Phylogenetic relationships of Andean-Ecuadorian populations of *Anastrepha fraterculus* (Wiedemann 1830) (Diptera: Tephritidae) inferred from COI and COII gene sequences

Bertha Ludeña [1,3], Rosa Bayas [1] & Jean-Christophe Pintaud [2]

[1] Pontificia Universidad Católica del Ecuador, Laboratorio de Genética Molecular de Eucariotes, Av. 12 de Octubre y Roca, Quito, Ecuador

[2] Institut de Recherche pour le Développement (IRD), UMR DIA-PC/DYNADIV, 911 Avenue Agropolis, BP 64501, F-34394 Montpellier Cedex, France

[3] Present address and correspondence: ECUAGENOMICA, Diego de Trujillo N54-153, Quito, Ecuador

Abstract. Phylogenetic relationships among Andean-Ecuadorian and other Neotropical populations of *Anastrepha fraterculus* and related species have been studied using two regions of mtDNA : 405 base pairs within Cytochrome Oxidase I (COI) and 224 base pairs within Cytochrome Oxidase II (COII). Phylogenetic relationships were inferred using Maximun Parsimony (MP) method and haplotype networks. Andean-Ecuadorian populations of *A. fraterculus* are monomorphic at the COI locus and fall within a clade of South-American lowland populations of *A. fraterculus*. They appear to be unrelated with populations of northern Andes of Colombia and Venezuela also assigned to *A. fraterculus*, meaning that this species, as currently circumscribed, is not monophyletic and is composed of different biological entities that are little differentiated morphologically. At the COII locus, Andean-Ecuadorian populations of *A. fraterculus* show a major haplotype with a few variants, and form a clade with the lowland populations of southern Brazil an Argentina, but are clearly differentiated from them. Andean-Ecuadorian populations of *Anastrepha fraterculus* appear to be homogeneous with respect to their mitochondrial genome and thus their identity as members of a single gene pool is confirmed by these results.

Résumé. Relations phylogénétiques entres les populations des Andes d'Equateur d'*Anastrepha frterculus* (Wiedemann 1830) (Diptera : Tephritidae) déduites des séquences géniques COI et COII. Les relations phylogénétiques des populations d'*Anastrepha fraterculus* des Andes d'Equateur avec celles d'autres régions néotropicales et avec diverses espèces affines ont été étudiées à l'aide du séquençage de deux fragments de l'ADN mitochondrial : un segment de 405 bp du gène Cytochrome Oxydase I (COI) et un segment de 224 bp du gène Cytochrome oxydase II (COII). Les relations phylogénétiques ont été étudiées à l'aide des méthodes de maximum de parcimonie et de réseaux d'haplotypes. Les résultats de COI placent les populations de *A. fraterculus* des Andes de l'Equateur (lesquelles sont monomorphes à ce locus) avec les populations sud-américaines de basse altitude, mais pas avec les populations des Andes septentrionales de Colombie et du Vénézuela, lesquelles forment un clade en distant, mettant en évidence la polyphylie de l'espèce *A. fraterculus*. Cette espèce est constituée d'entités biologiques distinctes mais morphologiquement peu différenciées. Au niveau du locus COII, les populations des Andes d'Equateur montrent un haplotype majeur et quelques variants, elles forment un groupe monophylétique avec des populations de basse altitude d'Amérique du Sud (sud du Brésil et Argentine), dont elles se différencient clairement néanmoins. Les populations andines équatoriennes de *Anastrepha fraterculus* sont finalement homogènes, et leur identité comme membres d'un même pool génétique est confirmée par les résultats obtenus.

Keywords: mtDNA, monomorphic, South-America, pest, fruit cultures.

Anastrepha (Schiner 1868) is the most notorious insect genus in the American tropics and subtropics for its economical impact on fruit cultures. Fifteen species have been listed as pests and other 28 have been reported to attack agriculturally important plant species (White & Elson Harris 1992; Aluja 1994; Caraballo 2001). The geographical range of this genus is very wide, spanning from the south of United States to central Argentina. It has been reported in Galapagos and most of the Caribbean islands also.

The species *Anastrepha fraterculus* (Wiedemann 1830), which is a pest across South America, is distributed in all the continental and insular (Galapagos islands) territory of Ecuador (Carrol *et al.* 2004; Steck 1999).

Despite 70 years of taxonomic work on this species, there is still no satisfactory definition for its taxonomic status. Studies regarding morphology (Stone 1942; Baker *et al.* 1944; Hernández Ortiz *et al.* 2004), molecular characters (Morgante *et al.* 1980;

E-mail: ecuagenomica@uio.satnet.net
Accepté le 25 mars 2010

Morgante & Malavasi 1985; Steck 1991; Steck & Sheppard 1993; MacPheron *et al*. 1999; Alberti *et al*. 1999, 2002, 2008; Smith-Caldas *et al.* 2001; Barr *et al.* 2005; Ruiz *et al.* 2007) and cytogenetics (Bush 1962; Solferini & Morgante, 1987; Morgante *et al*. 1993; Basso & Manso 1998; Basso *et al.* 2003; Morales & Cevallos 2003; Selivon *et al*. 2005; Cáceres *et al.* 2009) show a high variation within this fruit fly. Several authors suggest that under *A. fraterculus* name, a species complex is involved (Steck 1991; MacPheron *et al.* 1999; Smith-Caldas *et al.* 2001; Alberti *et al.* 2008; Cáceres *et al.* 2009). Recent studies based on eggshell morphology and karyotype of sex chromosomes have reported that at least four entities would compose the *A. fraterculus* complex: *A.* sp.1 *aff. fraterculus*, A. sp. 2 *aff. fraterculus* , A. sp.3 *aff. fraterculus* and *A.* sp.4 *aff. fraterculus* . The three first entities occur in Brazil (*A.* sp1 occurs in Argentina also) whereas *A.* sp.4 *aff. fraterculus* was sampled in Guayaquil, Ecuador (Selivon & Perondini 1997, 1998; Selivon *et al*. 2005). A molecular phylogeny based on the Transformer gene also identified four cryptic species within *Anastrepha fraterculus* (Ruiz *et al.* 2007) while a phylogeny based on COI mitochondrial sequences identified a highly divergent population from the Andes of Colombia and adjacent Venezuela (Smith-Caldas *et al.* 2001).

Ecuadorian populations of *Anastrepha fraterculus* have never been studied using molecular markers previously. Only karyotypical analysis of Andean-Ecuadorian populations have been done and it supports the presence of two biological groups: a northern and central group (Guayllabamba and Patate) with 2n = 12, and a southern group (Azuay and Loja) with 2n = 14 (Morales & Cevallos 2003). Moreover Selivon *et al*. (2005) reported a karyotypic form of 2n = 12 for specimens from Guayaquil (Ecuadorian Pacific coast). The present study aims at determining the taxonomic status of these populations through sequence comparison and phylogenetic analysis at mitochondrial loci documented for other South American populations (COI and COII). These mitochondrial markers have been useful to diagnose and delimit species previously (Farrel 2001;Roe & Sperling 2007) and they have been employed in previous studies of phylogenetic relationships of Tephritidae (Smith-Caldas *et al.* 2001; Alberti *et al* 2008).

The correct identification at the species level can help the implementation of control programs and management of pests, as in the case of *Anastrepha fraterculus* in Ecuador.

Material and Methods

Sample source. The specimens of *Anastrepha fraterculus* used in this work were adults emerged from fruits collected in four Ecuadorian provinces: Loja and Azuay in the southern Andes, Tungurahua (Patate) in the central Andes and Pichincha (Guayllabamba and Perucho) in the northern Andes. Some outgroup species also came from the tropical provinces of Guayas (Pacific coast) and Orellana (Amazonia) (Calles 2002).

The individuals analysed for the mitochondrial genes were the following: *A. fraterculus*: Azuay: no. 1848-1, 1891-1, 1892-5 on *Psidium guayava* (COII), no. 1881-35 on *P. guayava* (COI, COII), no. 1888-2 on *Juglans neotropica* (COII); Pichincha: no. 49-7-9 on *Inga edulis* (COII), no. 210-2 on *Annona cherimola* (COI, COII), no. 210-4 on *A. cherimola* (COII), no. 312-1, 640-2, no. 2489-1 on *P. guayava* (COI, COII), E002, 2479-2, 2489-2 on *P. guayava* (COII); Tungurahua: no. 2098-2 on *P. guayava* (COI, COII), no. 2098-3 on *P. guayava* (COII), no. 669 on *Prunus persica* (COI), 666-3, 667-4, 668-1-3 on *P. persica* (COII); Loja: no. 240-1, 240-2 on *Mangifera indica*, no. 1805-6-10 on *P. guayava*, no. 1807-13 on *P. guayava* (COI, COII), no. 1809-4-9-14, no. 1810-13 on *A. cherimola* (COII), 1810-14 on *A. cherimola* (COI, COII); *A. obliqua* (Guayas, on *Spondias mombin*, COI), *A. ornata* (no. 1045-2, Tungurahua on P. guayava, COI, COII); *A. striata* (no. 1811-11, Loja on *P. guayava*, COI, COII, no. 1816-7, Loja on *P. guayava*, COII, no. 627-2, COII, no. 627-3, Orellana on P. *guayava*, COI, COII); *A. serpentina* (Guayas, no. 2457-1 on *Chrysophyllum cainito*, COI, COII, no. 2288-4 on *Citrus paradisi*, COI, COII); *Ceratitis capitata* (Guayas, on *P. guayava*, COI).

DNA extraction. Flies were stored in ethanol until DNA extraction.Total DNA was extracted by hypotonic shock in the presence of proteinase K and Chloroform-Isoamilic Acid mix 24:1 and isolated by ethanol precipitation. The material obtained was stored in Robert's buffer (Ludeña 2006), after its control and quantification.

PCR reactions, genotyping and sequencing of COI and COII partial gene sequences —

Primers for PCR amplification were designed from the *Anastrepha fraterculus* mithocondrial gene sequence COI (Genbank accesion AF420642) and *Anastrepha striata* (Schiner 1868) COII (GenBank accession AY037528) using Primer Select (DNASTAR Inc.) program. COI primers are: IntAnF 5 CATATTTTACATCAGCWACTAT 3 and IntAnR 5TATCGTCGDGGTATACC3; COII primers are: Primer U5 ATGAACAGTCCCCGCTCTT3 and Primer L5 CTTGCTTTCAGTCATCTAAT 3.

PCR reactions were done in a final volume of 12,5µl with 40ng of DNA, 0.3µl of 20µm Primers, premix E buffer Failsafe 1 X and 1U of enzymatic Taq polymerase Failsafe mix, (Epicentre Biotechnologies).

The amplification program for COI proceeded as follows: initial denaturation for 1 minute followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 45 °C for 45 seconds and extension at 72 °C for 1 minute. A final extension step at 72 °C for 3 minutes was added to the program.

The amplification program for COII consisted of 32 cycles of denaturation at 94 °C (30 s), annealing at 51.5 °C (40 s), extension at 72 °C (50 s) and a final extension at 72 °C for 5 minutes.

Amplification was checked on 1% agarose gels. The amplicons were purified with the Qiaquick PCR Purification kit (Qiagen).

Sequencing reactions were carried out with the Silver Sequence kit (Promega). The template consisted of 25 ng of purified PCR product. The sequencing reaction products were run on 6% polyacrylamide gels and colored with silver nitrate.

Data analysis. The partial COI and COII sequences obtained in this study were aligned using the CLUSTALV option of MegAlign (DNASTAR Inc). Polymorphic positions were confirmed by eye.

In the COI analysis, sequences retrieved from GenBank of 15 species of *Anastrepha* were included. Much fewer comparative sequences were available in GenBank for the COII analysis, since this gene is less used in species-level phylogeny. Four sequences from Argentinean and South Brazilian *A. fraterculus* (from Alberti *et al*. 2008), one sequence of *A. grandis* (Macquart, 1846) and the one of *A. serpentina* (Wieldemann) were included in the COII analysis. *Ceratitis capitata* and *Bactrocera tau* (Walker 1849) were utilized as outgroups with sequences retrieved from GenBank for both COI and COII analyses. (Figs. 1–3).

The reconstruction of the phylogeny of *Anastrepha* was done by applying the Maximun Parsimony (MP) method, using a

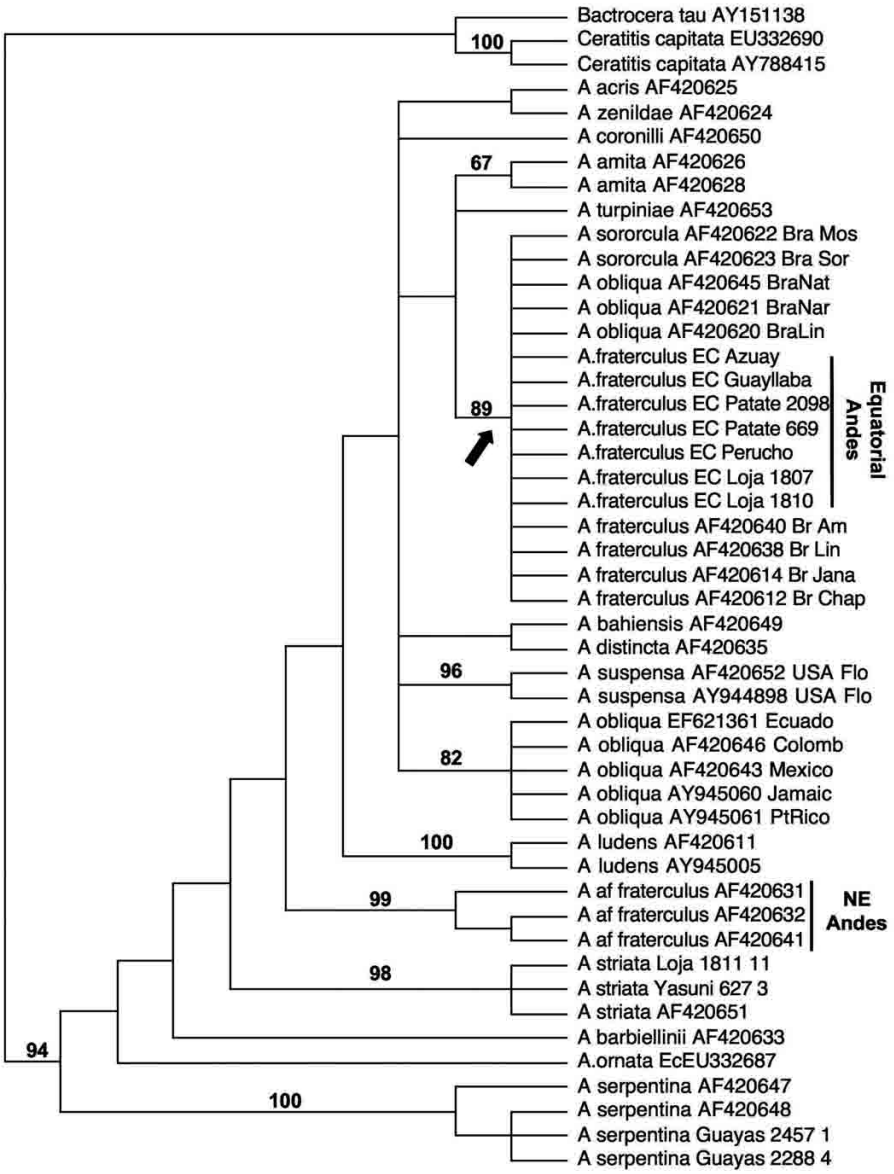

Figure 1
Strict consensus tree from MP analysis of COI sequences (with bootstrap scores on branches) of *Anastrepha spp*., with *Ceratitis capitata* and *Bactrocera tau* as outgroups. The solid arrow indicates the *A. fraterculus* species complex *sensu stricto*. Bars indicate Andean *Anastrepha* samples from Ecuador (Equatorial Andes) and northeastern Colombia and adjacent Venezuela (NE Andes).

heuristic search with TBR branch swapping and random taxon addition. Branch support was evaluated using the bootstrap method with 1000 replicates. All MP analyses were performed with PAUP 4.0b10 (Swofford 2002). Additionally, haplotype networks were constructed with TCS v.1.21 (Clement *et al.* 2000) and SplitsTree4 (Huson & Bryant 2006) for sequences within the clades including the Ecuadorian accessions recovered by the MP analysis.

Results

Cytochrome Oxidase I sequence. A fragment of 411 pb was sequenced in 7 individuals of *A. fraterculus* coming from four different Andean localities of Ecuador. This fragment showed no variation among these individuals. The corresponding sequence was deposited in GenBank under the Accession number EF621359. New sequences generated for *A. obliqua* (EF621361), *A. ornata* (EU332687), *A. striata* (EU332688, EU332689), *A. serpentina* (EF621362, EF621363) and *Ceratitis capitata* (EU332690) were also deposited in GenBank. The nucleotidic composition established for this region was: A = 32.1%, T = 36.8%, C = 16.8% and G = 14.3% consistent with the A-T richness of this mitochondrial sequence previously described for other insects (Bernasconi *et al*. 2000; Morlais & Severson 2002).

A matrix including the sequences obtained more 40 other accessions of 15 *Anastrepha* species from GenBank was constructed in order to assess the phylogenetic position of the Andean-Ecuadorian population.

More than 20 000 equally parsimonious trees were recovered from the MP analysis (L = 457, CI = 0, 676, RI = 0, 807). The Andean-Ecuadorian populations fall within a well-supported clade (89% Bootstrap Support) including South-American lowland populations of *A. fraterculus, A. sororcula* and *A. obliqua*. This clade appears distinct from both the main Neotropical clade of *A. obliqua* that includes the Ecuadorian accession of this species (82% BS) and the clade of Colombian-Venezuelan populations of the north-eastern Andes (99% BS) assigned to *A. fraterculus* (Fig. 1).

An haplotype network could not be reconstructed among these three clades with 95% confidence using TCS because of considerable mutational divergence, exceeding 10 steps. A phylogenetic network (Neighbour Net) showing the divergence of these three groups is instead provided (Fig. 2).

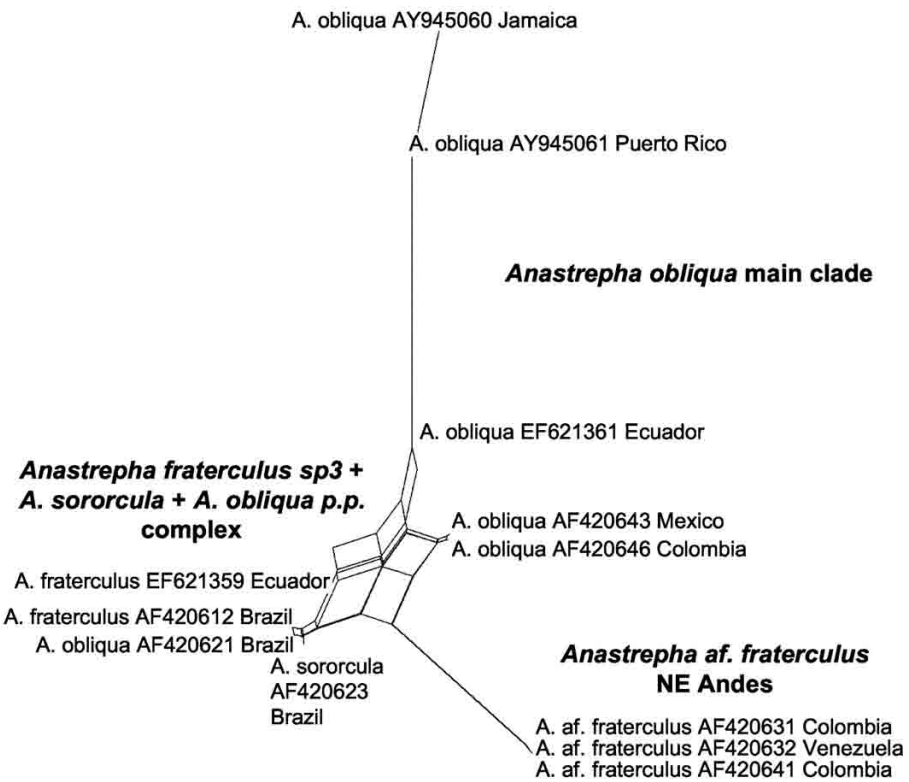

Figure 2
Phylogenetic network (Neighbour Net) generated with SplitsTree4 software showing relationships within the *Anastrepha fraterculus-obliqua-sororcula* complex. *A. fraterculus sp.3* refers to the nomenclature of cryptic species of Ruiz *et al.* (2007).

Cytochrome oxidase II sequence. A region of 224 pb was analysed in 31 individuals of *A. fraterculus* from five host and four different places and 9 individuals from 5 other species. In addition, four sequences from Argentinean and South Brazilian populations of *Anastrepha fraterculus* retrieved from GenBank were included in the analysis. The base composition established for this region was A = 34.38%, T = 33.93%, C = 14.78% and G = 16.96%. The MP analysis of the COII matrix resulted in 130 trees (L = 147, CI = 0.694 and RI = 0.761). In the strict consensus tree (Fig. 3), there is no internal resolution within the *Anastrepha fraterculus* clade (BS 54%), apart from the grouping of South Brazilian and Argentinean samples, supported by one synampomorphic substitution. Nine haplotypes of Ecuadorian *A. fraterculus* were found, each differing by one base (GenBank Accesions EF621367, EU332692-6). There was a dominant haplotype present in all populations, and eight minor variants. The haplotype network indicated that all these variants resulted from single, independent mutational events from the major haplotype, including a deletion (- T 160), an insertion (G 203) and six substitutions (Fig. 4). Five variants were encountered in southern Ecuador (Azuay and Loja) and three variants in northern Ecuador (Pichincha). The haplotype network also separated Andean-Ecuadorian and South Brazilian/Argentinean samples by two putative mutational steps.

Discussion

The COI analysis showed that Andean-Ecuadorian populations of *Anastrepha fraterculus* are closely related to lowland South-American populations of the *A. fraterculus* complex, including some populations of *A. sororcula* and *A. obliqua*. This grouping in supported by a high bootstrap support of 89% (Fig. 1). Consequently, Andean-Ecuadorian populations of *A.*

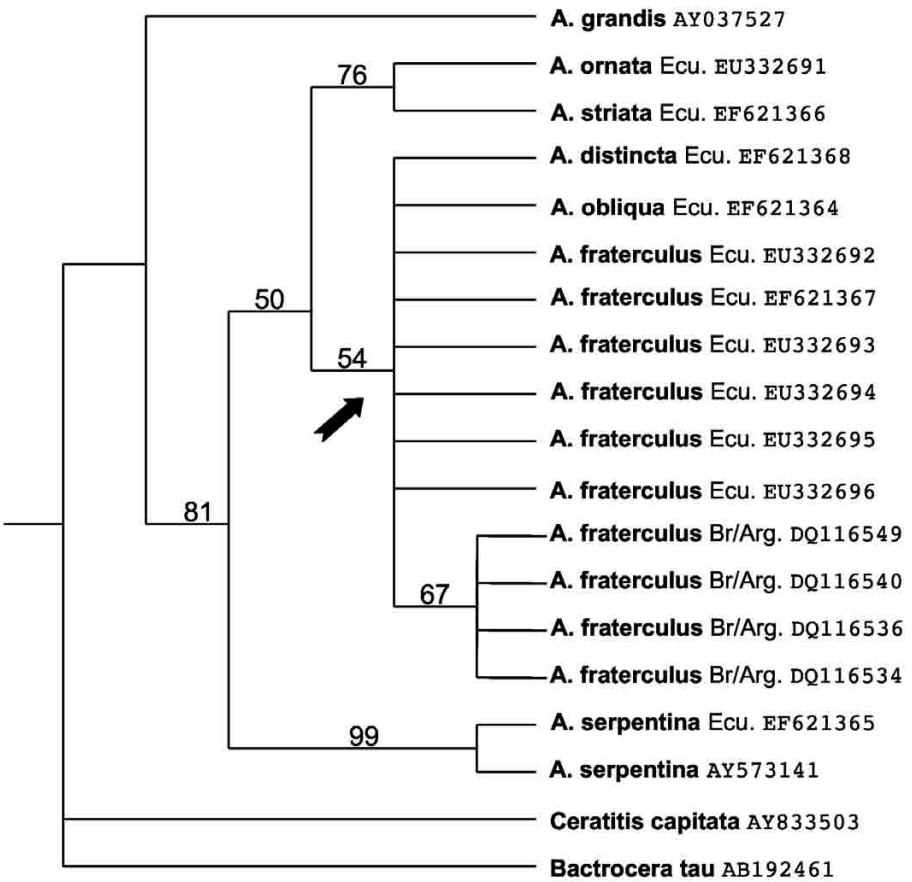

Figure 3
Strict consensus tree from MP Analysis of COII sequences (with bootstrap scores on branches) of *Anastrepha spp.* with *Ceratitis capitata* and *Bactrocera tau* as outgroups. The arrow indicates the *A. fraterculus* species complex.

fraterculus are not related to the north-eastern Andean Colombian and Venezuelan (Mérida) populations, which form a highly divergent and strongly supported clade (BS 99%), as noted by Smith-Caldas *et al.*(2001). The phylogenetic analysis done by Ruiz *et al.* (2007) using Transformer gene supports these relationships. The Andean Ecuadorian populations studied here correspond to the *A. fraterculus* sp.3 type from the Transformer gene analysis, which confirms that they belong to a genetic group distributed from the Andes to Brazil.

Polytomies in the COI tree mostly reflect lack of variation within several clades. The COII tree shows the monophyly of the Ecuadorian plus lowland South American populations of *A. fraterculus*, in agreement with the COI results. Divergence between the Ecuadorian and Argentinean groups indicates a marked geographic structure within *Anastrepha fraterculus*, which was not clear with COI data, and that is in need of further investigation at the continental scale.

On the other hand, populations of *A. fraterculus* seems homogeneous within smaller areas. Patterns of genetic variation are indeed similar in Argentina and adjacent Brazil (Alberti *et al.* 2008), and in Ecuador, with an haplotype at very high frequency at the COII locus, and minor variants of it.

It is to be noted that COII was more informative than the more widely used COI, the latter being invariable throughout Ecuador in the portion sequenced. COII showed nine haplotypes and even some geographical structure within Ecuador, with six haplotypes present in the south and four in the north of the country.

This study supports the monophyly of *A. fraterculus* in the Ecuadorian Andes. All samples are homogeneous in COI-II sequences.

It is necessary to apply multi-disciplinary approaches and to use more genetic markers in a larger *Anastrepha fraterculus* sampling throughout the Neotropics, in order to reach a comprehensive overview of the structure and evolution of this species complex, which is still incompletely understood.

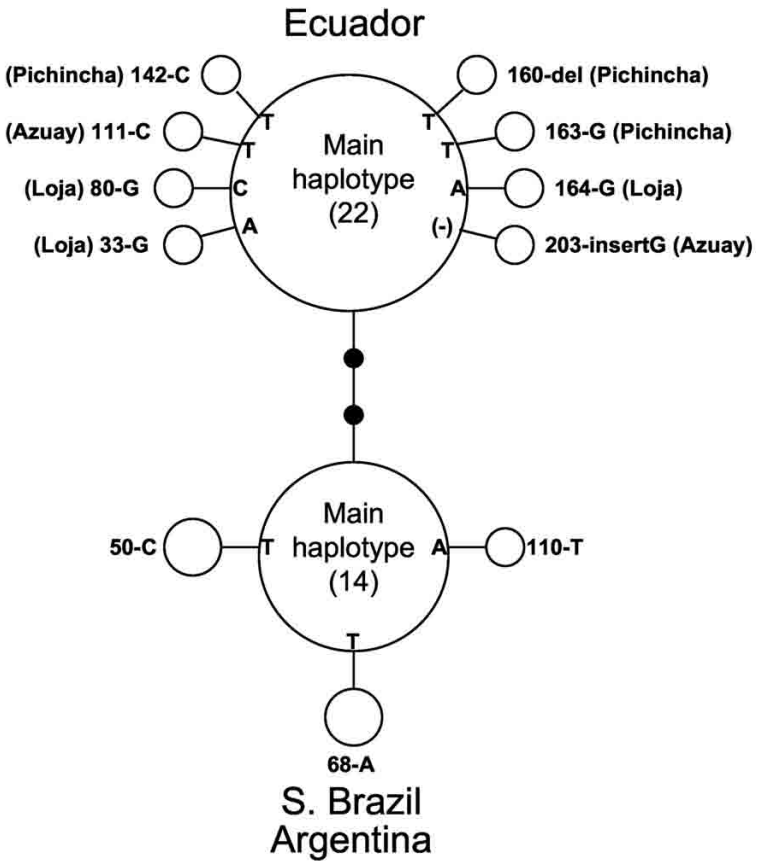

Figure 4
Haplotype network generated with TCS software of *Anastrepha fraterculus* COII sequences. Empty circles are proportional to the number of individuals having the corresponding haplotype identified by a point mutation, black circles represent unrecorded mutations necessary to connect observed haplotypes.

Acknowledgments. This study was partially financed by PROMSA (IQ-CT-004). We are grateful to Patricio Ponce and his students for providing specimens. Thanks are due to Stéphane Dupas for his comments on a previous version of this manuscript.

References

Alberti A. C., Calcagno G., Saidman B. O., Vilardi J. C. 1999. Analysis of the genetic structure of the natural population of *Anastrepha fraterculus* (Diptera: Tephritidae). *Annals of the Entomological Society of America* **92**: 731-736.

Alberti A. C., Confalonieri V., Zandomeni Ruben., Vilardi J. C. 2008. Philogeographic studies on the natural population of the South American fruit fly, *Anastrepha fraterculus* (Diptera:Tephritidae). *Genetica* **132:** 1-8.

Alberti A. C., Rodriguero M., Gómez P., Saidman B. O., Vilardi J. C. 2002. Evidence indicating that Argentine populations of Anastrepha fraterculus (Diptera: Tephritidae) belong to a single biological species. *Annals of the Entomological Society of America* **94**: 505-512.

Aluja M.1994. Bionomics and management of *Anastrepha*. *Annual Review of Entomology* **39**: 155-178.

Baker A. C., Stone W. E., Plummer C. C., and McPhail H. 1944. A review of studies in the Mexican fruit fly and related Mexican examples. *USDA Miscelaneous Publications* V. 531: 1-155.

Barr N. B., Cul L., McPheron B. 2005. Molecular Systematics of Nuclear gene *period* in Genus *Anastrepha* (Tephritidae). *Annals of the Entomological Society of America* **98 (2):**173-180.

Basso A., Manso F. 1998. Are *Anastrepha fraterculus* chromosomal polymorphisms an isolation barrier? *Cytobios* **83**: 237-244.

Basso A., Sonvico A., Quesada-Allue L. A., Manso. F. 2003. Karyotipic and Molecular Identification of Laboratory Stocks of the South American Fruit Fly *Anastrepha fraterculus* Wied. (Diptera: Tephritidae). *Journal of Economic Entomology*. **96**: 1237-1244.

Bernasconi M., Valsangiacomo C., Piffaretti J.-C., Ward P. 2000. Phylogenetic relationships among Muscoidea (Diptera: Calyptratae) based on mitochondrial DNA sequences. *Insect Molecular Biology* **9**: 67-74.

Bush G. L. 1962. The cytotaxonomy of some Mexican fruit flies in the genus *Anastrepha* (Diptera: Tephritidae). *Psyche* **69**: 87-101.

Cáceres C., Segura D., Vera M. T., Wornoayporn V., Cladera J., Teal P., Sapountzis P., Bourtzis K., Zacharopoulou A., Robinson A. 2009. Incipient speciation revealed in *Anastrepha fraterculus* (Diptera, Tephritidae) by studies on mating compatibility, sex pheromones, hybridization, and cytology. *Biological Journal of the Linnean Society* **97:** 152-165.

Calles J. 2002. Fluctuación poblacional de *Anastrepha spp*. y *Ceratitis capitata* (Diptera: Tephritidae) en los valles de Patate y Guayllabamba y notas sobre sus parasitoides. Tesis Ciencias Exactas y Naturales, Pontificia Universidad Católica del Ecuador. Quito-Ecuador.

Caraballo J. 2001. Diágnosis y clave pictórica para las especies del género *Anastrepha* Schiner, 1868 (Diptera; Tephritidae) de importancia económica en Venezuela. *Entomotropica* **16**: 157-164.

Carrol L. E., Norrbom A. L., Dallwitz M. J., Thompson F. C. 2004 onwards. *Pest fruit flies of the world – larvae.* http//delta-intkey.com.

Clement M., Posada D., Crandall K.A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657-1660.

Farrel B. D. 2001. Evolutionary assembly of the milkweed fauna: cytochrome oxidase I and the of *Tetraopes* beetles. *Molecular Phylogenetics and Evolution* **18**: 469-468.

Hernández-Ortiz V., Gómez-Anaya J. A. Sánchez A., McPheron B. A., Aluja M. 2004. Morphometric analysis of Mexican and South American populations of the *Anastrepha fraterculus* complex (Diptera: Tephritidae) and recognition of a distinct Mexican morphotype. **94**: 487-99.

Huson D. H., Bryant D. 2006. Applications of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* **23**: 254-267.

Ludeña B. 2006. Guía práctica para el Laboratorio de Biología Molecular. Centro de Publicaciones de la Pontificia Universidad Católica del Ecuador. Quito. Ecuador.

MacPheron B. A., Han H.-Y., Silva J. G., Norrbom A. L. 1999. Phylogeny of the genera *Anastrepha* and *Toxotrypana* (Trypetinae: Toxotripanini) based upon 16S rRNA mitochondrial DNA sequences, p. 343- 361 *in*: **Aluja M., Norrbom A. L. (eds.)**, *Fruit flies (Tephritidae): phylogenie and evolution of behavior*. CRC Press, Boca Raton, Florida, 944 p.

Morales D., Cevallos V. 2003. Variación cromosómica de *Anastrepha fraterculus* Wiedmann (Tephritidae) en poblaciones ecuatorianas. *Revista de la Pontificia Universidad Católica del Ecuador* **71**: 91-99.

Morgante J. S., Malavasi A., Bush G. L. 1980. Biochemical Systematics and evolutionary relationships of Neotropical *Anastrepha*. *Annals of the Entomological Society of America* **73**: 622-630.

Morgante J. S., Selivon D., Solferini V. N., Matioli S. R. 1993. Evolutionary patterns in specialist and generalist species of *Anastrepha*, p. 15-20. *in*: **Aluja M., Liedo P. (eds.)**, *Fruit flies Biology and Management*. Springer-Verlag, New York, NY, 492 p.

Morgante, J. S., Malavasi A. 1985. Genetic Variability in populations of the South Americas Fruit fly *Anastrepha fraterculus* (Tephritidae). *Revista Brasileira de Genetica* **8**: 241-247.

Morlais I., Severson D. 2002. Complete mitochondrial DNA sequence and aminoacid analysis of the cytochrome c oxidase subunit I (COI) from *Aedes aegypti*. *DNA sequence* **13**: 123-127.

Roe A., Sperling F. 2007.Patterns of evolution of mitochondrial cytochrome c oxidase I and II and implications for DNA barcoding. *Molecular Phylogenetics and Evolution* **44**(1): 325-345.

Ruiz M., Milano A., Salvemini M., Elrín-López J., Perondini A., Selivon D., Polito C, Saccone G. Sánchez L. 2007. The gene *Transformer* of *Anastrepha* fruit flies (Diptera, Tephritidae) and its evolution ijn insects. PloS ONE.**2**(11): e1239. dol:10.1371/journal.pone.0001239

Selivon D., Perondini A. L. P, 1997. Evaluation techniques for C and ASG banding of the mitotic chromosomes of *Anastrepha* species (Diptera: Tephritidae). *Brazilian Journal of Genetics* **20**: 651-653.

Selivon D., Perondini A. L. P. 1998. Eggshell morphology in two cryptic species of *Anastrepha fraterculus* complex (Wiedemann) (Diptera: Tephritidae). *Annals of the Entomological Society of America* **91**: 473-478.

Selivon D., Perondini A. L. P., Rocha L.S. 2005. Karyotype characterization of sex chromosomes of *Anastrepha* fruit flies (Diptera, Tephritidae). *Neotropical Entomology* **34**: 273-275.

Smith-Caldas M., McPheron B., Silva J., Zucchi R. 2001. Phylogenetic Relationships among species of the *fraterculus* group (*Anastrepha*: Diptera, Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase I. *Neotropical Entomology* **30**: 565-573.

Solferini V. N., Morgante J. S. 1987. Karyotype study of eight species of *Anastrepha* (Diptera: Tephritidae). *Caryologia* **40**: 229-241.

Steck G. J. 1991. Biochemical systematics and population genetic structure of *Anastrepha fraterculus* and related species (Diptera: Tephritidae). *Annals of the Entomological Society of America* **84**: 10-28.

Steck G. J., Sheppard W. S. 1993. Mitochondrial DNA variation in *Anastrepha fraterculus*, pp. 9-14 *in*: **Aluja M., Liedo P. (eds.)**, *Fruit flies Biology and Management*. Springer-Verlag, New York, NY, 492 p.

Steck G. J.1999. Taxonomic status of *Anastrepha fraterculus*, p. 13-20 *in*: Proceedings workshop, *The South American fruit fly Anastrepha fraterculus (Wied.): advances in artificial rearing, taxonomic status and biological studies*. IAEA-TECDOC-1064, Vienna, Austria.

Stone A. 1942. *The fruit flies of genus Anastrepha.* US Department of Agriculture Miscelaneous Publications 439, 112 p.

Swofford D. L. 2000. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4. Sinauer Associates, Sunderland, Massachusetts.

White I. M., Elson-Harris M. M. 1992. *Fruit flies of economic importance: their identification and Bionomics*. CAB International, Oxon, UK. 610 p.