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MOLECULAR CHARACTERIZATION OF *TUNGA TRIMAMILLATA* AND *T. PENETRANS* (INSECTA, SIPHONAPTERA, TUNGIDAE): TAXONOMY AND GENETIC VARIABILITY

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Summary:

A new species of the genus *Tunga*, *T. trimamillata* has recently been described on the basis of several morphological traits. To explore the taxonomic status of this flea with respect to *T. penetrans*, we undertook a molecular analysis of cytochrome oxydase II and 16S rDNA mitochondrial genes and of the internal transcribed spacer 2 nuclear marker on samples of both species. Maximum Parsimony evaluations of the three data set indicate a differentiation compatible with a specific rank between the two fleas with very high levels of divergence. Both mitochondrial and nuclear data are in line with a recent bottleneck in the Malagasy population of *T. penetrans*, possibly due to the recent colonisation of Africa *via* human transportation. Further, significantly lower mitochondrial variability in the Ecuadorian populations of *T. penetrans* with respect to the *T. trimamillata* ones is also evidenced.

KEY WORDS : cytochrome oxydase II, 16S rDNA, internal transcribed spacer 2, genetic variability, *Tunga penetrans, Tunga trimamillata.*

Résumé : CARACTÉRISATION MOLÉCULAIRE DE *TUNGA TRIMAMILLATA* ET DE *T. PENETRANS* (INSECTA, SIPHONAPTERA, TUNGIDAE) : TAXONOMIE ET VARIABILITÉ GÉNÉTIQUE

Une nouvelle espèce du genre Tunga, T. trimamillata, *a été récemment décrite sur la base de plusieurs traits morphologiques. Pour explorer l'état taxonomique de cette puce en ce qui concerne* T. penetrans, *nous avons entrepris une analyse moléculaire des gènes mitochondriaux de cytochrome oxydase II et 16S ARNr, et du marqueur nucléaire entretoise transcrite interne 2 sur des échantillons des deux espèces. Les évaluations de "Maximum Parsimony" des trois marqueurs indiquent une différentiation compatible avec le rang d'espèce entre les deux puces, avec une divergence très élevée. Les résultats des évaluations des marqueurs mitochondriaux et nucléaires sont en conformité avec un goulot d'étranglement récent dans la population de* T. penetrans *de Madagascar, probablement due à la colonisation récente de l'Afrique par l'homme. De plus, la variabilité des gènes mitochondriaux sensiblement inférieure dans les populations de l'Equateur de* T. penetrans *est également démontrée en ce qui concerne* T. trimamillata.

MOTS CLÉS : *cytochrome oxydase II, 16S ARNr, entretoise transcrite interne 2, variabilité génétique,* Tunga penetrans, Tunga trimamillata.

INTRODUCTION

The genus *Tunga* Jarocki, 1838 includes several species of sandfleas distributed in Central and South America, Sub-Saharian Africa, China and Japan. Adult females penetrate into the host's skin where, once fertilised, their abdomen increases enormously, owing to the development of up to 200 eggs: this can lead to harmful skin infections.

Most *Tunga* species are parasite of a single or a few closely related hosts, especially rodents, and show a geographically restricted distribution (Li & Chin, 1957; Smit, 1962, 1968; Barnes & Radovsky, 1969). On the contrary, *T. penetrans* (L., 1758) is a Afro- and Neotropical pest with a wide range of possible hosts, such as donkeys, horses, cows, pigs, dogs and humans (Linardi & Guimarães, 2000).

Recent morphological analyses (Pampiglione *et al.*, 2002, 2003, 2004) described a new species of *Tunga, T. trimamillata* Pampiglione, Trentini, Fioravanti, Onore & Rivasi, 2002. This ectoparasite has been recorded on goat, swine, cattle and man in Ecuador (Fioravanti *et al.*, 2003). *T. trimamillata* is morphologically similar to *T. penetrans,* the most evident diagnostic characters being three semi-spherical humps on the abdomen of gravid females of the former taxon. Other morphological differences concern the mean diameter of the gravid abdomen and the differential length of maxillary palp segments.

To evaluate the taxonomic status of *T. trimamillata* and its relationship with *T. penetrans*, we undertook a molecular investigation on 50 specimens of both species collected in five localities of Ecuador and Madagascar. We analysed two mitochondrial markers, cytochrome oxidase II (COII) and the large ribosomal subunit (16S) genes,

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and a nuclear one, the internal transcribed spacer 2 (ITS2). All these genes have been extensively used to address taxonomic and phylogenetic questions in insects, as well as in many other organisms, at different taxonomic levels (Caterino *et al.*, 2000; Hillis & Dixon, 1991).

MATERIALS AND METHODS

All samples were collected in Ecuador, but the Fort Dauphin (Madagascar) one. Gravid females were extracted from single hosts, while free-living males and females were taken at ground level, near to domestic animals. Specimens were fixed in absolute ethanol and morphologically determined as described in Pampiglione *et al.* (2003, 2004). All pertinent information on samples are given in Table I.

Genomic DNA was extracted from single specimens using a protocol involving guanidinium thiocyanate and diatomaceous silica (Gerloff *et al.*, 1995). PCR amplifications were performed in a 50 µl mixture using the Invitrogen Taq Polymerase kit following standard protocol. Thermal cycling was done in a Gene Amp PCR System 2400 (Applied Biosystems) programmable cyclic reactor, using the following program: initial denaturation at 94° C for five minutes, 30 cycles of 30 sec. at 94° C for, 30 sec. at 48° C, 30 sec. at 72° C, and a final extension for seven minutes at 72° C.

The amplified products were purified with the Nucleo Spin kit (Macherey-Nagel) and directly sequenced with the DNA sequencing kit (BigDye terminator cycle sequencing, Applied biosystems) in a 310 Genetic Analyzer (ABI) automatic sequencer. The primers utilised in both PCR amplification and sequencing reactions were mtD-13 = TL2-J-3034 (5'- AAT ATG GCA GAT TAG TGC A-3')/mtD-20 = TK-N-3785 (5'-GTT TAA GAG ACC AGT ACT TG-3') for the COII gene, and mtD-32 = LR-J-12887 (5'-CCG GTC TGA ACT CAG ATC ACG T-3')/mtD-34 = LR-N-13398 (5'-CGC CTG TTT AAC AAA AAC AT-3') for the 16S gene.

ITS2 sequences were amplified under the same conditions using the primers ITS2D (5'-CAC TCG GCT CGT GGA TCT AT-3') and ITS2R (5'-TTT AGG GGG TAG TCT CAC CTG-3'). Amplicons were ligated in pGEM-T Easy Vector (Promega) and used to transform *E. coli* DH5α competent cells. Recombinant clones were identified using the β-galactosidase gene blue-white colour system (Sambrook *et al.*, 1989) and directly sequenced. One or two positive colonies were sequenced for each individual, and in three specimens per population, from four to five recombinant colonies were screened to examine intragenomic variability.

Sequences were aligned with the CLUSTAL algorithm of the Sequence Navigator program (Version 1.0.1, Applied Biosystems); alignments were also edited by eye.

Haplotypes have been entered to Genbank under the accession numbers AF551751-AF551754 and AY425821-AY425830 (COII); AF551755-AF551757 and AY425831-AY425838 (16S); AY425818-AY425820 (ITS2).

Distance matrices following Kimura 2-parameter method (K2p), nucleotide diversities for all genes and translation to amino acids of the COII sequences with the *Drosophila* genetic code were obtained through Mega 2.0 package (Kumar *et al.*, 2001). Haplotype diversity and its variance were calculated with DnaSP (Rozas & Rozas, 1999). Maximum Parsimony analyses were performed using heuristic search with 100 random addition searches in PAUP* program (version 4.0b; Swofford, 2001). For 16S gene and ITS2 sequence analyses, gaps were considered as 5th state characters. Branch supports were calculated after 1,000 bootstrap replicates. It should be noted that in Figure 1 the bootstrap consensus trees are represented, since being an average of many bootstrap trees, they may be more reliable than the original ones (Felsenstein, 1985; Nei & Kumar, 2000)

Sequences of the fleas *Neopsylla mana* (AF257461; AF269115; AF353110) and *N. bidentatiformis* (AF251152; AF269111; AF353111) were drawn from GenBank and utilised as outgroups in COII, 16S and ITS2 analyses.

Haplotype networks for COII and 16S datasets were constructed with the algorithm described by Templeton *et al.* (1992), implemented on TCS version 1.3 (Clement *et al.*, 2000). The algorithm calculates the number of mutational steps by which pairwise haplotypes differ and computes the probability of parsimony for pairwise differences until the probability exceeds 95 %. The number of mutational differences associated with the probability just before the 95 % cut-off is then the maximum number of mutational steps between pairs of sequences justified by the 'parsimony' criterion (Templeton *et al.*, 1992; Clement *et al.*, 2000).

RESULTS

Sequencing analyses of the cytochrome oxidase II gene covered 606-667 bp, encoding for 202-222 amino acids.

The 14 haplotypes scored show the 73,5 % of the 98 variable sites at the third codon position, 19,4 % at the first and the 7,1 % at the second. 85 nucleotide substitutions unequivocally distinguish *T. trimamillata* samples from the *T. penetrans* ones.

Pairwise distances based on K2p methods range from 0.002 +/- 0.002 in intraspecific comparisons (e.g. haplotype c1 *vs* c2, or c11 *vs* c12), to 0.151 +/- 0.017 in the interspecific comparison between haplotypes c10 *vs* c11.

Ten out of 16 amino acidic replacements are diagnostic to distinguish the two analysed species, and three of such replacements are non-conservative (polar/apolar amino acids)
The haplotypes of the two *Neopsylla* species differ for 20 nucleotide substitutions (K2p distance 0.031 +/- 0.007), resulting in six polymorphic amino acidic sites with only one non-conservative replacement.
Sequencing analyses of the large ribosomal subunit gene covered 390-454 bp. The 11 haplotypes observed show 48 variable sites and six indels (insertions/deletions). Of the 48 variable sites, 36 identify the *T. tri-*

Taxon	Collecting site*	Host**	Acronym	Halotypes***		
				COII	16S	ITS2
Tunga trimamillata						
	Santa Isabel A (Ecuador)	goat	ecuA CAP1	c1	r1	i1
		goat	ecuA CAP2	c1	r1	i1
		cattle	ecuA *BOS*1	c1	r1	i1
		cattle	ecuA *BOS*2	c1	r1	i1
		*	ecuA FEM1	c2	r1	i1
		*	ecuA FEM2	c1	r1	i1
		*	ecuA FEM3	n.d.	r1	i1
		*	ecuA FEM4	c3	r2	i1
		*	ecuA FEM5	c4	n.d.	i1
		*	ecuA FEM6	c5	r1	i1
		*	ecuA FEM7	c1	n.d.	i1
	Catacocha (Ecuador)	*	ecuC FEM1	c1	r1	i1
		*	ecuC FEM2	c1	r1	i1
		*	ecuC FEM3	c1	r1	i1
		*	ecuC FEM4	c6	r3	i1
		*	ecuC FEM5	c1	r4	i1
		*	ecuC FEM6	c7	n.d.	i1
		*	ecuC FEM7	c8	r5	i1
		*	ecuC FEM8	c9	r1	i1
	Machala (Ecuador)	cattle	ecuM *BOS*1	c1	r6	i1
		cattle	ecuM *BOS*2	c1	r1	i1
		cattle	ecuM *BOS*3	c10	r7	i1
		cattle	ecuM *BOS*4	c1	r4	i1
		cattle	ecuM *BOS*5	c1	r1	i1
		cattle	ecuM *BOS*6	c1	r8	i1
Tunga penetrans						
	Santa Isabel B (Ecuador)	*	ecuB FEM1	c11	r9	i2
		*	ecuB FEM2	c11	r9	i2
		*	ecuB FEM3	c11	r9	i2
		*	ecuB FEM4	c12	r9	i2
		*	ecuB FEM5	c11	r9	i2
		*	ecuB FEM6	c11	r9	i2
		*	ecuB FEM7	c12	r9	i2
		*	ecuB MAL1	c12	r9	i2
		*	ecuB MAL2	c11	r10	i2
		*	ecuB MAL3	c12	r9	i2
		*	ecuB MAL4	c13	r9	i2
		*	ecuB MAL5	c11	r9	i2
		*	ecuB MAL6	n.d.	r9	i2
	Pelileo (Ecuador)	swine	ecuP *SUS*1	c11	r9	i2
		swine	ecuP *SUS*2	c11	r9	i2
		swine	ecuP *SUS*3	c11	r9	i2
		swine	ecuP *SUS*4	c11	r9	i2
		swine	ecuP *SUS*5	c11	r9	i2
		swine	ecuP *SUS*6	c11	n.d.	i2
		swine	ecuP *SUS*7	c11	n.d.	i2
		swine	ecuP *SUS*8	c11	r11	i2
	Fort Dauphin (Madagascar)	man	mad HOM1	n.d.	r9	n.d.
		man	mad HOM2	c14	n.d.	i3
		man	mad HOM3	c14	r9	i3
		man	mad HOM4	c14	r9	i3

* Santa Isabel A and B refer to *Tunga trimamillata* and *T. penetrans* sampling, respectively.
** asterisks mark free-living males and females taken at ground level.
*** n.d. indicates haplotypes not determined.

Table I. – List of analyzed specimens: collecting sites, hosts, acronyms and related haplotypes are given.

mamillata haplotypes from the *T. penetrans* ones. Pairwise K2p distances between 16S haplotypes range from 0.002 +/- 0.002 in the intraspecific comparisons (f.i. haplotype r1 *vs* r3, r4, r6 and r8), to 0.095 +/- 0.015 between haplotypes r8 and r11, pertaining to *T. trimamillata* and *T. penetrans,* respectively.

16S rDNA sequences of the two outgroup sequences, *N. mana* and *N. bidentatiformis*, differ for only five nucleotide substitutions and two indels (K2p distance 0.011 +/- 0.005).

Intrapopulation and intraspecific variability for the two mitochondrial markers are given in Table II. In *T. trimamillata* populations, haplotype diversity (h_D) for COII gene range from 0.3330 +/- 0.0460 (Machala) to 0.7860 +/- 0.0220 (Catacocha), while for 16S sequences values are comprised between 0.2220 +/-0.0276 (Santa Isabel A) and 0.7140 +/- 0.0327 (Catacocha). Within *T. penetrans* populations decidedly lower h_D values can be observed: from 0 (Fort Dauphin and Pelileo) to 0.5910 +/- 0.0120 (Santa Isabel B) for COII, and between 0 (Fort Dauphin) and 0.3330 +/- 0.0463 (Pelileo) for 16S. Nucleotide diversity (π) within populations and the mean values for each taxon follows the same pattern (Table II). On the whole, it appears clear that *T. trimamillata* experiences a higher variability. Even excluding from the variability analyses in *T. penetrans,* the monomorphic sample of Fort Dauphin, a comparable picture can be depicted (not shown).

The sequencing of ITS2 rDNA covered 470-473 bp. All analysed specimens of *T. trimamillata* are identified by genotype i1. In *T. penetrans*, the two Ecuadorian populations share the same genotype i2 and the Fort Dauphin one shows a private genotype i3; the latter two genotypes differ for one substitution and two indels and show a K2p distance value of 0.002 +/- 0.002. Twenty-three polymorphic sites and seven indels characterise the three genotypes with 21 substitutions and two indels distinguishing the two *Tunga* species. K2p distance in the comparison i1 *vs* i2-3 is 0.049 +/- 0.011. The two *Neopsylla* ITS2 sequences differ for two nucleotide substitutions and one indel; K2p value is 0.005 +/- 0.003.

Heuristic search for Maximum Parsimony analysis on COII nucleotide sequences results in 12 equally parsimonious trees. As expected on the basis of sequence characterisation, Maximum Parsimony dendrogram (Fig. 1A) split the haplotypes in two well-defined clusters (100 % bootstrap value). The first one embodies specimens morphologically identified as *T. penetrans*, and it is further divided in the Fort Dauphin haplotype branch and a highly supported sub-cluster containing Santa Isabel B and Pelileo samples. The second cluster contains *T. trimamillata* samples, with haplotypes from different populations completely intermingling.

Maximum Parsimony analysis for 16S gene gave three equally parsimonious trees; a clear-cut differentiation of the two *Tunga* taxa (100 % bootstrap value) is again evident, with the only difference that the Malagasy haplotype clusters within the main *T. penetrans* group (Fig. 1B).

The same analysis performed on the nuclear marker ITS2 results in one most parsimonious tree whose topology, given the three genotypes scored, obviously shows a splitting between the *T. trimamillata* sequence and the two *T. penetrans* genotypes (data available from the authors).

The TCS program on COII haplotypes computed two haplotype networks, corresponding to *T. penetrans*

T. trimamillata	**Population**	**S.ta Isabel (ecuA)**		**Catacocha (ecuC)**		**Machala (ecuM)**		**Total**	
	Sample size	N = 11		N = 8		N = 6		N = 25	
		COII	16S	COII	16S	COII	16S	COII	16S
	Number of sequences	10	9	8	7	6	6	24	22
	Number of haplotypes	4	2	5	4	2	5	9	8
	Haplotype diversity (h_D)	0.6440	0.2220	0.7860	0.7140	0.3330	0.9330	0.6160	0.602
	Variance of h_D	0.0230	0.0276	0.0220	0.0327	0.0460	0.0148	0.0130	0.0145
	Polymorphic sites (S)	3	1	6	4	1	5	10	9
	Nucleotide diversity (π)	0.0016	0.0009	0.0027	0.0026	0.0005	0.0038	0.0018	0.0023
	Variance of π	0.0009	0.0006	0.0010	0.0012	0.0005	0.0018	0.0005	0.0008
T. penetrans	**Population**	**S.ta Isabel (ecuB)**		**Pelileo (ecuP)**		**Fort Dauphin (mad)**		**Total**	
	Sample size	N = 13		N = 8		N = 4		N = 25	
		COII	16S	COII	16S	COII	16S	COII	16S
	Number of sequences	12	13	8	6	3	3	23	22
	Number of haplotypes	3	2	1	2	1	1	4	3
	Haplotype diversity (h_D)	0.5910	0.1540	0.0000	0.3330	0.0000	0.0000	0.5490	0.1770
	Variance of h_D	0.0120	0.0159	0.0000	0.0463	0.0000	0.0000	0.0100	0.0113
	Polymorphic sites (S)	2	1	0	1	0	0	5	2
	Nucleotide diversity (π)	0.0011	0.0004	0.0000	0.0006	0.0000	0.0000	0.0020	0.0004
	Variance of π	0.0008	0.0005	0.0000	0.0006	0.0000	0.0000	0.0009	0.0003

Table II. – Intrapopulation and intraspecific data for mitochondrial COII and 16S genes of *Tunga trimamillata* and *T. penetrans*.

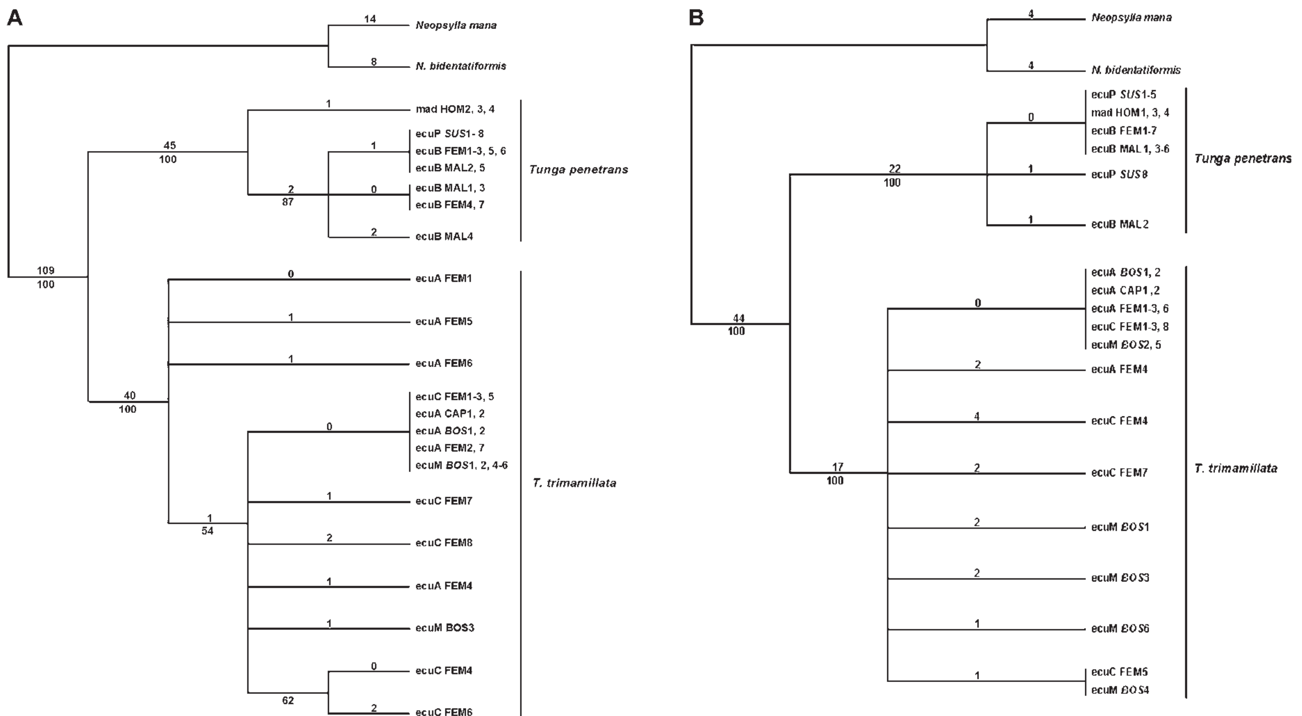

Fig. 1. – Maximum Parsimony bootstrap consensus dendrograms computed on: A) COII haplotypes (TL = 233; CI = 0.974; HI = 0.026); B) 16S haplotypes (TL = 107; CI = 0.981; HI = 0.019). Numbers above and below branches indicate mutational steps and bootstrap percentages, respectively. Acronyms are as in Table I.

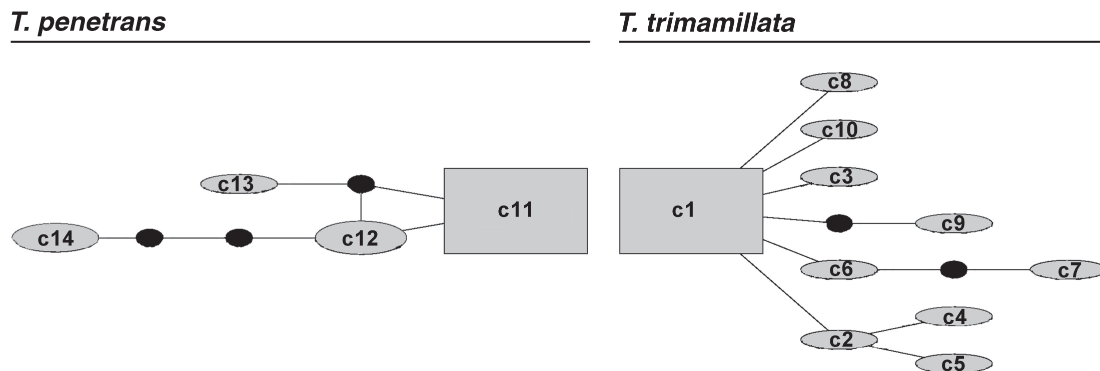

Fig. 2. – Networks for COII haplotypes observed in *Tunga penetrans* and *T. trimamillata* populations. The size of grey areas is proportional to the haplotype frequency; rectangles represent the estimated ancestral haplotype; black dots represent missing/ideal haplotypes. Haplotypes are as in Table I.

individuals, one side, and to *T. trimamillata* specimens, the other (Fig. 2). These networks are not connected since the genetic distance between the two sets of haplotypes largely exceed the maximum number of steps (11) allowed at 95 % probability threshold. In the *T. trimamillata* network, the 10 haplotypes have a maximum divergence of three steps. The four *T. penetrans* haplotypes have a maximum divergence of four steps, the Malagasy one being the most differentiated. A comparable picture emerges when the TCS program is run on the 16S data set (data available from the authors).

DISCUSSION

Both mitochondrial and nuclear datasets indicate that the two *Tunga* species here analysed are genetically distinct entities: notwithstanding morphological affinities, specimens determined as *T. trimamillata* or *T. penetrans* are unambiguously placed in two different and well supported clusters. The non-connected haplotype networks obtained further support such divergence. The reliability of the morphological characters chosen to distinguish the two species is supported.

Further, the level of genetic divergence observed between the two taxa is very high if compared with the range of differentiation observed between the two species used as outgroups. *Neopsylla mana-N. bidentatiformis* genetic distances are from 5-fold (COII) to 10-fold (ITS2) lower than those calculated between the two *Tunga* species. Unfortunately, no other *Tunga* taxa or molecular data on them are available for intrageneric comparisons: it is not possible therefore to argue at present if *T. penetrans* and *T. trimamillata* are distantly related species or if a high level of divergence characterises the specific entities of the genus.

At the intraspecific level, it is to be noted that in all phylogenetic analyses, *T. trimamillata* haplotypes clusters together without any particular geographical and/or host provenience pattern; also in the COII network a particular grouping cannot be observed. On the contrary, for *T. penetrans* this is true only for the analyses involving 16S rDNA. In fact, in both COII (either Maximum Parsimony or haplotype network) and ITS2 dataset the Malagasy population of Fort Dauphin shows consistent and unique differences. It has been reported that *T. penetrans* colonised Africa following the commercial trade with the Americas in the late 18th/ early 19th century (Hoepli, 1963; Connor, 1976). According to this event, the recent colonisation of Madagascar, possibly with a bottleneck effect, could explain the complete lack of 16S differentiation from the American specimens and the fixing of a particular COII haplotype. However, it should be considered that the very limited sampling could have biased this estimate.

As any other repeated sequences, ITS2 evolves following the so-called concerted evolution (Smith, 1976) through a process known as molecular drive (Dover, 2002). Molecular drive, involving genomic turnover mechanisms and population dynamics processes, make it possible to homogenise and fix a particular repeat variant within each single reproductive units. This leads to a lower degree of divergence within than between populations and/or species.

Repeated sequence dynamics clearly explains the lack of nucleotide variation within analysed populations, but also strongly evidences that in the Fort Dauphin sample a private variant has been fixed. The probability and the time necessary to homogenise and fix a particular repeat within a reproductive unit are mainly linked to population size and rates/biases of genomic turnover mechanisms. Since the dynamics of genomic turnover mechanisms are assumed to be approximately equal within the same species, the fixation of a particular repeat variant in less than 300 years could be explained with a population size-dependent process. The African colonisation of a small number of sand fleas could have lead to the random fixation of i3 haplotype within the new populations of *T. penetrans*. It will be interesting to check if i3 ITS2 variant characterises only the Fort Dauphin population, or the entire African and Old World populations of *T. penetrans*: in fact, this could clarify if the African/Asian spreading is the result of only one or more colonisation event(s).

The two species show quite different variability levels: even disregarding the Malagasy sample, the Ecuadorian specimens of *T. penetrans* have very limited mitochondrial haplotype diversity, while *T. trimamillata* populations evidence a significantly higher degree of variability. The lower genetic differentiation found in *T. penetrans* is difficult to explain: the two taxa are both unspecialised ectoparasites sharing a wide trophic niche; further, even if the number of population sampled is limited, the analysed *T. trimamillata* specimens are geographically closer than the *T. penetrans* ones and the number of analysed specimens per populations are comparable between the two taxa. Therefore, it does not seem possible to ascribe the lower variability found in *T. penetrans* to either an ecological specialisation or a bias in population sampling. Its limited variability could be the outcome of bottlenecks encountered in Ecuador. Among the driving forces there could be a higher susceptibility of *T. penetrans* with respect to *T. trimamillata* to antiparasitary treatments, which are mainly performed in swine breeding. It is in fact to be considered that the Pelileo population showing the lowest variability was sampled on pigs. However, this possibility should be specifically addressed with further studies.

As a general remark, it will be of interest to check mitochondrial and nuclear markers variability in a wider "taxon sampling" of *T. penetrans* to verify if its low diversity is shared with other South-American populations or if it is limited to the Ecuadorian area, where also an ecological competition with *T. trimamillata* cannot be excluded.

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