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Rapid identification of non-neosomic *Tunga penetrans* and *Tunga trimamillata* (Insecta Siphonaptera) specimens through PCR-RFLP method

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Abstract

Tunga penetrans (L.) and *T. trimamillata* Pampiglione *et al*. are the unique species of the genus parasiting men. While morphological identification of gravid females is possible, the diagnosis on non-gravid females, males or body fragment is quite difficult, time consuming and often requires taxonomic experience. In this paper we present a protocol for the diagnosis of these species based on the PCR-RFLP method. We used the ribosomal intergenic spacer 2 (ITS2) region that was invariable within species but variable among the two taxa. PCR-RFLP confirmed DNA sequence data and demonstrate the reliability of this rapid and relatively inexpensive method to unambiguously discriminate *T. penetrans* and *T. trimamillata*.

Key words: *Tunga penetrans*; *T. trimamillata*; intergenic spacer 2; PCR-RFLP; molecular diagnosis.

Introduction

The genus *Tunga* Jarocki, 1938 includes ten species of sand fleas distributed around the tropical belt. Adult females burrow into the host's skin where, once fertilised, their abdomen increases up to ten-fold its original dimension (neosomy), owing to the development of up to 200 eggs. This can lead to harmful skin infection called "tungiasis". The majority of *Tunga* species are specialised parasite of a single or a few hosts, especially rodents. However, the two species *T. penetrans* (L., 1758) and *T. trimamillata* Pampiglione, Trentini, Fioravanti, Onore and Rivasi, 2002 have a wider range of possible hosts, such as wild and domestic mammals, and are the unique sand fleas that parasitise humans (Linardi and Guimarães, 2000; Fioravanti *et al*., 2003). Notwithstanding the tropical distribution of these sand fleas, a number of cases of tungiasis have been reported in Italy as well as in other temperate countries (D'Antuono *et al*., 1990; Fein *et al*., 2001): these cases are due to individuals infected during their permanence in endemic areas, or to inhabitants of these areas that imported the parasite.

Gravid *T. penetrans* and *T. trimamillata* females are easily diagnosed, the most notably difference between the two taxa being the three humps surrounding the *T. trimamillata* head and torax (Pampiglione *et al*., 2003). Furthermore, the two sand fleas are differentiated by a number of other small differences, such as the length and the shape of maxillary palps, the presence/absence of spines on the surface of the 3rd leg tibia, the shape of the abdominal spiracles, the length of claspers and aedeagus and the profile of oedegal apodeme (Pampiglione *et al*., 2004).

Even if all these morphological characters are available, the diagnosis of non-neosomic individuals (both males and females) is quite difficult and time consuming. Furthermore, the determination of body fragments is impossible.

Recently, a molecular study was performed in order to assess taxonomic status and genetic variability of *T. penetrans* and *T. trimamillata*; the analyses of both mitochondrial and nuclear markers showed a clear differentiation of specific rank (Luchetti *et al*., 2005). Notably, nuclear intergenic spacer 2 (ITS2) rDNA region is invariable in intraspecific comparisons while 24 point mutations and one indel occurs between sequences of the two species.

The restriction fragment length polymorphism (RFLP) method consists of digesting DNA, followed by the analysis of restricted fragments obtained in order to discriminate genetic entities (species, subspecies, strain, etc.). Although this method is rapid and quite inexpensive, it requires large amounts of genomic DNA. This problem can be solved by combining RFLP with the polymerase chain reaction (PCR) which allows to obtain great quantities of a specific DNA fragment, even from small samples. This PCR-RFLP assay has been used in a number of studies, including insects (f.i. Otranto *et al*., 2003; Szalanski *et al*., 2003).

In this paper, we present a PCR-RFLP analysis of the ITS2 region on Ecuadorian samples of *T. trimamillata* and *T. penetrans* in order to provide a rapid and low-cost protocol for the correct identification of these two sand fleas in human tungiasis.

Materials and methods

Free living, ground collected fleas of *T. trimamillata* and *T. penetrans* were sampled in three localities of Ecuador (table 1), and preserved in absolute alcohol.

Table 1. Sampling data for the analysed species.

Species	Locality	Acronym	No.
T. penetrans	S.ta Isabel	TpISA	10
	Pelileo	TpPEL	10
T. trimamillata	S.ta Isabel	TtISA	10
	Machala	TtMAC	10

Voucher specimens are deposited in the Insect Collection of the Department of Evolutionary and Experimental Biology, University of Bologna.

Total DNA was extracted by grinding an entire flea in 20 µl of quick extraction buffer (0.1X PCR buffer, 0.1X SDS, 15 units of proteinase K), then frozen 5 minutes in liquid nitrogen, warmed 1 hour at 65 °C and for 10 minutes at 95 °C.

PCR was performed in a 50 µl mixture using the Taq Polymerase Recombinant kit (Invitrogen), using the manufacturer protocol. Amplifications were done in a Gene Amp PCR System 2400 (Applera) thermocycler, with the following program: initial denaturation at 95 °C for 5'; 30 cycles of 30" at 95 °C, 30" at 48 °C, 30" at 72 °C; final elongation step at 72 °C for 7'.

The primers for PCR amplifications were ITSD (5'- CAC TCG GCT CGT GGA TCT AT -3') and ITSR (5'- TTT AGG GGG TAG TCT CAC CTG -3').

A restriction map was generated with BioEdit software (Hall, 1999) on previously sequenced ITS2 regions (A.N.: AY425818 - AY425820; Luchetti *et al*., 2005).

The restriction enzyme reactions were performed on at least 200 ng of amplicon with 1 µl of restriction enzyme (either *Msp*I or *Rsa*I, Promega, see below), for 2 hour at 37 °C.

To check the restriction patterns, the digested DNA was electrophoresed on a 1.5% agarose gel stained with ethidium bromide.

Results and discussion

A PCR-RFLP protocol was assessed in order to rapidly identify the two *Tunga* species parasiting men and domestic animals.

The restriction analysis through BioEdit of ITS2 sequences in both species gave a number of restriction sites with both degenerated and non-degenerated sequence. Among the latter, we choose two enzymes, *Msp*I and *Rsa*I, on the basis of the unambiguity of predicted restriction bands produced. In particular, this analysis showed that for *T. penetrans* ITS2 sequence, *Msp*I digest gave three bands of 346, 115 and 51 bp, while the *Rsa*I cleavage resulted in two bands of comparable sizes (266 and 246 base pairs; table 2).

On the contrary, *T. trimamillata* ITS2 sequence is cleaved once by *Msp*I, with the production of two bands 396 and 115 bp long, while the *Rsa*I cut produces three bands of 266, 161 and 84 base pairs (table 2).

The digests with the two restriction enzymes of the 511 (*T. trimamillata*) and 512 (*T. penetrans*) bp ITS2 amplicons gave the expected pattern.

The digest of *T. penetrans* ITS2 (figure 1) with *Msp*I produces two well defined bands (346 and 115 base pairs) and a faint band corresponding to the 51 base pairs long. The *Rsa*I digest resulted in a single band. This is composed of two fragments of 266 and 246 bp which overlap when resolved on a 1.5% agarose gel.

Table 2. Banding pattern for ITS2 of the analysed species.

Species	*M s p* I		*R s a* I	
	Cut positions	Band size	Cut positions	Band size
T. penetrans	115; 166	51; 115;346	266	246; 266
T. trimamillata	115	115; 396	266; 427	84; 161; 266

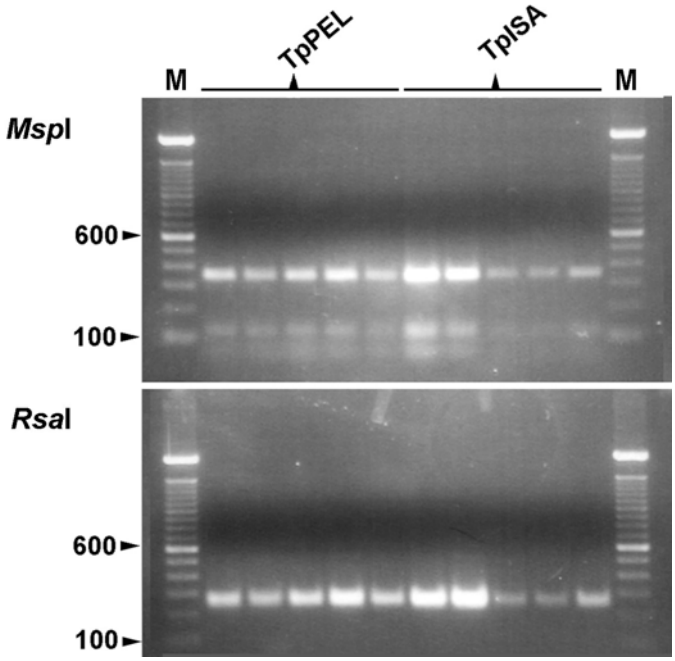

Figure 1. Agarose gel electrophoresis of PCR-RFLP on *T. penetrans* ITS2. *Msp*I digestion is shown in the upper panel, while *Rsa*I digestion is depicted in the lower panel. Acronyms as in table 1; M indicate 100 bp molecular weight marker.

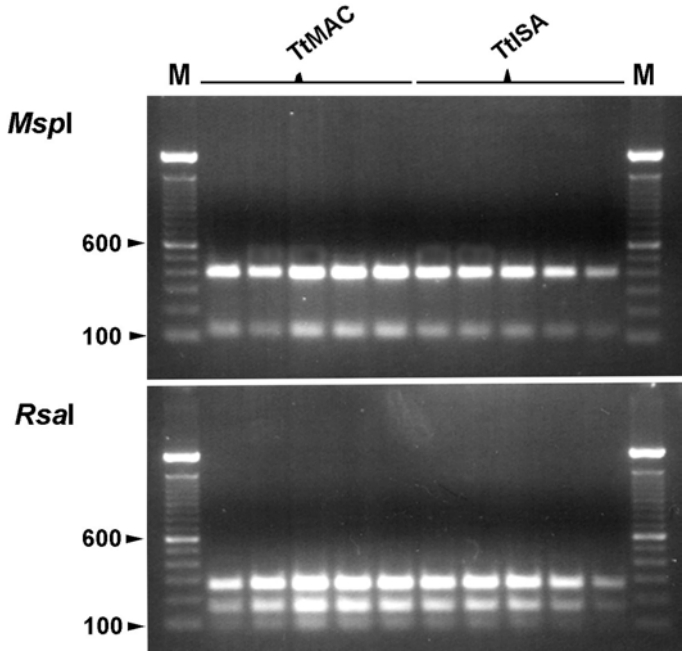

Figure 2. Agarose gel electrophoresis of PCR-RFLP on *T. trimamillata* ITS2. *Msp*I digestion is shown in the upper panel, while *Rsa*I digestion is depicted in the lower panel. Acronyms as in table 1; M indicate 100 bp molecular weight marker.

The digest of *T. trimamillata* ITS2 (figure 2) reflected the predicted RFLP pattern: the *Msp*I digest produced the predicted fragments of 396 and 115 bp. The *Rsa*I restriction results in two fragments, 266 and 161 bp, and a faint fragment of 84 bp.

The same pattern was observed for all analysed samples of each species for each restriction enzyme, indicating that this is a good method to discriminate individuals pertaining to the considered species. This also confirms the lack of intraspecific variability, as previously observed through direct sequencing of the ITS2 region (Luchetti *et al*., 2005).

Finally, it should be noted that a software analysis performed on ITS2 sequences of *T. penetrans* from Madagascar (Luchetti *et al*., 2005) gave the same results as those from Ecuador. In fact, the Malagasy ITS2 genotype differs from the South-American one for one point mutation and two insertions, but the cutting sites of *Msp*I and *Rsa*I are conserved. Unfortunately, no specimens from Madagascar were available for PCR-RFLP analysis.

The PCR-RFLP assay is widely used to rapidly and unambiguously identify species that are nearly or completely indistinguishable through a simple morphological analysis. The results here presented demonstrate the reliability of this approach to diagnose non-neosomic *T. penetrans* and *T. trimamillata* samples in 8-12 hours and it is less expensive than the sequencing. Given the use of PCR, this method can also be utilised when only small tissue fragments are available.

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References

D'ANTUONO A., GATTI M., NEGOSANTI M., PASSARINI B., PAULUZZI P., REGGIANI M., 1990.- Tungiasis. A clinical case.- *Giornale Italiano di Dermatologia e Venereologia*, 125 (6): 259-261.

FEIN H., NASEEM S., WITTE D. P., GARCIA V. F., LUCKY A., STAAT M. A., 2001.- Tungiasis in North America: a report of 2 cases in internationally adopted children.- *Journal of Pediatry*, 139 (5): 744-746.

FIORAVANTI M. L., PAMPIGLIONE S., TRENTINI M., 2003.- A second species of *Tunga* (Insecta, Siphonaptera) infecting man: *Tunga trimamillata*.- *Parasite*, 10 (3): 282-283.

HALL T. A., 1999.- BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.- *Nucleic Acids Symposium Series*, 41: 95-98.

LINARDI P. M., GUIMARÃES L. R., 2000.- *Sifonapteros do Brasil*.- FAPESP, São Paulo, Brasil.

LUCHETTI A., MANTOVANI B., PAMPIGLIONE S., TRENTINI M., 2005.- Molecular characterisation of *Tunga trimamillata* and *T. penetrans* (Insecta, Siphonaptera, Tungidae): taxonomy and genetic variability.- *Parasite*, 12 (2): in press.

OTRANTO D., TRAVERSA D., TARSITANO E., STEVENS J., 2003.- Molecular differentiation of *Hypoderma bovis* and *Hypoderma lineatum* (Diptera, Oestridae) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).- *Veterinary Parasitology*, 112 (3): 197-201.

PAMPIGLIONE S., TRENTINI M., FIORAVANTI M. L., ONORE G., RIVASI F., 2003.- Additional description of a new species of *Tunga* (Siphonaptera) from Ecuador.- *Parasite*, 10 (1): 9-15.

PAMPIGLIONE S., TRENTINI M., FIORAVANTI M. L., GUSTINELLI A., 2004.- Differential diagnosis between *Tunga penetrans* (L., 1758) and *T. trimamillata* Pampiglione *et al*., 2002 (Insecta, Siphonaptera), the two species of the genus *Tunga* parasitic in man.- *Parasite*, 11 (1): 51-57.

SZALANSKI A. L., AUSTIN J. W., OWENS C. B., 2003.- Identification of *Reticulitermes* spp. (Isoptera: Reticulitermatidae) from south central United States by PCR-RFLP.- *Journal of Economic Entomology*, 96 (5): 1514-1519.

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