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Molecular analyses reveal two geographic and genetic lineages for tapeworms, *Taenia solium* and *Taenia saginata*, from Ecuador using mitochondrial DNA.

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**Abstract**

Tapeworms *Taenia solium* and *Taenia saginata* are the causative agents of taeniasis / cysticercosis. These are diseases with high medical and veterinary importance due to their impact on public health and rural economy in tropical countries. The re-emergence of *T. solium* as a result of human migration, the economic burden affecting livestock industry, and the large variability of symptoms in several human cysticercosis, encourage studies on genetic diversity, and the identification of these parasites with molecular phylogenetic tools. Samples collected from the Ecuadorian provinces: Loja, Guayas, Manabí, Tungurahua (South), and Imbabura, Pichincha (North) from 2000 to 2012 were performed under Maximum Parsimony analyses and haplotype networks using partial sequences of mitochondrial DNA, cytochrome oxidase subunit I (COI) and NADH subunit I (NDI), from Genbank and own sequences of *Taenia solium* and *Taenia saginata* from Ecuador. Both species have shown reciprocal monophyly, which confirms its molecular taxonomic identity. The COI and NDI genes results suggest
phylogenetic structure for both parasite species from south and north of Ecuador. In *T. solium*, both genes gene revealed greater geographic structure, whereas in *T. saginata*, the variability for both genes was low. In conclusion, COI haplotype networks of *T. solium* suggest two geographical events in the introduction of this species in Ecuador (African and Asian lineages) and occurring sympatric, probably through the most common routes of maritime trade between the XV-XIX centuries. Moreover, the evidence of two NDI geographical lineages in *T. solium* from the north (province of Imbabura) and the south (province of Loja) of Ecuador derivate from a common Indian ancestor open new approaches for studies on genetic populations and eco-epidemiology.

**Keywords**: mtDNA lineages, cysticercosis, taeniasis, COI, NADH-I, *Taenia solium*, *Taenia saginata*, Ecuador.

1. **Introduction**

Infestations by *Taenia* spp., and *Echinococcus* spp., are common, not only in developing countries but also in industrialized countries, where apart from being a threat to health, they represent a socio-economic impact. Cysticercosis causes a debilitating disease in humans as well as losses in the meat industry due to the condemnation of meat from infected animals (Jiménez et al., 2002; Raether and Hänel, 2003; Rodriguez Hidalgo, 2007; Schantz, 1999; Tsai et al., 2013)

The Andean region of Ecuador was described as hyperendemic for taeniasis / cysticercosis, with prevalences in rural communities up to 1.60% for taeniasis and 14.4% for *T. solium*-cysticercosis by. The prevalence of *T. saginata* in Ecuador is not well established, however, a moderate prevalence of bovine cysticercosis with 0.37% on veterinary inspection and 4.03% by serological techniques has been reported (Cayo-Rojas et al., 2011; Cruz et al., 1989; Rodriguez-Hidalgo et al., 2006; Rodríguez-Hidalgo et al., 2010, 2003; Rodriguez Hidalgo, 2007).

As a result of their great importance in Ecuador, these two cestodes species have been extensively studied. However, the strategies aiming to control these parasitoses have major limitations i.a. it has not been possible to accurately identify the prevalence of *Taenia* spp. and the determination of strains by
means of ecological, biological or morphological criteria is difficult (Ito et al., 2003). The efficiency of control depends on detailed epidemiological information including identification and precise characterization of the causative agent in each endemic area (Gasser et al., 1999; Jia et al., 2010; McManus, 1990).

The genus *Taenia* has been successfully identified using, enzyme electrophoresis and mitochondrial molecular markers to differentiate between species and also to infer phylogenies (Hoberg et al., 2000; Nakao et al., 2010; Queiroz and Alkire, 1998). However, little is known about the genetic intra-specific variation in cestodes (Pawlowsky Zbigniew, 2002) and considerable research indicates a great heterogeneity in pathology caused by *Taenia solium* but there is a misunderstanding about the role of genetic diversity and adaptability of species (Del Brutto, 2013; Finsterer and Auer, 2012; Marquez and Arauz, 2012; Román, 2014; Sotelo, 2011). Based on this issue, mitochondrial DNA analysis provides complementary tools for characterization of a population. Gene fragments or complete genome of mitochondrial DNA have been successfully used in population genetics, ecology and identification of tapeworms (Jia et al., 2010). Genetic variation associated with different hosts is a well-known fact in several cestodes species e.g. *Echinococcus granulosus* and Ito et al., (Ito et al., 2003) suspected that for *Taenia saginata* and *Taenia solium* it may well be equally the case.

Nakao et al., (Nakao et al., 2002), using the complete genes Cytochrome Oxidase subunit I and Cytochrome b of the mitochondrial DNA, showed the existence of two lineages of *Taenia solium* worldwide: an Asian group and an African and Latin-American group of strains. A sequence from Ecuador was included in these studies, showing minimal variation with the rest of the sequences in the analysis, albeit this sequence represents only a small portion of the Ecuadorian gene pool. Yanagida et al., (Yanagida et al., 2014) using different mitochondrial genes, report two genetic sympatric lineages of *T. solium* in Madagascar close related with Asia and Africa/Latin America as consequence of historical human migration. These authors shown the Africa/Latin America lineage connected by haplotypes network with the Ecuadorian haplotype AB066491 here also used.
It is important to complete more detailed variability studies for this species, even more so when genetic variation of tapeworms from various geographic locations might be linked to clinical and pathological differences found in human cysticercosis (Maravilla et al., 2003; Vega et al., 2003).

The aim of this research is to determine the genetic diversity within populations of *Taenia solium* and *Taenia saginata* collected in six locations, from the north and the south of Ecuador. We also hypothesized that different geographical origins or introductions, can be assessed by means of presence of geographic phylogenetic structuration within sequences-populations and/or haplotypes networks analysis. The results in this study can underpin epidemiological research and the control of these parasites. Furthermore, it provides a great source of information and support for new diagnostic methods and reinforces vaccines developing in the fight against these important diseases (Assana et al., 2010; Fernadez et al., 2006; Maravilla et al., 2008; Sciutto et al., 2013; Tsai et al., 2013).

2. **Materials and methods**

2.1. **Source of *Taenia* spp. specimens.**

Specimens used in this research belong to the biological bank of the Centro Internacional de Zoonosis (CIZ) at Universidad Central del Ecuador from localities of a former study and control program. Samples have been conserved in ethanol solution 70%, and kept frozen at -20°C. Samples were collected from the Ecuadorian provinces of: Loja, Guayas, Manabí, Tungurahua (South), and Imbabura, Pichincha (North) from 2000 to 2012 (Supplementary data 1). Specimens were isolated from human faecal material after anthelmintic treatment. Patients became from both urban and rural areas. The vouchers of remains specimens are deposited in the bank of CIZ.

2.2. **DNA extraction, amplification and sequencing of COI and NDI genes.**

For DNA extraction of *Taenia*’s proglottids, the Wizard Genomic DNA Purification of Promega® commercial kit was used (Promega, 2010) following the manufacturer’s protocol. The DNA samples obtained from proglottids of *Taenia* spp. were analysed in agarose gel 0.8% to ascertain presence, quality and size of the extracted material. The quantification of genomic DNA extracted from *Taenia*
spp. was performed using the Invitrogen fluorometer QUBIT®. We used the Quant-iT™ Broad-Range DNA Assay Kit according to the manufacturer's instructions (Data not shown).

The reactions were performed in 25 µL, using a thermo cycler TECHNE TC-412, using the primers for NDI sequence (Bowles and McManus, 1993) and JB3-JB4.5 for COI (Bowles et al., 1992). For each reaction buffer PCR 1X, 3mM of MgCl₂ 0.5 µM of oligonucleotide, 0.15 mM of dNTP's and 0.5 U of Taq Polymerase (Invitrogen) was added. The procedure was as follows: 92 °C for 5 minutes for initial denaturation; 35 cycles of 94 °C, 30 s (denaturation); 55 °C (COI)/ 57 °C (NDI), 30 s (annealing); 72 °C, 30 s/ 1 min (extension), followed by a final extension at 72 °C for 5 minutes. For each batch of reactions, a negative control (molecular biology grade water) and a positive control (DNA from T. saginata or T. solium) were included.

Amplified fragments of COI and NDI were purified using PureLink® PCR Purification Kit (Invitrogen) according to the manufacturer's instructions, subsequently the fragments were sent to Macrogen Inc. (Korea) for sequencing in duplicate (forward and reverse).

2.3. Phylogenetic analysis and haplotype networks

Own sequences were contig assembly performed using the Sequencer 4.2.2 (Gene Codes, Ann Arbor, MI) software and identity confirmed by BLAST in NCBI resources. The consensus sequences of gene cytochrome oxidase subunit 1 (COI) and NAD dehydrogenase subunit 1 (NDI) from the two groups (Taenia solium and Taenia saginata) were edited in MacClade software (Maddison and Wayne, 2011), resulting sequences of 404 bp (COI) and 459 bp (NDI). Sequences deposited in GenBank NCBI from other species of Taenia were included as reference/external groups and outgroups (Nixon and Carpenter, 1993) (Supplementary data 2) in order to get a wide geographic diversity covering distinct continents and to test the problem species monophyly. DNA sequences were aligned using MacVector 7.2 (Accelrys, Madison, WI) by ClustalW algorithm with gap creation and extension penalties by default (Supplementary data 3 and 4). Parsimony analyses were implemented in PAUP 4.0b10 (Swofford, 2001) using the heuristic search option with a Tree Bisection Reconnection branch-
swapping algorithm with at random stepwise addition of 10 replicates for each search and 100-1,000 replications per analysis. Gaps were treated both as missing data and as a fifth character state. The characters were treated as unordered, and equally weighted, after that the characters were weighted by consistence index. The robustness of the trees was estimated using parsimony bootstrap with 500 pseudoreplicates after excluding uninformative characters (Carpenter, 1996). We also performed a Maximum Likelihood (ML, substitution model estimated by ModelTest, Posada and Crandall, (1998) on PAUP) and a distance analysis (Neighbour-joining, NJ) using PAUP 4.0b10 (Swofford, 2001).

Later, a Nexus Matrix of sequences for each species was used to construct haplotype networks for TCS 2.1.1 with 95% of connection limit (Clement et al., 2000). We use the monophyly (based on % of bootstrapping, location in clades on the tree, and a genetic divergence intra species bigger than inter species) and phylogenetic species concept to delimit the different taxa. Then, the paraphyletic position in the tree topology of sequences from GenBank under with the same label means a different species and as consequence a misidentified specimen.

3. Results and Discussion

3.1. PCR amplification of genes COI and NDI

A total of 53/68 specimens of *T. solium* and *T. saginata* were successfully amplified. Most samples were complete adult specimens (without scolex), with one third of the samples were proglottids only. Tapeworms were previously characterized using morphology and RFLP analysis of 12S gene (Rodriguez-Hidalgo et al., 2002).

3.2 Phylogenetic Analysis

3.2.1 Cytochrome oxidase subunit I gene (COI)

The maximum parsimony analysis of the 67 sequences used, characters re-weighted, yielded a single parsimonious tree whose length values, consistency index and retention index values of L= 364.82, CI= 0.84 and RI= 0.91 (Fig. 1). The ML and NJ yielded the same topology of MP. The substitution model
that fit with the matrix alignment calculated by ModelTest with Bayesian Information Criteria (BIC) for ML was TrN+G (Supplementary data 5).

The topologies of phylogenetic trees obtained using COI gene (weight and reweighted) reveal the internal grouping of clades, corresponding to sequences of a monophyletic derivated clade *Taenia solium, Taenia saginata* in a monophyletic clade plus *T. asiatica* and the outgroups sequences in a basal location. The *Taenia saginata* group does not show a phylogenetic structure, therefore samples from distant locations like Japan, Belgium, Kenya and Ecuador present values of divergence between 0% and 0.26%, evidenced in the tree as a polytomy. A more detailed analysis of the alignment showed two changes: E42.Tsag. Tungurahua, G by A in position 303, and Tsag. FRANCE T by C in position 243.

In a similar fashion, the internal distribution of the *Taenia solium* clade shows a phylogenetic structure. Within the internal or derivated clade of *T. solium*, the sequences showed two subclades with geographical correspondence: an ancestral Asian subclade + Peruvian sequence (Fig. 1, in yellow and green colours) and a derivated African + America subclade (Fig. 1, in blue colour) with Ecuadorian sequences included in a politomy (not-resolved branches). These findings are consistent with the results found by Nakao et al. (Nakao et al., 2002). The distance *(p*-uncorrected) matrix (Table 1, Supplementary data 6) shows that genetic divergence of the COI gene in samples from Imbabura (North Ecuador) and Loja (South Ecuador), varies between 0 % and 1.06%, therefore, no significant difference was observed among Ecuadorian samples from different geographic locations.

The difficulty of taxonomic identification of metacestodes of *Taenia* spp. is evidenced when some particular sequences found in gene databases do not correspond to the species labelled based on their location in our trees. In our study, two COI sequences: *T. solium*-Mexico (EU747650) and *T. solium*-Peru (AF360866), appear as different species from those originally published. In the first case (EU747650), the sequence shows a clear homology and sister relationship with *T. hydatigena*, which has pigs as a common intermediate host (Conlan et al., 2009) suggesting a misidentify. The sequence
(AF360866) is presented as a particular case, the topology analysis locates it as an ancestor of *T. solium*, however the divergence with this species is considerably high: 11.94%. The divergence between *T. solium* and the closest sequence *T. hydatigena* is 12.40%, therefore, this specimen (AF360866) could be a genetic variation of *T. solium* from Peru (conservative point of view), or an intermediate species between *T. solium* and *T. hydatigena*, this however, requires further research.

In order to resolve the polytomies observed in the tree and to detect mutational ancestor-descendant changes between geographical sequences, we have performed the haplotype network (TCS) shown in Fig. 2. We found eleven haplotypes of worldwide distribution grouped into two different (not connected) networks with Ecuadorian haplotypes located in both linked with African-American and Asian haplotypes.

3.2.2. *NAD dehydrogenase subunit I* gene (*ND1*)

The maximum parsimony analysis of the 31 sequences and re-weighted yielded a single tree with values of L= 344.89, CI= 0.76 e RI= 0.83 (Fig. 3). The ML and NJ yielded the same topology of MP. The substitution model that fit with the matrix alignment calculated by ModelTest with Bayesian Information Criteria (BIC) for ML was TVM+G (Supplementary data 7).

The topology of the tree shows three consecutive basal clades corresponding to the outgroups taxa. Then, internally two sister and reciprocal monophyletic clades: *T. solium* clade + *T. asiatica* (*T. saginata* clade plus *T. krabbei* and *T. multiceps*). In the derivate group of *T. saginata* we found a polytomy with no geographic structure. The sequences of *T. asiatica*, *T. krabbei* and *T. multiceps* are located as ancestral taxa to *T. saginata*.

The *T. solium* clade shows a geographical structure, forming two groups: one group with all sequences of America (Fig. 3, in red colour), Ecuador included; and another group with Asian sequences (Fig. 3, in yellow colour). The strict consensus from three topologies shows that the distribution of the clades has the same geographical pattern. The bootstrap analysis with 1,000 repetitions shows clades with strong support by statistical re-sampling of the alignment matrix (Fig. 3).
In addition, topologies of phylogenetic trees obtained using NDI gene show a similar distribution to the one found in COI gene trees, however, within the clade of *Taenia solium* two subclades were found from the north (Imbabura) and from the south (Loja) of Ecuador. The distance (*p*-uncorrected) matrix (Table 1, Supplementary data 8) shows that genetic divergence of the NDI gene in samples from Imbabura and Loja is around 1.2% which is much less than in case of an interspecies divergence (e.g. 12%, between *T. asiatica* and *T. saginata* it is 12%). These findings demonstrate higher variability in NDI than COI in the mtDNA of *Taenia* spp., in accordance with Jia *et al.* (Jia *et al.*, 2010). The clade of *Taenia saginata* as well as the results shown in the COI gene did not show evidence of significant geographic differentiation.

In order to resolve the polytomies observed in the reweighed tree and to detect ancestor-descendant mutational changes between geographical sequences (Fig. 4) we used the TCS software. The haplotype network constructed is shown in Fig. 4. Five haplotypes worldwide distributed were grouped into a single network with H4 and H5 from South Ecuador and North Ecuador respectively with the India haplotype H1 as close related based on mutational changes.

### 3.3. Haplotype Networks

Searching for deeper lineage structure than in phylogenetic analysis, a network of haplotypes was performed, in order to discriminate patterns of genetic divergence through specific mutational changes. The network analysis using COI molecular marker resulted in two not connected networks of eleven haplotypes. The first network consists of haplotypes from Brazil, Cape Verde (South Africa), Colombia and Southern Ecuador (Loja) showing the detailed connection of mutational changes that the polytomies in the phylogenetic tree does not, for instance, the ancestor-descendent mutations relationship among Colombia and Cape Verde haplotypes with Loja-Ecuador. The second network is formed by haplotypes from France, Mexico, Haiti, South Korea, China, India and our North-South Ecuador (Imbabura + Loja) including the Ecuadorian sequence in GenBank. Those results showing a clear correlation of the Ecuadorian samples with African and Europe-Asian sequences both occurring
sympatric in the Country similar to Yanagida et al., (2014) results. The two networks of haplotypes analysed are correlated with one of the most common routes of maritime trade between the XV-XIX centuries, supporting the hypothesis of Nakao et al, (Nakao et al., 2002) on the introduction of *Taenia solium* from Europe to Africa and Latin America.

The haplotype network obtained from NDI shows a most probable introduction of *Taenia solium* to Ecuador from a haplotype from India and subsequently the sequences from Ecuador (north and south) show two separate lineages of mutational changes, with 2 changes to North Ecuador lineage and four mutations to South Ecuador lineage. This network is consistent with the results reported by Martinez-Hernández et al. (Martinez-Hernandez et al., 2009) using COI and Cytb, who found a strong correlation among sequences from Latin America (Peru and Mexico) and India.

In conclusion, both networks COI and NDI, suggest that the introduction of *Taenia solium* to Ecuador occurred probably in two different events and geographical origins. Those differences might have been maintained in spite of anthropogenic pressure such as intensive human migrations, and trading of animals and meat products. This meta-population seems to have undergone two colonization processes, with differentiation between northern and southern Ecuador. The Southern colonization influenced from Asia and Africa haplotypes introductions and the Northern Ecuador mainly influenced by Asian introductions. In summary, this research reports the pattern of the genetic variability and gene flow among Ecuadorian localities of both *Taenia* species. This study provides important genetic baseline data and new approaches for studies on genetic populations, eco-epidemiology and control.

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**References**


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Figure legends

Fig. 1. Phylogenetic tree with characters reweighed from the COI gene showing the phylogenetic relationships of *Taenia* spp. Right side brackets show the species outgroups, and the taxonomic nomination of sequences. Numbers on branches are the bootstrap values. Lines-branches in colors shown the geographical association into each subclades.

Fig. 2. Global Network haplotypes of *Taenia solium* for COI gene. Two not connected networks shows the two geographical lineages. Ecuador haplotypes are located in both networks (Africa-Latin America and European-Asia networks).
**Fig. 3.** Phylogenetic tree with NDI gene and characters reweighted showing the phylogenetic relationships of *Taenia* spp. Right side brackets show the species outgroups, and the taxonomic nomination of sequences. Numbers on branches are the bootstrap values. Lines-branches in colours shown the geographical association into each subclades.

**Fig. 4.** Global Network haplotypes of *Taenia solium* using data from NDI gene. Ecuador haplotypes are located in two separate lines (Southern and Northern Ecuador) after mutational steps from India haplotype.
<table>
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<th>Collection Place</th>
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<td>T. saginata (E55)</td>
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Figure 1. Phylogenetic tree with characters reweighed from the COI gene showing the phylogenetic relationships of *Taenia* spp.
Figure 2. Global Network haplotypes of *Taenia solium* for COI gene.
Figure 3. Phylogenetic tree with NDI gene and characters reweighted showing the phylogenetic relationships of Taenia spp.
Figure 4. Global Network haplotypes of *Taenia solium* using data from NDI gene.
• *Taenia solium* and *Taenia saginata* cause taeniasis/cysticercosis, a NTD in Ecuador.

• Maximum Parsimony analyses in *Taenia solium* revealed greater geographic structure.

• COI haplotype networks suggest two geographical events in the introduction of *T. solium* in Ecuador.

• Two NDI geographical lineages in *T. solium* derivate from a common Indian ancestor.