected from cats), 2 isolates 6–12 (collected from humans), 3 isolates 13–15 (*T. trimamillata* collected from pigs), 4 isolates 16–17 (collected from rats), 5 isolates 18–19 (collected from pigs), 6 isolates 20–22 and 24–26 (collected from dogs), 7 isolate 23 (collected from dog). M 100 bp ladder (Peqlab)



**Fig. 2** Alignment of the partial sequence of the hypervariable section of the 16S rDNA of *Tunga* isolates. Sequences of *Tunga* isolates 1–5 (cats), 20–22 (dogs) and 7–9 (humans) were identical, so that only one is shown representative (named Basic). Sequences of isolates 16–17 and 18–19 were identical in sequence, so that only one is shown representative (named rats and pigs, respectively). Isolates 13–15 were identical in sequence, so that only one is shown representative (named Trima). Congruence in sequence for all isolates is marked grey, a dash indicates a deletion

Basic	TTGTAATTTATATATTTTAAAGAAATATTTA-TTAATATATAAATAAATATTTTTATT	60
Human (6)	TTGTAATTTATATATTTTAAAGAAATATTTTC-TTAATATATAAATAAATATTTTTATT	60
Trima	TTGTAATTTATATATTTTATAATAAATAAATTA-ATAATATTTATATGAAAAATTTTTATT	60
Dog (23)	-----CGGCATACGGCGG-GCGCCTCTTGGATAACAGCGCTT----	60
Human (10)	----CTATGACTTTTTAATCCGCCATTAGGGGTGTATGGGTGGAATTGACTACAGATTTT	60
Human (11)	----CTA-AACTTCCAAACCTGTT-TTTGGGGTGTCTGGT-GGGTAACTTCACTTTTT	60
Human (12)	----CTA-AACTACCAAACCTGTCATTAGAAAT-TTCGGTTGGGGCGACTCGGTATCAT	60
Dog (24)	----AAATATCTCCTTTATATGGGGTAGCAA-TTTTGGTTGGGGTACCTCGGAATATA	60
Dog (25)	----AAATATCACCTTTATATGGGGTAGCAA-TTTTGGTTGGGGTACCTCGGAATATA	60
Dog (26)	----AAATATCTCCTTTATATGGGGTAGCAA-TTTTGGTTGGGGTACCTCGGAATATA	60
Dog (27)	----AAATATCTCCTTTATATGGGGTAGCAA-TTTTGGTTGGGGTACCTCGGAATATA	60
Pigs	----AAAAATA-ACCTAACATGGACTAGCAA-TTTCGGTTGGGGTACCTCGGAGTA-C	60
Rats	--CTAAAAA-A-AATAAATATGAACTAAAAAATTTTCGGTTGGGGTACCTCGGAGAA-T	60
Basic	GGGGTGATAGGAAAATTAATAAACTTTTTTAT-TTTTAAACATAG--ATTA-ATGATT	120
Human (6)	GGGGTGATAGGAAAATTAATAAACTTTTTTAT-TTTTCAACATAG--ATTA-ATGATT	120
Trima	GGGGTGATAGGAAAATTAATAAACTTTTTTATTTTATATTTTGAACATAA--ATTA-ATGAAT	120
Dog (23)	GCCGTGATAGGAAAATTAATAAACTTTTTTAT-TTTTAAACATAG--ATTA-ATGATT	120
Human (10)	TTCTTAACCTCCGAGCATTACTGGCTAAGAGTTCCCACTGAGCGTATTACTATACCT	120
Human (11)	TTCTTTTCCATACATTACTGGTTTATTTGTTCCCACTGAGCTATTACTACTT	120
Human (12)	AACCCAACTCCGAGCAGTACATGCTAAGACTTCCACAGTCCGAGCACTACTACTCT	120
Dog (24)	AAAAAATCCTGAG--TGATTAAGATTTAGACCCAGTAG-TAAAAAT--ACTACATCACT	120
Dog (25)	AAAAAATCCTGAG--TGATTAAGATTTAGACCCAGTAGATAAAAAAT--ACAACATCACT	120
Dog (26)	AAAAAATCCTGAG--TGATTAAGATTTAGACCCAGTAG-TAAAAAT--ATTATATCATT	120
Dog (27)	AAAAAATCCTGAG--TGATTAAGATTTAGACCCAGTAG-TAAAAAT--ATTATATCATT	120
Pigs	AAAAAATCCTCCGAGTATTTTAACTTAGACAAACAGTCAAATAAACATAGCATCACT	120
Rats	AAAAAATCCTCCGAGTATTTTAACTTAGACTCACAAGTCAAAGTAACTAATATC--T	120
Basic	TATTG-ATCCA-TAAATTTGTGATTATAAGATTAAGTTACTTTAAGGGATAACAGCGCAATC	180
Human (6)	TA---ATCCA-TAAATTTGTGATTATAAGATTAAGTTACTTTAAGGGATAACAGCGCAATC	180
Trima	TATTG-ATCCA-TAAATTTATGATTAAAAGATTAAGTTACTTTAAGGGATAACAGCGTAATA	180
Dog (23)	TATTG-ATCCA-TAAATTTGTGATTATAAGATTAAGTTACTTTAAGGGATAACAGCGCAATC	180
Human (10)	AATTG-ATCGGATAACTTGCGCCAACGGCAACTAGTTACCTAGGGATAACAGCGCAATC	180
Human (11)	TACTGTATCGGATAACTTGCCCAACGGCAACAAGTTACCTAGGGATAACAGCGCAATC	180
Human (12)	AATTGTATCCAATAACTTGCACCAACGGCAACAAGTTACCTAGGGATAACAGCGCAATC	180
Dog (24)	TATTG-GTCCAATAATTTTTGATCAACGGAAACAAGTTACCTAGGGATAACAGCGCAATC	180
Dog (25)	TATTG-GTCCAATAATTTTTGATCAACGGAAACAAGTTACCTAGGGATAACAGCGCAATC	180
Dog (26)	TATTG-GTCCAATAATTTTTGATCAACGGAAACAAGTTACCTAGGGATAACAGCGCAATC	180
Dog (27)	TATTG-GTCCAATAATTTTTGATCAACGGAAACAAGTTACCTAGGGATAACAGCGCAATC	180
Pigs	TATTG-ATCCA--AAATTTTGTGATCAACGGAAACAAGTTACCTAGGGATAACAGCGCAATC	180
Rats	TATTG-ACCCA--AT-TATTGATCAACGGAAACAAGTTACCTAGGGATAACAGCGCAATC	180
Basic	ACAA	184
Human (6)	ACAA	184
Trima	TTTT	184
Dog (23)	ACAC	184
Human (10)	ACAA	184
Human (11)	ACAA	184
Human (12)	ACAA	184
Dog (24)	ACAA	184
Dog (25)	ACAA	184
Dog (26)	ACAA	184
Dog (27)	ACAA	184
Pigs	ACAA	184
Rats	ACAA	184

**Fig. 3** Phylogenetic tree of 27 *Tunga* isolates based on DNA sequence of hypervariable section of mitochondrial 16S subunit. The tree was generated using Clustal X program for sequence alignment and by neighbour joining method using Kimura's two-parameter distance (Kimura 1980) in MEGA 2.1 software. Numbers at nodes indicate percent bootstrap values of 1,000 replicates. Numbers in brackets indicate isolate number according to Table 1



**Fig. 4** Flea taken from human skin showing a rounded back side of the head

pect. However, further characteristics have to be checked in specimens treated with a KOH-solution in order to get further insights.

## Discussion

We investigated genetic diversity of *Tunga* sp. isolates—the correct species names will be given, when morphological data are also available—by sequencing a hypervariable section of 16S rDNA of 24 isolates all captured in the wild in Brazil, as well as of three isolates of the recently described *T. trimamillata* (Trentini et al. 2001; Pampiglione et al. 2002, 2003). This region was previously described as AT-rich within holometabolous insects (Whitfield and Cameron 1998). Within *T. penetrans* of humans as well as in other flea species (e.g. *Ctenocephalides felis*), a 200-bp sized hypervariable segment has already been reported (Vobis et al. 2004). Both facts were also confirmed for all isolates tested in our study. However, our study revealed a high level of variation in this hypervariable segment within isolates of



**Fig. 5** Flea originating from human skin showing a curved dorsal side of the head

*T. penetrans*, that has not even been found between different flea species like *C. felis*, *Ctenocephalides canis*, *Archeopsylla erinacei* or *Pulex irritans* (Vobis et al. 2004).

The phylogenetic tree that we present here is the first constructed on 16S rDNA sequences for the genus *Tunga*. Regardless of the hosts where the isolates were collected from, all showed two regions of major congruence, the smaller one of 6-bp size from nucleotide 154–159, and the larger one of 12-bp size from nucleotide 163–174. Apart from these regions, sequence differences within *Tunga* populations were very high, e.g. 49% between isolates collected from cats or humans compared to isolates collected from rats or pigs. Also isolates collected from dogs only showed significant differences in sequence. These values are even more remarkable, as such differences were not reached in a recent study even between different flea species, e.g. *C. felis* and *A. erinacei* (Vobis et al. 2004). It is also noteworthy, that 13 isolates collected from cats, dogs and humans in Brazil seem to be closer related to *T. trimamillata* from Ecuador, than to other isolates from *Tunga* collected also from dogs or humans in the same region of Brazil.

As the phylogenetic tree in Fig. 3 shows, the studied isolates can roughly be divided in four clades: one ranging from human isolate 6 to dog isolate 22, two others containing the remaining dogs (isolate 24–27) and human isolates (isolate 10–12), respectively, and a last one containing isolates from pigs and rats. The significant differences in sequences among the isolates were not expected in this dimension, especially in consideration of the small geographical area, where the isolates were collected. Because 13 isolates and four pairs of isolates

showed 100% congruence in sequence to each other, these differences cannot be explained by assuming, that individuals of a population are differentiated with the used primer pair Sen-mt16S and Rev-mt16S.

Until now, no detailed morphological studies have been carried out on all isolates, but at least it is mentionable, that one probable clade (human isolates 10, 11 and 12) shows differences in the shape of the head. That this finding is in addition to the presented phylogenetic tree maybe a hint for a necessary subdivision of the species *T. penetrans*. Further investigations on morphological, biological and genetic traits are needed to address this question. This should include an examination of a different gene region such as the internal transcribed spacer regions 1 and 2 (ITS1/ITS2), that have been proven useful for analysing genetic relationships among species (Baldwin et al. 1995) or also within species (Essig et al. 1999; Marcilla et al. 2002). The construction of phylogenetic trees for *T. penetrans* isolates based on ITS1 and ITS2 sequences has already been completed and results will soon be published (Vobis et al. 2005).

It should be acknowledged that the relationships of the *Tunga* populations presented here are based on a mtDNA gene tree that may not necessarily reflect the true species phylogeny. However, the maternal transmission and haploid nature of the mitochondrial genome increases the likelihood of recovering more recent population level history and is more likely to convey a gene tree that is congruent with the species tree (Moore 1995, 1997). But even if true species phylogeny may not be accurately reflected, the existence of one or more new species of *Tunga* may be presumed—or the studied specimens belong to one of the already described species such as *T. caecata*, *T. bondari* or *T. travassosi* (Barnes and Radovsky 1969; Lewis 1972).

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## References

- Baldwin B, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann Mo Bot Gard* 82:247–277
- Barnes AM, Radovsky FJ (1969) A new *Tunga* (Siphonaptera) from the nearctic region with description of all stages. *J Med Entomol* 6:19–36
- Brothers WS, Heckmann RA (1980) Tungiasis in North America. *Cutis* 25:636–638
- Essig A, Rinder H, Gothe R, Zahler M (1999) Genetic differentiation of mites of the genus *Chorioptes* (Acari: Psoroptidae). *Exp Appl Acarol* 23:309–318
- Feldmeier H, Heukelbach J, Eisele M, Sousa AQ, Barbosa LM, Carvalho CB (2002) Bacterial superinfection in human tungiasis. *Trop Med Int Health* 7:559–564

- Feldmeier H, Eisele M, Moura RCS, Heukelbach J (2003) Severe tungiasis in underprivileged communities: case series from Brazil. *Emerg Infect Dis* 9:949–955
- Fiovaranti ML, Pampiglione S, Trentini M (2003) A second species of *Tunga* (Insecta, Siphonaptera) infecting man: *Tungatrimamillata*. *Parasite* 10(3):282–283
- Greco JB, Sacramento E, Tavares-Neto J (2001) Chronic ulcers and myiasis as ports of entry for *Clostridium tetani*. *Braz J Infect Dis* 5:319–323
- Heukelbach J, Oliveira FAS, Hesse G, Feldmeier H (2001) Tungiasis: a neglected health problem of poor communities. *Trop Med Int Health* 6:267–272
- Heukelbach J, Wilcke T, Eisele M, Feldmeier H (2002) Ectopic localization of tungiasis. *Am J Trop Med Hyg* 67:214–216
- Heukelbach J, Costa AML, Wilcke T, Mencke N, Feldmeier H (2004) The animal reservoir of *Tunga penetrans* in severely affected communities of north-east Brazil. *Med Vet Entomol* 18:329–335
- Hopkins GHE, Rothschild M (1953) An illustrated catalogue of the Rothschild collection of fleas (Siphonaptera) in the British Museum (National History). *Brit Mus Nat Hist* 36–69
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 17:1244–1245
- Lewis RE (1972) Notes on the geographical distribution and host preferences in the order Siphonaptera. 1. Pulicidae. *J Med Entomol* 9:511–520
- Marcilla A, Bargues MD, Abad-Franch F, Panzera F, Carcavallo RU, Noireau F, Galvao C, Jurberg J, Miles MA, Dujardin JP, Mas-Coma S (2002) Nuclear rDNA ITS-2 sequences reveal polyphyly of *Panstrongylus* species (Hemiptera: Reduviidae: Triatominae), vectors of *Trypanosoma cruzi*. *Infect Genet Evol* 1:225–235
- Moore WS (1995) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution* 49:718–726
- Moore WS (1997) Mitochondrial-gene trees versus nuclear-gene trees, a reply to Hoelzer. *Evolution* 51:627–629
- Pampiglione S, Trentini M, Fioravanti ML, Onore G, Rivasi F (2002) A new species of *Tunga* (Insecta, Siphonaptera) in Ecuador. *Parassitologia* 44(Suppl 1):127
- Pampiglione S, Trentini M, Fioravanti ML, Onore G, Rivasi F (2003) Additional description of a new species of *Tunga* (Siphonaptera) from Ecuador. *Parasite* 10:19–15
- Parker A, Kornfield I (1996) An improved amplification and sequencing strategy for phylogenetic studies using the mitochondrial large subunit rRNA gene. *Genome* 39:793–797
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 24:4876–4882
- Trentini M, Pampiglione S, Marini M, Gianetto S (2001) Observations about specimens of *Tunga* sp. (Siphonaptera, Tungidae) extracted from goats of Ecuador. *Parassitologia* 42(Suppl 1):65
- Vobis M, D'Haese J, Mehlhorn H, Mencke N, Blagburn BL, Bond R, Denholm I, Dryden MW, Payne P, Rust MK, Schroeder I, Vaughn MB, Bledsoe B (2004) Molecular phylogeny of isolates of *Ctenocephalides felis* and related species based on analysis of ITS1, ITS2 and mitochondrial 16S rDNA sequences and random binding primers. *Parasitol Res* 94:219–226
- Vobis M, D'Haese J, Mehlhorn H, Feldmeier H, Heukelbach J, Mencke N (2005) Phylogenetic relationship of isolates of *Tunga penetrans* from Brazil based on ITS1 and ITS2 sequences. *Parasitol Res* (in press)
- Whitfield JB, Cameron SA (1998) Hierarchical analysis of variation in the mitochondrial 16S rRNA gene among Hymenoptera. *Mol Biol Evol* 15:1728–1743