Phylogeography of the Mountain Tapir (*Tapirus pinchaque*) and the Central American Tapir (*Tapirus bairdii*) and the Origins of the Three Latin-American Tapirs by Means of mtCyt-B Sequences

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1. Introduction

The Perissodactyla order is a very old group of mammals (around 60 Millions years ago, MYA). In the fossil record, there are representative specimens from five main superfamilies (Tapiroidea, Rhinocerotoidea, Chalicotheroidea, Equoidea and Brontotheroidea) including 14 different families (Savage and Long 1986; Holbrook 1999), although the phylogenetic relationships among these superfamilies are not well resolved. The order had its maximum diversity peak during the Eocene, but during the upper Oligocene, 10 of the 14 families became extinct (Radinsky 1969; Froehlich 1999; MacFadden 1992; Metais et al. 2006). Currently, only three superfamilies and three families are present (Tapiridae, Rhinocerotidae and Equidae).

The first species of the Tapiroidea superfamily appeared in the last phase of the Paleocene and in the lower Eocene (55 MYA) at the same time as the original species of Equidae and Chalicotheriidae and before the apparition of Brontotheriidae and Rhinocerotidae, which appeared close to the end of the Eocene. Some of these original Tapiroidea forms (*Heptodon*, Helaletidae family from the lower Eocene of Wyoming, Radinsky 1963, 1965), showed very similar morphologic resemblances to the current tapirs (*Tapirus*). Some families in the fossil record, such as Hyrachyidae (*Hyrachyus*) of the middle Eocene (45 MYA) of North America

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and Europe have been conformed to a transition family between Tapiroidea and Rhinocerotoidea (Radinsky 1967, 1968). However, other authors (Holbrook 1999; Colbert 2005) consider Tapiroidea as a taxon which excludes forms related to Rhinocerotoidea. Several families within Tapiroidea are well recognized by all the authors. These are the cases of Deperetellidae (Middle Eocene-Lower Oligocene from Asia; Tsubamoto et al. 2005), Lophiodontidae (Lower and Upper Eocene from Europe and Middle Asian Eocene; McKenna and Bell 1997), Lophialetidae (Middle-Upper Asian Eocene; McKenna and Bell 1997), and Helaletidae (Lower Eocene-Middle Oligocene from North America and Upper Eocene-Lower Oligocene from Asia).

The current Tapiridae family (Gray 1821) is composed of a unique genus, *Tapirus* (Brünnich 1772). The oldest fossil records of this family are dated from the Oligocene of Europe (33-37 MYA), and their fossils have been frequently found in Europe, North America and Asia (Hulbert 1995). Following Radinsky (1965), the Tapiridae descended from the Helaletidae, through the genus *Colodon*. Colbert (2005) defined the Tapiridae family as the clade conformed by the most recent common ancestor of *Protapirus* until the current *Tapirus*. The family has been around since the Lower Oligocene and includes the genera *Protapirus* and *Tapirus*, *Miotapirus* (North-America), *Megatapirus* (Asia), *Tapiravus* (North-America), *Tapiriscus* (Europe), *Eotapirus* (Europe), *Palaeotapirus* (Europe) and *Plesiotapirus* (Asia).

The oldest record of *Tapirus* comes from the European Oligocene, where the fossil remains are found until the Pleistocene (McKenna and Bell, 1997). In North America, the *Tapirus* records indicate that they were present in the Middle Miocene through the present (Hulbert 1995), while for Asia the records indicate that *Tapirus* has been in existence since the Lower Miocene (Deng 2006). Around 20 different *Tapirus* species are recognized for North-America, Europa and Asia.

Of the current four species, two are present in South-America (*T. terrestris* and *T. pinchaque*), one in Central America (*T. bairdii*) and another is present in Asia (*T. indicus*). *T. terrestris* is widely distributed across a great part of South America, including Colombia, Venezuela, Surinam, Guyana, French Guyana, Ecuador, Peru, Bolivia, Brazil, Paraguay and Argentine. *T. pinchaque* is geographically found in the Northern and Central Andes, and is adapted for living at high altitude mountains, in Venezuela, Colombia, Ecuador and northern Peru. *T. bairdii* is distributed from south-eastern Mexico and throughout all of Central America (excluding El Salvador) to the western Andes, in the Colombian and Ecuadorian Chocó. *T. indicus* lives on a very fragmented area of Vietnam, Cambodia, Burma, Sumatra, Thailand, Malaysia and Toba islands (Brooks et al. 1997).

From a molecular genetics point of view, only a few works have been published with *Tapirus*. The first one was the work of Ashley et al. (1996), where the genetics relationships among the *Tapirus* species were analyzed by means of the mitochondrial Cytochrome Oxidase subunit II (mtCOII) gene sequences collected from *T. terrestris*, (two samples) *T. bairdii* (two samples) *T. indicus* (two samples) and *T. pinchaque* (one sample). The second work, completed by Norman and Ashley (2000), included a new sequence of *T. pinchaque*. The authors partially sequenced the 12S rRNA for the eight *Tapirus* specimens that they studied. These two mt genes that were used by previous studies contradict each other so that there is no consensus of the relationship between South/Central American tapirs.

Norton and Ashley (2004a,b) published two DNA microsatellite works with wild and captive *T. bairdii* populations. Very recently, a new work on the genetics biogeography of *T. terrestris* has been published (Thoisy et al. 2010) showing dispersion of this species from the western Amazon to the rest of South America.

However, no study has included a large amount of samples of the three Latin American *Tapirus* species. Among the molecular markers relevant for phylogeography, biosystematics, and genetic structure studies in mammal populations, the mtCyt-b gene is commonly used (Patton et al. 2000; Cortez-Ortiz et al. 2003). Herein, we sequenced 201 individuals belonging to the three *Tapirus* species presented in South America (*T. terrestris, T. pinchaque*) and in Central America (*T. bairdii*) for the mtCyt-b.

The main aims of the present study were as follows: 1- To determine the gene diversity levels for the three Latin America *Tapirus* species at the mtCyt-b gene and the degree of genetic divergence among these three species; 2- To analyze the possible demographic historical changes (population expansions or bottlenecks) in the three Latin American *Tapirus* species; 3- To provide new data on the phylogenetics relationships of the three *Tapirus* species; 4- To search for possible correlations among the time splits among the haplotypes found within *T. pinchaque* and within *T. bairdii* (for *T. terrestris*, this is shown elsewhere) and 5- To analyze the possible spatial genetic structure in two species (*T. pinchaque* and *T. bairdii*).

2. Material and methods

A total of 201 *Tapirus* samples were analyzed. Of these samples, 141 belonged to *T. terrestris* from different regions of Colombia [41 animals; one from Bajo Sinú-Tierra Alta, (Córdoba Department), 2 from Mesay River (Caquetá Department), one from Fondo Canaima, (Vichada Department), 18 from Leticia to San Juan de Atacuarí (Amazonas Department), 7 from Eastern Colombian Llanos (Meta Department), 3 from Pto. Inirida (Guania Department), 3 from Palomino River-Sierra Nevada de Santa Marta and 6 from Antioquia Department)], Venezuela (5 from El Zulia, Maracaibo), French Guiana (11 from Carnopi River), Ecuador (7 animals; 4 from Limoncocha, Sucumbios and 3 from Coca, Sucumbios), Peru [30 animals; one from Arica (Curaray River), 2 from Napo River (Nueva Vida and Mazán), 7 from Nanay River, one from Requena (Ucayali River), one from Bretaña (Canal del Puhinauva-Ucayali River), 4 from Pucallpa (Ucayali River), and 15 from Pto. Maldonado (Madre de Dios River)], Bolivia (11 animals; 9 from Mamoré River, one from Chimoré River and one from Villa Bella at the Beni River), Brazil [24 animals; 2 from Yavarí River, 12 from Tabatinga, 2 from Negro River, 2 from Santarem (Pará state) and 6 from Amazon mounth (Pará state)], Paraguay (one animal from Hernandarias) and Argentina (4 animals from the Yungas area in Salta-Jujuy) (Figure 1). Additionally, one animal from the Barcelona Zoo (Spain), 5 animals from the Cincinnati Zoo (Ohio, USA) and one animal of unknown origin were also analyzed. More details of origins of these samples are recorded in Ruiz-Garcia et al. (2012b). Of the remaining samples, 30 belonged to *T. pinchaque* and 30 to *T. bairdii*. The geographical origins of the *T. pinchaque* samples in Columbia were as follows: six samples from Los Nevados National Park at the Risaralda Department, 14 samples from the Tolima Department (one from the Resguardo Vereda La Bella-Planadas, Marquetalia; three from

Gaitania; four from the Vereda San Miguel-Planadas; two from the Ereje-Blanco River basin; one from La Azulena; one from Peñas Blancas; and two from the Saldaña River basin), and two samples from the Huila Department (Vereda Marengo). Samples within Ecuador came from Papallacta (two samples, Provincia Napo), Cosagua (one sample, Provincia Napo), La Bonita (one sample, Provincia Sucumbios) and Sangay National Park (four samples, Las Culebrillas locality). The samples of this very elusive and rare species were composed of small pieces of skins, bones and teeth of specimens collected by F. Kaston and M. Ruiz-García in Colombia and A. Castellanos in Ecuador.

The 30 *T. bairdii* samples were collected as follows: 14 animals were sampled at the Darien region in Panamá (near to the Columbian frontier), nine animals were sampled at the Braulio Carrillo National Park in Costa Rica, three exemplars were sampled at the Petén region in Guatemala and another four specimens were sampled near Campeche in the Mexican Yucatán. These samples consisted of small amounts of blood (the Darien samples) and hairs with roots (for the remainder animals). Figure 2 shows the sampling localities for *T. pinchaque* and *T. bairdii*.

Fig. 1. Map of the geographical distribution of *Tapirus terrestris* and the sampling points. Into parentheses, the number of individuals sampled in each point.

Fig. 2. Map of the geographical distribution of *Tapirus bairdii* and *Tapirus pinchaque* and the sampling points. Into parentheses, the number of individuals sampled in each point.

2.1 Molecular analyses

DNA from teeth, bones, muscle, and skins, were obtained with the phenol-chloroform procedure (Sambrook et al. 1989), while DNA samples from hair and blood were obtained with 10% Chelex® 100 resin (Walsh et al. 1991). Amplifications for mtCyt-b gene were performed with the primers L7 (5' ACC AAT GAC ATG AAA AAT CAT CGT T 3') - H6 (5' TCT CCA TTT CTG GTT TAC AAG AC 3'), which had been designed for perissodactyles (Tougard et al. 2001). The PCRs were performed in a 50-µl volume with reaction mixtures including 10 µl of 10x Buffer, 7 µl of 25mM MgCl$_2$, 2 µl of dNTPs (dNTP Mix Promega, 40mM), 4 µl (100 µM) of each primer, one unit of Taq DNA polymerase (Gotaq, Promega), 2 µl of DNA from blood, skin or muscle tissue or 2-10 µl of DNA from hairs and teeth and a variable quantity of H$_2$0. PCR reactions were carried out in a Geneamp PCR system 9600 (Perkin Elmer) and in an iCyclerTM BioRad thermocycler. The temperatures employed were as follows: 94 °C for 5 minutes, 35 cycles of 50 s at 94 °C, 50 s at 53 °C and 1.5 minutes at 72 °C and a final extension time for 10 minutes at 72 °C. All amplifications, including positive and negative controls, were checked in 2 % agarose gels, employing the molecular weight marker ϕX174 DNA digested with *Hind* III and *Hinf* I and HyperLadder IV and the gels were visualized in a Hoefer UV transiluminator. Those samples that amplified were purified

using membrane-binding spin columns (Qiagen). The double-stranded DNA was directly sequenced in a 377A (ABI) automated DNA sequencer. The samples were sequenced in both directions and all the samples were repeated to ensure sequence accuracy.

2.2 Data analyses

2.2.1 Genetic diversity and heterogeneity analyses

The statistics employed to determine the genetic diversity among the three neotropical tapir species were the number of polymorphic sites (S), the number of haplotypes (H), the haplotypic diversity (H_d), the nucleotide diversity (π), the average number of nucleotide differences (k) and the θ statistic by sequence.

Different tests were carried out to measure genetic heterogeneity, and possible gene flow estimates, among these tapir species. These tests were those of Hudson et al. (1992a,b) (H_{ST}, K_{ST}, K_{ST}^*, Z, Z*), Hudson (2000)'s Snn test and the chi-square test on the haplotypic frequencies with permutation tests with 10,000 replicates as well as the G_{ST} statistic from the haplotypic frequencies and the γ_{ST}, N_{ST} and F_{ST} (Hudson et al. 1992a) statistics from the nucleotide sequences.

2.2.2 Demographic genetics analyses in the three Neotropical tapir species

Diverse strategies were used to determine possible demographic changes across the natural history of the three Neotropical tapir species. The procedures employed were as follows: 1- The mismatch distribution (pairwise sequence differences) was obtained following the method of Rogers and Harpending (1992) and Rogers et al. (1996). Two theoretical curves were obtained (population growth and bottleneck show characteristic signatures in histograms yielding the relative frequencies of individual pairs that differ by *i* nucleotide sites), one assuming a constant population size and another assuming a population expansion with a θ_0 (= $2N_{e0}\mu$). N_{e0} is the female effective number before growth and μ is the mutation rate per generation for the population before the expansion. And, θ_1 (= $2N_{e1}\mu$) with N_{e1} as the female effective number after growth for the same population after the expansion and when $\tau = 2\mu t$ (t = number of generations). This is the time elapsed from the population expansion in a mutational temporal scale. The empirically observed distribution was compared to these two theoretical curves. Some coefficients were used to determine the similarity between the observed and the theoretical curves. These were the raggedness *rg* statistic (Harpending et al. 1993; Harpending 1994), the Mean Absolute Error (MAE) between the observed and the theoretical mismatch distribution (Rogers et al. 1996) and the R_2 statistic of Ramos-Onsins and Rozas (2002). 2- The Fu & Li D and F tests (Fu and Li 1993), the Fu F_S statistic (Fu 1997) and the Tajima D test (Tajima 1989a), originally created to detect natural selection affecting DNA sequences, were also used to determine possible population size changes (Simonsen et al. 1995; Ramos-Onsins and Rozas, 2002). All these statistics, tests, and analyses were obtained by means of the DNAsp 4.1.03 and Arlequin 3.1 programs.

2.2.3 Phylogenetic analyses and molecular temporal splits among the *Tapirus* species

The mtCyt-b sequence alignments were carried out manually and with the DNA Alignment program (Fluxus Technology Ltd).

To reconstruct the phylogeny and the split times of the three neotropical tapirs, several analyses were undertaken. The first was the application of the FindModel program to determine, among 28 different evolutionary nucleotide models, which one was the most probable for the tapir sequence set.

A Bayesian procedure was employed with the BEAST v. 1.4.8 program (Drummond and Rambaut 2007) to determine the phylogenetic relationships and the temporal splits among the three neotropical tapirs. In this analysis, we employed the sequences of 11 *T. bairdii*, 14 sequences of *T. pinchaque*, 40 sequences of *T. terrestris* and one sequence of *T. indicus*. We reduced the number of exemplars in this analysis to obtain some manageable trees. These animals basically represented all the haplotypes found. The precise and detailed genetic relationships among the 141 *T. terrestris* can be observed in Ruiz-García et al. (2012b). We performed this analysis to estimate the time to most recent common ancestor (TMRCA) for the different tapir clades found. Analysis was performed using a GTR (General Time Reversible) model of nucleotide substitution with gamma distributed rate variation among sites, and four rate categories (GTR+G) because it was determined to be the better model using the FindModel program. We employed diverse calibration points following Ashley et al. (1996) and Norman and Ashley (2000) and several paleontological records (Patterson and Pascual 1968; Simpson 1980; Webb 2006) in the same Bayesian tree. For this, we used a combination of a temporal split between the ancestor of *T. indicus* and the ancestor of the three neotropical tapirs of 18 ± 1 MYA (mtCOII gene; Ashley et al. 1996) and a temporal separation of 2.7-3.1 ± 0.5 MYA between the ancestors of *T. terrestris* and *T. pinchaque*, during the Great American Biotic Interchange after the reestablishment of the land bridge between North and South America. A Yule process of speciation and a relaxed molecular clock with an uncorrelated log-normal rate of distribution was assumed (Drummond et al. 2006). Results from the two independent runs (20,000,000 generations with the first 2,000,000 discarded as burn-in and parameter values sampled every 100 generations) were combined. The Bayesian trees showed the posterior probability values, which provide an assessment of the degree of support of each node on the tree, the estimations of temporal splits in each node and the lower and upper 95 % highest posterior densities (HPD). Monophyletic constraints were imposed for nodes that were used to calibrate the evolutionary rates. To analyze the autocorrelation tree (ACT) and effective sample size for parameter estimates (ESS), the program Tracer version 1.4 (Rambaut and Drummond 2007) was employed. The final tree was estimated in the TreeAnnotator v1.4.5 software and visualized in the FigTree v. 1.2.2 program.

To estimate other possible independent divergence times among the haplotypes found in the four *Tapirus* species, a Median Joining Network (MJ) (Bandelt et al. 1999) was applied by means of the software Network 4.2.0.1 (Fluxus Technology Ltd). Once the haplotype network was constructed, the ρ statistic (Morral et al. 1994) was estimated. This statistic measures the age of an ancestral node in mutational units. This value is transformed into years by multiplication with the mutation rate. Additionally, the standard deviation (σ) was calculated (Saillard et al. 2000). The ρ statistic is unbiased and highly independent of past demographic events. These events could have influenced the shape of a given evolutionary tree, but these events only influence the error of the time estimated and do not increase or decrease the time. We employed a mutation rate of 5.6 x 10^{-3} substitutions per site per

million years. It was a mean value estimated by Ruiz-García (unpublished result) for a variety of neotropical species (pink river dolphin, Andean bear, deer, and several genera of Neotropical Primates as *Saimiri, Cebus, Alouatta*, *Ateles*, *Lagothrix* and *Aotus*). For *T. pinchaque*, this mutation rate represented one mutation each 196,881 years and for *T. bairdii* and the set of all the *Tapirus* sequences taken together, this equaled one mutation each 203,384 years.

2.2.4 Spatial genetics analyses applied to *Tapirus pinchaque* and to *Tapirus bairdii*

Several strategies were applied to determine if *T. pinchaque* and *T. bairdii* presented some significant spatial genetic trend because this could help to understand the evolutionary events that have determined the natural history of these two species. These strategies were as follows:

1. A Mantel's test (Mantel 1967) was used to detect possible overall relationships between a genetic matrix among individuals (Log-Det genetic distance with different pattern heterogeneity among lineages and different rates among sites with a Gamma distribution, Nei and Kumar 2000) and the geographic distance matrix among the individuals analyzed. In this study, Mantel's statistic was normalized according to Smouse et al. (1986). This procedure transforms the statistic into a correlation coefficient. The geographic distances were measured with the Spuhler's (1972) procedure, where

$$D = arcos \left(\cos X_{(i)} . \cos X_{(j)} + \sin X_{(i)} . \sin X_{(j)} \cos |Y_{(i)} - Y_{(j)}| \right) ,$$

where $X_{(n)}$ and $Y_{(n)}$ are the latitude and longitude of the *nth* individual sampled, respectively. The significance of the correlations obtained was tested using a Monte Carlo simulation with 5,000 permutations.

2. To determine possible isolation by distance among the haplotypes within the geographical area analyzed in *T. bairdii* and in *T. pinchaque*, the IBD version 1.2 software (Bohonak 2002) was employed. In this analysis, we used the quoted genetic distance against the geographical distance among the individuals sampled. The intercept and the slope of this relationship was calculated using Reduced Major Axis (RMA) regression (Sokal and Rohlf 1981; Hellberg 1994). Ten thousand randomizations (jackknife over population pairs and bootstrapping over independent population pairs) were executed to determine 95 and 99 % confidence intervals. The calculations were completed with non-transformed data and with log transformed data (genetic distance & geographical distance) jointly and separately.

3. A spatial autocorrelation analysis (Sokal and Oden 1978ab; Sokal and Wartenberg 1983; Sokal et al. 1986, 1987, 1989; Sokal and Jacquez 1991; Epperson 1990, 1993; Ruiz-García 1998, 1999 and Ruiz-García and Jordana 1997, 2000) was applied to the different haplotypes found in both species (separately, of course). The most frequent haplotype was weighted as 1, while the rest of the haplotypes were differentially weighted depending on the number of nucleotide substitutions differing from the most frequent haplotype. Autocorrelation coefficients and correlograms were estimated. For this, the Moran's I index and the Geary's c coefficient (Moran 1950; Durbin and Watson 1950) were employed

in the current study. In the case of *T. bairdii*, three distance classes (DC) were defined (3 DC: 0-161 km; 161-776 km; 776-1,893 km), while in the case of *T. pinchaque*, four DC were defined (4 DC: 0-71 km; 71-163 km; 163-494 km; 494-772 km). The criteria used, to choose these particular distance classes, was a relatively equal number of point pairs, among distance classes. To use these statistics, individuals must be connected using some type of network, which simulates as realistically as possible, the relationships existing between them. In this case, three network connections were used. The first method was binary, with all pairs of individuals connected at different specified distance classes. These connections were determined by using the possible gene flow routes between the individuals considered (Trexler 1988). Also, the Gabriel-Sokal network (Gabriel and Sokal 1969; Matula and Sokal, 1980) and the Delaunay's triangulation with elimination of the crossing edges (Ripley 1981; Upton and Fingleton 1985; Isaaks and Srivastava, 1989) were used. However, the results were very similar in each case. The Bonferroni (Oden 1984), Oden's Q and the Kooijman's tests were calculated with SAAP 4.3 software to determine the statistical significance of autocorrelation.

4. Another different spatial autocorrelation analysis was carried out. In this case, for *T. bairdii*, seven variables (the polymorphic nucleotide sites) were employed, while 47 variables were used for *T. pinchaque*. Distograms (Degen and Scholz 1998; Vendramin et al. 1999) and correlograms were estimated in both cases among individuals. For the distograms, two procedures were employed: the Gregorious's (1978) genetic distance and the number of common haplotypes (Hamrick et al. 1993). For the correlograms, the Moran´s I index and the Geary´s c coefficient were employed as before. Three and four DC were employed in both *Tapirus* species. In this case, the significance of distograms, correlograms and autocorrelation coefficients was calculated by means of 1,000 Monte-Carlo simulations (Manly 1997) and 95 % confidence intervals were estimated (Streiff et al. 1998). Also, the Bonferroni procedure was employed to determine the significance of these autocorrelation coefficients. For this analysis, the SGS version 1.0d software was applied (Degen et al. 2001).

3. Results

3.1 Gene diversity, genetic heterogeneity and demographic changes in the three Neotropical tapirs

The mutation model, which best fitted for all the *Tapirus* sequences, was the Jukes-Cantor model if we consider the AIC criteria (AIC = 8.3177) or the GTR model of nucleotide substitution with gamma distributed rate variation among sites if we consider the maximum likelihood criteria (LnL = -3.2603).

Of the three *Tapirus* species studied, *T. terrestris* clearly showed the highest levels of genetic diversity (Table 1): $S = 107$, $H = 80$, $H_d = 0.984 \pm 0.003$, $\pi = 0.0114 \pm 0.0003$, $k = 10.335 \pm 4.743$ and $\theta_{per\ gene} = 19.376 \pm 4.739$. *T. pinchaque* showed the second level of gene diversity, while *T. bairdii* showed the lowest. Therefore, taking into account some relative gene diversity statistics, such as π, that are not affected by the sample size, *T. terrestris* presented 1.46 times more genetic diversity than *T. pinchaque* and 4.56 times more genetic diversity than *T. bairdii*. *T. pinchaque* showed 3.12 times more genetic diversity than *T. bairdii*.

	S	NH	Hd	π	K	θ per sequence
Tapirus terrestris	107	80	0.984 ±0.003	0.0114 ±0.0003	10.335 ±4.743	19.376 ±4.739
Tapirus pinchaque	47	10	0.895 +0.070	0.0078 +0.0046	7.029 +4.498	14.455 +5.565
Tapirus bairdii	7	6	0.800 ±0.114	0.0025 ±0.0005	2.182 ±1.306	2.390 ±1.251

Table 1. Genetic diversity statistics estimated for the three *Tapirus* species studied (*T. terrestris, T. pinchaque, T. bairdii*). S = number of polymorphic sites; NH = Number of Haplotypes determined; π = Nucleotide diversity; K = Average number of different nucleotides within each group analyzed; θ per sequence (= $2N_e\mu$), being N_e, the effective female population size, and μ, the mutation rate per generation.

The genetic divergence among the three neotropical *Tapirus* species by means of the mtCyt-b gene was highly significant (Table 2). For instance, the γ_{st}, N_{st} and F_{st} statistics yielded extreme genetic differentiation (0.789, 0.916 and 0.911, respectively) among the three species considered, with virtually no gene flow among them (Nm = 0.13, 0.05, 0.05, respectively). When the genetic heterogeneity was estimated by species pairs (Table 3), it was observable that the genetic differentiation of *T. bairdii* was noteworthy higher with regard to the two South-American species (F_{st} = 0.952 respect to *T. pinchaque* and F_{st} = 0.941 respect to *T. terrestris*), whereas between the two South-American species, the genetic differentiation was considerably lower (F_{st} = 0.491).

Genetic differentiation estimated considering the three *Tapirus* species analyzed	P	Gene flow	
χ^2 = 132.000 df = 88	0.0017*		
H_{ST} = 0.0485	0.0000*	γ_{ST} = 0.7898	Nm = 0.13
K_{ST} = 0.7851	0.0000*	N_{ST} = 0.9159	Nm = 0.05
K_{ST}* = 0.3789	0.0000*	F_{ST} = 0.9108	Nm = 0.05
Z_S = 462.7719	0.0000*		
Z_S* = 5.6642	0.0000*		
S_{nn} = 0.9849	0.0000*		

Table 2. Diverse genetic heterogeneity statistics (χ^2, H_{ST}, K_{ST}, K_{ST}*, Z_S, Z_S*, S_{nn}) and their associated probabilities taken together the three *Tapirus* species analyzed (*T. terrestris, T. pinchaque, T. bairdii*). All the probability values were significant (* P < 0.00001). Also some gene flow estimates (Nm) and the genetic heterogeneity statistics from which derived are shown. All the Nm estimates were lower than 1 indicating that genetic isolation exists between the three *Tapirus* species analyzed (*T. terrestris, T. pinchaque, T. bairdii*).

F_{ST}	γ_{ST}	1	2	3
1 *Tapirus terrestris*			0.280	0.803
2 *Tapirus pinchaque*	0.491			0.906
3 *Tapirus bairdii*	0.941	0.952		

Table 3. Genetic heterogeneity statistics (F_{ST}, γ_{ST}) among all the pairs of the different *Tapirus* species considered.
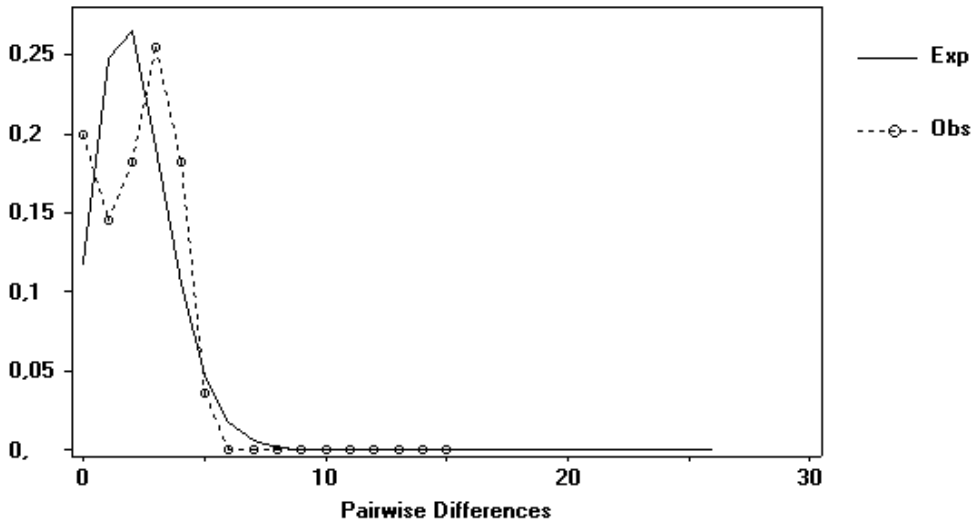
The results of the historical demographic analyses are provided in Figure 3 and Table 4. *T. terrestris* was the species which undoubtedly crossed a population expansion during its natural history. The mismatch distribution, as well as all the statistics applied, showed significant evidence of population expansion for this species. In the case of *T. pinchaque*, the evidence of population expansion is relatively weaker. Only three statistics showed significant evidence of a possible population expansion (Fu and Li D*, Fu and Li F* and Tajima D statistics), but the mismatch distribution analysis (and the *rg* statistic) as well as the Fu's F_s and the R2 statistics (the two last being the most powerful tests to detect demographic changes) did not reveal any significant trend. In the case of *T. bairdii*, the situation is no clear. The mismatch distribution (and the *rg* statistic) revealed a significant population expansion, but neither of the other statistics revealed a significant population change trend. Therefore, only *T. terrestris* showed an undisputable pattern of population expansion, whereas the other two *Tapirus* species showed inconclusive results with regard to this topic.
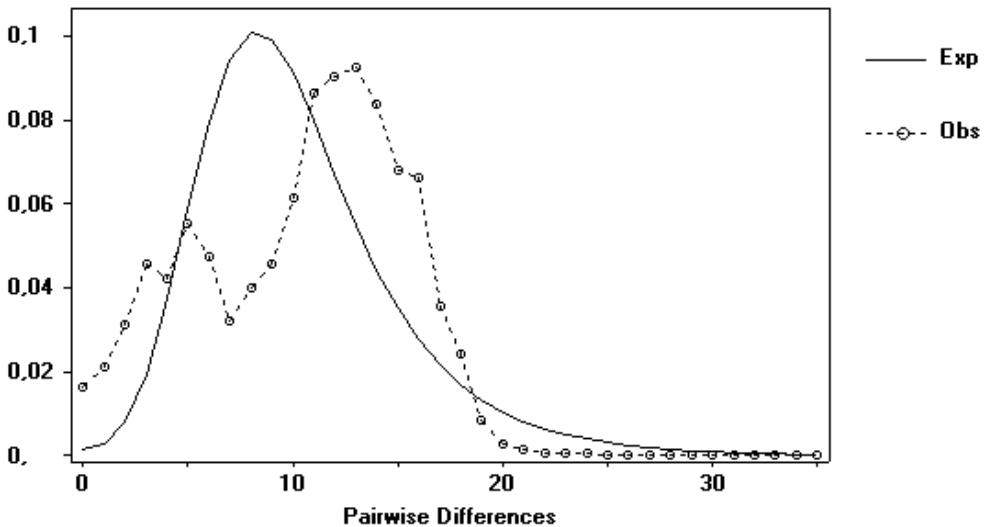
Tapirus terrestris
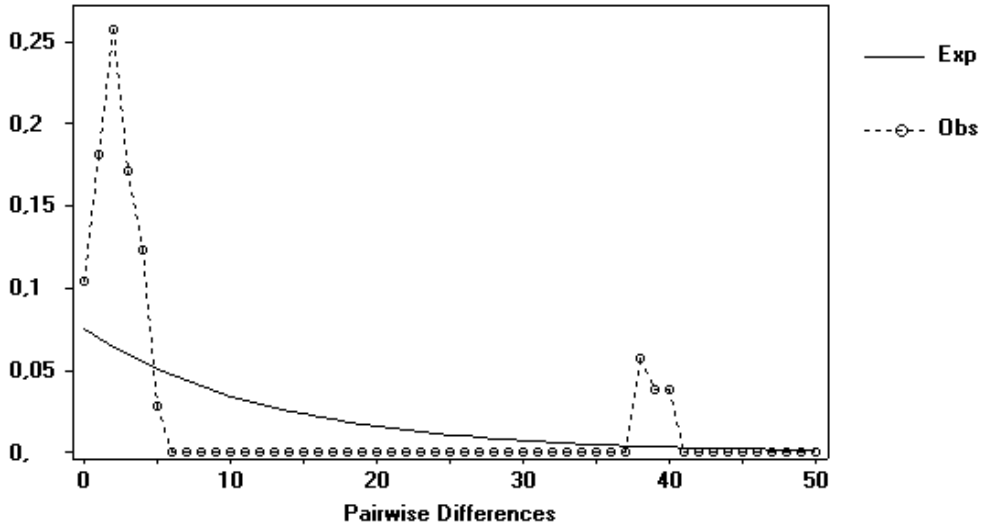
Tapirus pinchaque

Tapirus bairdii

Fig. 3. Historical demographic analyses by means of the mismatch distribution procedure (pairwise sequence differences) with mtCyt-b gene sequences in the three *Tapirus* species studied.

	Tajima D	Fu & Li D*	Fu & Li F*	Fu's Fs	raggedness *rg*	R2
Tapirus terrestris	P[D ≤ -1.642] = 0.018*	P[D* ≤ -4.00] = 0.004**	P[F* ≤ -3.59] = 0.004**	P[Fs ≤ -34.02] = 0.000**	P[rg ≤ 0.0035] = 0.0009**	P[R2 ≤ 0.0453] = 0.031*
Tapirus pinchaque	P[D ≤ -2.207] = 0.002**	P[D* ≤ -2.843] = 0.004**	P[F* ≤ -3.071] = 0.003**	P[Fs ≤ -1.028] = 0.302	P[rg ≤ 0.0362] = 0.195	P[R2 ≤ 0.1955] = 0.932
Tapirus bairdii	P[D ≤ 0.353] = 0.384	P[D* ≤ -0.193] = 0.402	P[F* ≤ 0.263] = 0.389	P[Fs ≤ -1.331] = 0.146	P[rg ≤ 0.0374] = 0.024*	P[R2 ≤ 0.1331] = 0.111

Table 4. Demographic statistics applied to the three neotropical *Tapirus* species studied. * P < 0.05; ** P < 0.01, significant population expansions.

3.2 Phylogenetics, temporal splits and phylogeography in *T. pinchaque* and *T. bairdii*

The phylogenetic relationships and the possible temporal splits among the four *Tapirus* species were analyzed by means of the BEAST v. 1.4.8 software (Figure 4). All four *Tapirus* species were monophyletic, with the first split between *T. indicus* and the three neotropical *Tapirus* species, the second split between *T. bairdii* and the clade *T. terrestris-T. pinchaque* and the third split between *T. terrestris* and *T. pinchaque*. All the probabilities of the main clades of each one of the *Tapirus* species are equal or almost equal to 1.

This analysis offered the following temporal separation estimations: the ancestor of *T. indicus* diverged around 17 MYA (95 % HPD: 15.1-19 MYA) from the ancestor of the three neotropical tapirs, while the ancestor of *T. bairdii* diverged around 10.9 MYA (95 % HPD: 6.3-16.3 MYA) from the *T. terrestris-T. pinchaque* clade and the ancestors of *T. terrestris* and *T. pinchaque* diverged around 3.8 MYA (95 % HPD: 3.1-4.7 MYA). Additionally, the temporal diversification of the current *T. bairdii* began around 2.4 MYA (95 % HPD: 0.8-4.5 MYA), the diversification of the current *T. terrestris* around 3.5 MYA (95 % HPD: 2.3-4.4 MYA) and the diversification of *T. pinchaque* around 2.1 YA (95 % HPD: 1-3.3 MYA).

The mutation rate was around 3.9×10^{-3} during the separation of the ancestors of the Asian tapir and the neotropical ones and during the separation of the ancestor of the Central-American tapir against the ancestor of the South-American tapir. This mutation rate accelerated nearly three times during the separation of the ancestors of *T. terrestris* and *T. pinchaque* (1.03×10^{-2}) and in the initial lineage diversification within *T. bairdii* (1.01×10^{-2}). However, within the diversification of the *T. terrestris* lineages and within the diversification of *T. pinchaque* lineages (and also in recent *T. bairdii* lineages), the mutation rates were similar to those of the initial separation branches. Therefore, a period of mutation acceleration seems to have occurred during the ancestral divergence between the two South-America tapir species and during the initial lineage diversification within *T. bairdii*.

The time splits within *T. pinchaque* and *T. bairdii* are as follows: 1- As it was mentioned earlier, the ancestor of the *T. pinchaque* haplotypes began to diversify 2.11 MYA. Two
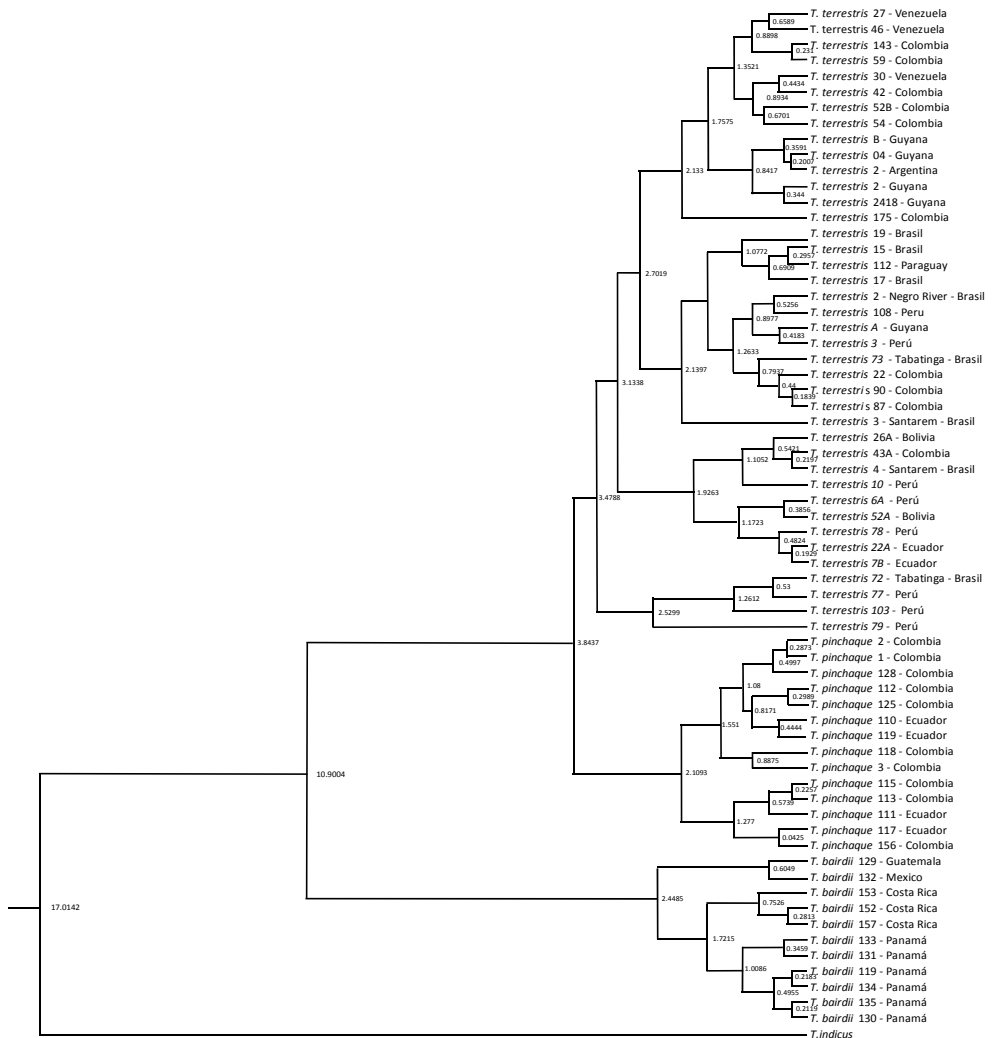
Fig. 4. Possible phylogenetic relationships among the four *Tapirus* species by means of the BEAST v. 1.4.8 software.

lineages were generated, one, with an ancestor around 1.51 MYA and other with an ancestor around 1.28 MYA. Both lineages were inter-dispersed within Colombia and Ecuador. The first lineage was subdivided in other two ensembles. One of them began its diversification around 1.08 MYA and the other around 0.89 MYA. This last one was composed of haplotypes only found in Colombia. The first one was comprised of haplotypes simultaneously found in Colombia and Ecuador and that diverged 0.87, 0.50, 0.44, 0.30 and 0.29 MYA. The other main *T. pinchaque* lineage had an ancestor which began to diversify

1.28 MYA and internal ensembles that began to diversify around 0.57, 0.42 and 0.23 MYA. 2-As commented before, the ancestor of the *T. bairdii* haplotypes began its diversification 2.44 MYA and determined one lineage, which is found in Mexico and Guatemala (and that internally diversified around 0.60 MYA in the northern area of the distribution), and another lineage, in the southern range of the distribution, whose diversification began 1.72 MYA, originating the animals from Costa Rica (with a common ancestor 0.75 MYA and with additional haplotype diversification 0.28 MYA) and the animals from Panama, with a common ancestor 1 MYA. Within the Panamanian animals, different haplotype split times were 0.50, 0.35 and 0.22-0.21 MYA.

By employing another approximation (MJ with the ρ statistic), the following temporal splits were estimated (Figure 5): 1- The divergence between the haplotype of *T. indicus* and two of the main *T. terrestris*'s haplotypes were 19.037 ± 0.282 MYA and 17.542 ± 0.153 MYA, or between the first haplotype and the more frequent one in *T. pinchaque* was 19.830 ± 0.448 MYA, very similar to the split time estimated for the initial divergence time between the Asian tapir and the Neotropical tapir species with the best Bayesian hypothesis tree. The two most frequent haplotypes of *T. terrestris* diverged from the ancestor of the current *T. bairdii* since 9.582 ± 0.157 MYA and 7.931 ± 0.076 MYA (and this divergence estimate was of 9.559 ± 0.102 between the ancestors of *T. bairdii* and *T. pinchaque*), while these same two *T. terrestris*'s haplotypes diverged from the main *T. pinchaque*'s haplotype 1.582 ± 0.299 MYA and 1.525 ± 0.336 MYA, respectively. 2- Within *T. bairdii*, some interesting split divergence times were as follows: the ancestor of the main Panamanian's haplotype diverged from the ancestors of the main Costa Rican, Guatemalan and Mexican haplotypes 33,897 ± 33,897 YA, 116,219 ± 58,110 YA and 135,589 ± 33,897 YA while the two Costa Rican's haplotypes diverged 67,795 ± 67,795 YA and the Guatemalan and the Mexican's haplotypes diverged 101,692 ± 19,692 YA. 3- Within *T. pinchaque*, the main Tolima's haplotype separated from the major part of the other haplotypes found in Colombia and Ecuador around 65,627 ± 32,813
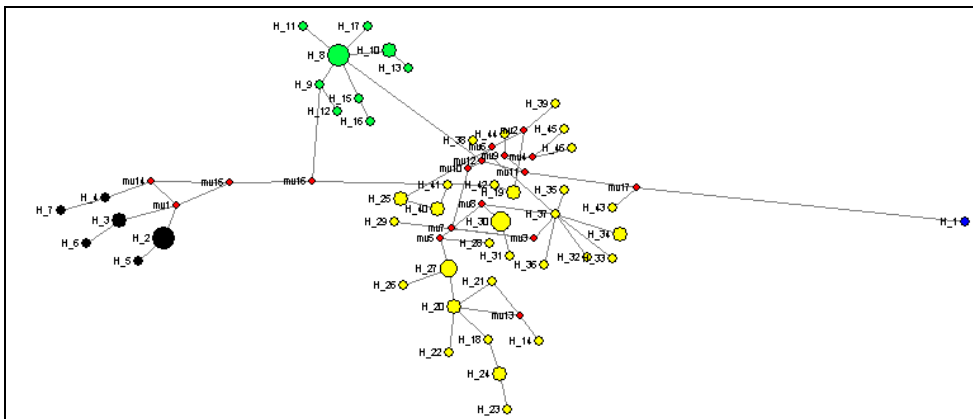

Fig. 5. Median Joining Network (MJ) applied by means of the software Network 4.2.0.1 (Fluxus Technology Ltd) to the haplotypes found in four *Tapirus* species. In blue = *Tapirus indicus*; In yellow = *Tapirus terrestris*; In green = *Tapirus pinchaque*; In black = *Tapirus bairdii*. In red, those hypothetical connecting haplotypes that were not detected in our study.

YA, with the lowest value around 32,813 ± 32,813 YA with another Ecuadorian's haplotype and the highest value around 98,440 ± 46,405 YA with Huila's haplotype. In whatever case, all the haplotype splits within *T. bairdii* and *T. pinchaque* were during the last phase of the Quaternary, mainly since the last glacial period to the beginning of the Holocene.

3.3 Spatial genetic patterns in *T. pinchaque* and in *T. bairdii*

The first spatial analysis revealed that *T. pinchaque* did not show any significant spatial trend in the distribution of its haplotypes (r = -0.119, approximate Mantel t-test = -0.660, p = 0.254; out of 5000 random permutations, 2112 were < Z, 0 = Z and 2888 > Z; one-tail probability p = 0.423). On the contrary, the *T. bairdii*'s haplotypes showed a very significant spatial genetic trend (r = 0.819, approximate Mantel t-test = 3.611, p = 0.0002; out of 5000 random permutations, 5000 were < Z, 0 = Z and 0 > Z; one-tail probability p = 0.0004).

The second analysis by means of the IBD software confirmed that *T. pinchaque*'s haplotype distribution did not follow an isolation by distance model [lineal model: intercept (± standard error) = 0.02587 ± 0.00227, slope (± standard error) = -0.007058 ± 0.000691, R^2 = 0.0142; 99% confidence intervals with 10,000 bootstraps over all the individuals: intercept = (-0.01207, 0.03452), slope = (-0.009161, -0.008307), R^2 = (0.0000, 0.0985)], while the individual spatial distribution of the *T. bairdii*'s haplotypes clearly fit with an isolation by distance pattern [lineal model: intercept = 0.000636 ± 0.000199, slope = 0.0004294 ± 0.0000338, R^2 = 0.670; 99% confidence intervals with 10,000 bootstraps over all the individuals: intercept = (0.0002354, 0.001088), slope = (0.0003631, 0.0005143), R^2 = (0.454, 0.820)]. These results were obtained with normal data, but the results with the log genetic or log geographical distances or both log data simultaneously were very similar.

The first spatial autocorrelation analysis (Table 5) did not show any spatial trend in *T. pinchaque.* Neither the overall correlogram (p = 0.455, for the Morans's I index; p = 0.521, for the Geary´s c coefficient) nor any individual autocorrelation coefficient were significant. Even, the most negative value for the Moran´s I index of the four defined distance classes (DC) was the first one (-0.20) and the most positive was the fourth DC (0.02). Therefore, this means that the most differentiated haplotypes were those geographically closest, whereas the more similar haplotypes were at greater geographical distances. Contrarily, this first spatial autocorrelation analysis showed a striking significant result for the overall correlogram in *T. bairdii* related to the monotonic clinal pattern for both the Moran's I index and the Geary's c coefficient (Table 5), with the first DC highly positive (Moran's I index 1 DC = 0.500, p = 0.000; Geary's c coefficient 1 DC = 0.05, p = 0.000) and the third DC highly negative (Moran's I index 3 DC = -0.68, p = 0.000; Geary's c coefficient 3 DC = 2.29, p = 0.000). Similarly, the same was found for *T. pinchaque* with no spatial autocorrelation (Table 5) with the distogram with the Gregorious (1978)'s distance or the distogram with the number of haplotypes shared (SGS software). Contrarily, again, in *T. bairdii* the spatial patterns were highly significant for the distogram with the Gregorious (1978)'s distance or the distogram with the number of haplotypes shared.

Moran´s I index	1CD	2CD	3CD	4CD	Overall Probability Correlogram
Tapirus pinchaque	0-71 km -0.20	71-163 km -0.03	163-494 km -0.08	494-772 km 0.02	0.455
Tapirus bairdii	0-161 km 0.50**	161-776 km -0.09	776-1893 km -0.68**		0.000**

A

Geary's c coefficient	1CD	2CD	3CD	4CD	Overall Probability Correlogram
Tapirus pinchaque	0-71 km 1.74	71-163 km 0.89	163-494 km 0.85	494-772 km 0.53	0.521
Tapirus bairdii	0-161 km 0.05**	161-776 km 0.59	776-1893 km 2.29**		0.001**

A

Distogram with Gregorious (1978)'s distance	1CD	2CD	3CD	4CD	Overall Probability Correlogram
Tapirus pinchaque	0-1.9175 units	1.9175-3.835 units	3.835-5.7525 units	5.7525-7.67 units	
	0.1713 (95% interval: 0.0691-0.1878)	0.0745 (95% interval: 0.0213-0.7021)	0.1654 (95% interval: 0.0696-0.4004)	0.0940 (95% interval: 0.0359-0.4199)	1 CD: (95% interval: 0.494-0.506) 2 CD: (95% interval: 0.497-0.503) 3 CD: (95% interval: 0.221-0.779) 4 CD: (95% interval: 0.700-0.300)
Tapirus bairdii	0-4.37 units	4.37-8.74 units	8.74-13.110 units	13.11-17.48 units	
	0.0601 (95% interval: 0.1955-0.3759)	0.3571 (95% interval: 0.2142-0.4365)	0.5476 (95% interval: 0.1429-0.5000)	0.5195 (95% interval: 0.1688-0.5195)	1 CD: (95% interval: 1.000-0.000)** 2 CD: (95% interval: 0.180-0.820) 3 CD: (95% interval: 0.020-0.980)* 4 CD: (95% interval: 0.017-0.983)*

B

Table 5. A- Spatial autocorrelation analysis with 4 Distance Classes (DC) for the individuals analyzed of *Tapirus pinchaque* and with 3 DC for the individuals analyzed of *Tapirus bairdii*. * $P < 0.05$, ** $P < 0.01$, significant probability. This analysis was carried out with the SAAP 4.3 program. B- Distograms with the Gregorious's (1978) distance carried out with the SGS software for the individuals analyzed of *Tapirus pinchaque* and *Tapirus bairdii*. * $P < 0.05$, ** $P < 0.01$, significant probability.

4. Discussion

4.1 Genetic diversity, genetic heterogeneity, possible demographic changes and spatial structure

It was clear that *T. terrestris* was the species with the highest gene diversity level, which agrees quite well with the fact that it is the Latin American *Tapirus* species with the widest geographical distribution and thus with the highest potentially effective numbers. This coincides with the elevated polymorphism found for this species with other markers (Tapia et al. 2005). Curiously, *T. pinchaque*, a species with a very restrictive geographic distribution and a small population size of no more than a thousand individuals, which could be on the brink of extinction (Ashley et al. 1996), presented a gene diversity that was more than three times higher than that of *T. bairdii* which historically occupied a distribution from southern Mexico to the Pacific area of Colombia and Ecuador (historically in Ecuador until the Guayaquil Gulf and currently in Guayas-Bucay, Cotacachi-Cayapas Reserve and Awá Reserve; Tirira 2008). Thus, although *T. pinchaque* has a very small census population size and a very restrictive geographical distribution, within disturbed and fragmented ecosystems, the species is not depauperated from a genetics point of view. This is a good new for conservation purposes. However, this is the first genetics analysis reported for this species and other genetic markers, such as DNA microsatellites, must be analyzed for this elusive species to corroborate that its genetic diversity levels are high although it has an extremely small population size. If this affirmation is correct, this means that the reproductive system of this species, and its gene flow capacity dispersion, is enough to maintain these elevated gene diversity levels. The case of the *T. bairdii* seems to be more dramatic. Although its geographic distribution is wide, it has been dramatically reduced and fragmented in the last two centuries and today no more than 6,000 individuals are left in the wild (Ashley et al. 1996). Its mitochondrial genetic diversity levels were extremely low compared with the other two neotropical tapirs and other neotropical mammals studied for the same or similar mitochondrial genes (Primates, Lavergne et al. 2010; Ruiz-García and Pinedo-Castro 2010; Ruiz-García et al. 2010, 2011a,b, 2012a; jaguars, pumas and other felids, Ruiz-García et al. 2006, 2009a, Cossios et al. 2009; artiodactyls, Ruiz-García et al. 2007, 2009b; river dolphins, Banguera-Hinestroza et al. 2002; Ruiz-García 2010a,b; Ruiz-García et al. 2008; Caballero et al. 2010). This could mean that this species suffered from a bottleneck and/or the gene drift has been more intense on this species by natural or human constrictions. In fact, this species has intensely declined in the last century by habitat destruction and hunting and has been extinct in El Salvador and in a major fraction of its original distribution range in Colombian and Ecuador (Brooks et al. 1997). Additionally, this species also showed a low gene diversity level for DNA microsatellite markers, with an expected heterozygosity ranging from 0.37 (Costa Rica sample) to 0.43 (Panama sample) and the average number of alleles oscillating from 2.5 (Costa Rica) to 3.33 (Panama) (Norton and Ashley 2004a). These microsatellite gene diversity levels were among the lowest found for mammals and similar to other genetically depauperated neotropical mammals, such as the Andean bear (Ruiz-García 2003, 2007, 2012; Ruiz-García et al. 2005) or the Andean cat (Cossios et al. 2012). These levels, together with the low mitochondrial gene diversity herein reported, agree quite well with a history of isolation, gene drift or bottlenecks for this population. Nei et al. (1975) showed that the populations which did not quickly recover

their population sizes following a bottleneck will experience a greater loss of the gene diversity levels and will take longer to recover heterozygosity (or in this case, nucleotide diversity). Moreover, the mutation process to add new genetic variants could take thousands of generations resulting in the maintenance of low allele or haplotypic diversity for thousands of years following the original bottleneck. Norton and Ashley (2004a) did not find clear evidence of bottlenecks in the Costa Rican Corcovado National Park *T. bairdii* population nor in the Panamanian population because they did not observe a heterozygosity excess expected in populations experiencing a recent bottleneck. Nevertheless, in the Costa Rican population, they determined an allele frequency mode shift which is consistent with a bottleneck. In our case, the mtCyt- gene sequences yielded, for *T. bairdii*, the weakest evidence of a possible population expansion relative to the cases of the other two neotropical tapir species, which is concordant with the fact that this is probably the most bottlenecked species. Contrarily, *T. terrestris* experienced a clear historical population expansion during the Pleistocene, becoming probably the most successful large herbivore in South America, which survived the last glacial extinction. In the case of *T. pinchaque*, there is less conclusive evidence of population expansion and it seems to have a constant population size throughout its history.

The genetic heterogeneity among the three *Tapirus* species was highly significant, showing that, effectively, they were three separated species. However, the two South-American species were highly related, while the Central American species was considerably divergent from the two South-American ones. This agree quite well with the morphological classification of Hershkovitz (1954), who had recognized the two South-American tapir species as belonging to the sub-genus *Tapirus*, while the *T. bairdii* was located in the sub-genus *Tapirella*. This is clear evidence that in the Neotropics, there are currently two different molecular *Tapirus* lineages.

Other results which should be commented upon are the absence of any spatial pattern in the case of *T. pinchaque* and the striking spatial pattern discovered in *T. bairdii*. Although the current geographical distribution of *T. pinchaque* is restrictive and the populations could be severely fragmented, the intense climatic changes in the Andes during the Pleistocene probably provoked the stochastic mixing of different haplotypes that were generated in diverse areas when the conditions were sufficiently adequate for population expansion. Additionally, the population size of this species could be higher in the past than it is currently, although several Andean mammal species have shown to have small effective numbers throughout their histories (Andean bear, Ruiz-García 2003, 2007, 2012; Ruiz-García et al. 2003, 2005; Andean cat, Cossios et al. 2012). Also, the females of *T. pinchaque* could have a great capacity for dispersion, which did not provide for the generation of an appreciable spatial pattern during the history of this species. In contrast, in the case of *T. bairdii*, the geographical distribution, and the minor extension, of the Central American forests could restrict the dispersion of this large herbivorous mammal compared to the South American forests. Thus, an isolation-by-distance pattern has been generated for *T. bairdii*. Norman and Ashley (2004a), by using microsatellites, did find an elevated F_{ST} value between Panamanian and Costa Rican tapirs, although they were not differentiated in two different populations by the Structure software. The results obtained seem to put forward the possible existence of Management Units (MU) in the case of *T. bairdii* but not for *T.*

pinchaque. One aspect which should be analyzed is if this significant spatial pattern and the limited gene diversity for mt sequences (this study) and for DNA microsatellites (Norton and Ashley, 2004a) is the result of the Pleistocene history or of the more recent history of this species.

4.2 Temporal splits within *T. pinchaque* and *T. bairdii*, climatic and geological changes and phylogenetics relationships among the Neotropical tapirs

Tapirus has been in South America since the lower Pleistocene, or Plio-Pleistocene, (around 3 MYA; Cione and Tonni 1996; Nabel et al. 2000) in the Ensenadan South American Land Mammal Age (SALMA), during the Great American Biotic Interchange (GABI). Our results showed that the ancestor of *T. terrestris* and *T. pinchaque* lived 3.8 MYA and the haplotypic diversification within *T. terrestris* and within *T. pinchaque* occurred 3.5 MYA and 2.1 MYA respectively. Thus, the diversification of the ancestors of the current South-America *Tapirus* coincides with the climatic changes that originated the completion of the Panamanian land bridge (2.8-3.5 MYA; Coates and Obando, 1996) or slightly earlier coinciding with the Chocó-Panamá island bridge (Galvis 1980), which could have been used by the ancestors of the current *Tapirus* to colonize northern South America from Central America. During the upper Pliocene orogeny, the present Tuira, Atrato and Sinú river basins as well as near lowlands were raised above sea level. Thus, the mountains of southern Central America and of the northern Andes were uplifted to about their present elevation (Van der Hammen 1961). Even if the divergence split was slightly prior to the completion of the Panamanian land bridge, and although the Nicaraguan, Panamanian and Colombian portals remained open (upper Miocene-Middle Pliocene), numerous volcanic island existed from the lower Atrato Valley and the Tuira river basin of eastern Panama to the Nicaraguan portal, which could have been used by the South-American *Tapirus*'s ancestor to migrate southward. The Cuchillo bridge of the Urabá region, connecting the Tertiary Western Colombian Andes with the Panamanian islands was probably above sea level during this period. Simpson (1950, 1965) claimed that many mammals were "island hoppers". Tapirs have a high capacity to swim wide zones of the Amazon River (Brooks et al. 1997).

The haplotypic time divergence within *T. terrestris* began around 3.5 MYA, when the *Tapirus* genus penetrated in South-America. It's likely that the *T. terrestris*' ancestor generated the ancestor of *T. pinchaque*, whose haplotypic diversification began more recently (2.1 MYA) than that of *T. terrestris*. Therefore, *T. pinchaque* could be a mountain specialized descendent of some *T. terrestris* lineage in the transition of the western Amazon and the Eastern Andes Cordillera. In fact, the northern Andes, where the current *T. pinchaque* lives, are a hot point of speciation for taxa (Sedano and Burns 2010) and a top biodiversity hotspot (Orme et al. 2005). Furthermore, the haplotype diversification of *T. pinchaque* began after the completion of the northern Andean uplift. Effectively, after rising slowly for millions of years, the Central Andes had a rapid and final uplift in the last 6-10 MYA (Garzione et al. 2008). The final uplift of the northern Andes in the Eastern Cordillera in Colombia was in the last 3-6 MYA (Hooghiemstra and Van der Hammen 2004), while the beginning of the haplotype diversification in *T. pinchaque* was around 2.1 MYA. This molecular result did not agree with the fact that *T. pinchaque* could be more primitive and to be in origin of *T. terrestris* as certain authors claimed (Hershkovitz 1954).

In the evolution of *T. pinchaque* some ages seems to be especially important for haplotypic diversification. These temporal haplotypic splits were around 2.1-1.5, 0.85-1.3, 0.4-0.6 and 0.2-0.3 MYA. The ages between 2.1 and 1.5 MYA coincide with the end of the Pliocene and the beginning of the Pleistocene (1.6-2.5 MYA). Therefore, the initial Pleistocene changes could have generated the fragmentation of the *T. pinchaque* population. During that epoch, the Andean forests (where *T. pinchaque* lives) were transformed into open cold dry savannah ("paramo"), which could have potentially isolated ancestor populations of *T. pinchaque* in Colombia. This was also the epoch of the last upheaval of the central and northern Andes. For instance, during this time in Huancho (north of Lima-Peru) little lakes and the Ventanilla bay were formed. Also the Andean cordillera between Cajamarca and Huancavelina (Peru) was created by volcanism in this period of intense climatic and geographic changes. Van der Hammen (1992) demonstrated that the mean temperature in the Colombian Andes was 4 °C lower than today. He also stated that the rain level was lower than the 500-1,000 mm reported for today. This epoch was characterized by a great fauna change with the beginning of the Marplatense epoch in Argentina or the Villafranquense epoch in Europe.

The Milankovitch's cycles (each 19,000-24,000, 43,000 or 90,000-100,000 years) occurred across the Quaternary with its cold and dry phases and generated forest refuges in South America (Haffer 1997; Whitmore and Prance 1987). Therefore, the Pleistocene forest refugia invoked by Haffer (1969, 1982) could be very important for understanding the evolution of the three current Latin American *Tapirus* species. Environmental conditions in Central and South America were influenced by these alternating dry and wet climatic periods and by sea level fluctuations. During the glacial periods, sea levels were lower by about 100 m, while sea level rose by about 30 to 50 m above the present level during the interglacials (Haffer 1967).

The next haplotype diversification peak occurred around 0.8-1.1 MYA. The Pre-Pastonian glacial period (0.80-1.30 MYA), which was the highest glacial peak of the first Quaternary glaciation (Nebraska-Günz), could have separated to a certain degree, some of these *T. pinchaque*'s haplotypes. Another mammal, the Pampas cat (*Leopardus colocolo*) (Cossios et al. 2009), suffered an intense genetic population fragmentation in this same epoch.

The *T. pinchaque* haplotype differentiation around 0.4-0.6 MYA agrees quite well with the coldest epoch of the Mindel-Kansas glacial period (Elster glacial period for Scandinavia, Bonaerense period for Argentina and Kamasiense I for Eastern Africa; 0.3-0.6 MYA). Also, the *T. pinchaque* haplotype differentiation that occurred around 0.2-0.3 MYA coincides with the Riss I glacial period for central Europe, the Illinois glacial period for North America, the Saale for the Nordic glaciations and the Kanjeriense for Eastern Africa. Finally, other haplotype diversification periods were 98,000, 66,000 and 33,000 YA. These different ages corresponded to the last glacial period which originated 130,000 to until 10,000 YA. The Eemiense interglacial period (130,000 to 80,000 YA), was characterized by high temperatures, high rain precipitations and extensive forests of *Aliso, Vallea* and *Weinmannia* (Van der Hammen 1992). However, this epoch had some short but very intense cold periods (one event 95,000-100,000 YA), where the Andean forests disappeared around the Cundiboyacense highlands in Colombia. These brief, intensely cold periods were recorded by oxygen isotopes in Greenland's ice. Agreeing quite well with the haplotype diversification that occurred 66,000 YA, the first big cold period began during the last glacial

epoch (Earlier Pleni-glacial period) around 60,000-70,000 YA. Around 26,000-35,000 YA, during the middle-upper Pleni-glacial period, the climate was extremely dry. For instance, the Bogotá and the Fúquene lakes, in the Colombian highlands, disappeared (Van Geel and Van der Hammen 1973) and typical dry vegetation such as *Symplocos, Myrica, Myrsine* and *Alnus* appeared in the northern Andes (Van der Hammen 1980). During this epoch, the Amazon was colonized by dry vegetation such as *Ilex*. Van der Hammen (1992) demonstrated that the upper Pleni-glacial (30,000-16,500 YA), when the Amazon was characterized as having a dry- ambient climate, coincided with the most cold and dry period in the Andes (26,000-14,000 YA) as well as with the most extensive ice period in the Northern Hemisphere. Thus, another *T. pinchaque* haplotype period of diversification could exist at the beginning of this period.

For *T. bairdii*, the situation is similar to *T. pinchaque*. The first haplotypic divergence processes in *T. bairdii* (1.7-2.4 MYA) coincided with the beginning of the Pleistocene. A second period of haplotype diversification in this species was around 0.6-0.75 MYA. This period agrees quite well with a high glacial peak of the second glacial period (Kansas-Mindel), which could promote population differentiation within *T. bairdii*. A third moment of haplotype diversification was around 0.28 MYA. As in the case of *T. pinchaque*, this coincides with the Riss I glacial period for central Europe and with the Illinois glacial period for North-America. Also, in the last glacial period (130,000-10,000 YA), there was an intense haplotype diversification in *T. bairdii* (136,000, 116,000, 102,000, 68,000, 34,000 YA), coinciding with some of the cold peaks in the Eemiense interglacial period, the beginning of the Earlier Pleni-glacial period and with the beginning of the Upper Pleni-glacial period.

Thus, the climatic changes during the Pleistocene could be decisive in understanding the haplotype differentiation within *T. pinchaque* and *T. bairdii*.

All the phylogenetic analyses carried out with the mtCyt-b gene showed the close genetic relationship between both South-America species, *T. terrestris* and *T. pinchaque*, while the Central American species, *T. bairdii*, seems to belong to another *Tapirus* lineage, but more related with the other neotropical tapirs than to the Asian tapir. This result disagrees with that obtained by Ashley et al. (1996) and Norton and Ashley (2000) with the mtCOII gene, which showed a clade conformed by *T. terrestris* and *T. pinchaque* and another clade integrated by *T. bairdii* and *T. indicus* (with 55 % bootstrap support). Nevertheless, the results obtained by Norton and Ashley (2000) with the 12S rRNA gene supported monophyly of neotropical tapirs (83 % bootstrap), with the South-American tapirs and the Central American ones as the current forms of two different tapir lineages. When both genes were employed by Norton and Ashley (2000), the monophyly of the neotropical tapirs was also supported (62 % bootstrap). Thus, similar to the two sequence sets provided by Norton and Ashley (2000), the molecular results we show herein are closely related to the paleontological data of South-American tapirs.

The fossil remains of *Tapirus* in South America are scarce and fragmentary. This fragmentation is important because in many cases it makes it difficult to obtain taxonomic conclusions. But, all the taxa seem to be highly related and they could belong to a unique tapir colonization of South America, just as the genetics results seem to indicate. The most representative *Tapirus* fossil records in South America are as follows: Ameghino (1902) found the left mandible with three pre-molars of a *Tapirus* taxon larger than the current *T.*

terrestris from the lower Pleistocene of Tarija (Bolivia). He named this taxa as *T. tarijensis*. Rusconi (1928) and Cattoi (1951) described, from some teeth material, two supposed *Tapirus* species of the lower Pleistocene (Ensenadense age: 2-0.5 MYA) located in the south eastern section of the Buenos Aires Province. They named these taxa as *T. australis* and *T. dupuyi*. Nevertheless, Ubilla (1983) and Tonni (1992) lately considered that these materials really did not constitute new species (*Tapirus* sp.). Cattoi (1957) described another *Tapirus* form (*T. rioplatensis*), larger than *T. terrestris*, also from Ensenadense in the north-western area of the Buenos Aires Province. Tonni (1992) described a *T. terrestris's* mandible from the Lujanense age (upper Pleistocene Lujanense period to present, the last 130,000 YA) collected in the Colon Department of the Entre Rios Province, as being the last and most southern record for this species. Also, Noriega et al. (2004) and Ferrero et al. (2007) found fossil fragments of *T.* cf. *terrestris* (a right hemi-maxille) for the El Palmar Formation at the El Boyero locality (upper Pleistocene) from the Entre Rio Province. Ferrero and Noriega (2003, 2005, 2007) registered another *Tapirus* species from the analysis of a complete skull collected from outcrops of the Arroyo Feliciano Formation in the Diamante Department (Upper Pleistocene; Lujanense period) in the Argentine area of Mesopotamia. This skull was morphometrically compared, with a cladistic analysis, to several Tapiridae such as *Miotapirus, Paratapirus, Plesiotapirus,* five North American species (*T. veroensis, T. haysii, T. johnsoni, T. webbi* and *T. polkensis*) as well as the fourth current living *Tapirus* species. This taxon has been named *T. mesopotamicus*. This species seems to be closely related with *T. pinchaque*, and both species are related to *T. terrestris*. Therefore, four taxa of *Tapirus* have been determined for Argentina (Forasiepi et al. 2007): *T. terrestris*, *T.* sp, *T. mesopotamicus*, and *T. rioplatensis*. In whatever case, the existence of fossil records of tapirs in these Argentinean areas is associated with climatic conditions hotter and more humid than climatic conditions today. In Uruguay, Ubilla (1983) determined another *Tapirus* species for the Libertad Formation (Lower Pleistocene) in the Montevideo Department. This species was named *T. oliverasi* and had a larger size than *T. terrestris* but smaller than *T. rioplatensis*. Ubilla (1996) described *T. terrestris* fossils from the Tacuarembó and Salto Departments and other materials classified as *Tapirus* sp. Additionally, Ubilla and Rinderknecht (2006) found a complete skull from the Tacuarembó Department, which could be a new species. Therefore, the presence of *Tapirus* is associated with tropical forests and the extension and diversification of the genus during a great part of the Pleistocene was higher than it is today (especially during the Ensenadense period). It is in Brazil where more fossil *Tapirus* records have been found. Winge (1906) determined another species, *T. cristatellus*, from the fossils of Lagoa Santa, Minas Gerais state. This species has a skull and teeth greater than the current *T. terrestris*. Many fossil remains of *T. terrestris* or *T.* sp. have been described in Iraí (Rio Grande do Sul; Souza-Cunha 1959), in Arroio Touro Passo (Bombin 1976), Quaraí River (Oliveira 1992), in Bom Jardim (Pernambuco state; Rolim 1974), in Arroio Chuí (Soliani 1973) and in the Upper Jurua River (Acre state; Rancy 1981). An abundance of *T. terrestris* fossils have also been found in Areia Preta, Jacupiranga (Sao Paulo state; Paula-Couto 1980), Barauna (Rio Grande do Norte state; Porpino and Santos 2003), Cavernas do Bauxi (Mato Grosso; Hirooka 2003), Cavernas do Japones e Nascente do Formoso (Serra da Bodoquena, Mato Grosso do Sul; Salles et al. 2006) and the Cavernas do Vale do Rio Rocha (Gramados, Parana state; Sedor et al. 2005). Holanda et al. (2005) described some new findings in three Brazilian localities. The molar length and the width indices showed dimensions within the

proportions of *T. terrestris*, with the exception for the PM1 (a specimen of the Rondonia State) and for the M2 and M3 (the second specimen from the Rio Grande do Sul State) which are more quadrangular like in *T. veroensis* and *T. haysii* (Plio-Pleistocene of North America). All these paleontological records were dated in less of 3.0 MYA. Recall that our genetics results pointed out that the *T. terrestris* diversification began around 3.5 MYA.

Very recently, Holanda and Couzzol (2006) described three specimens from the Pleistocene at Acre and Rondonia. Their morphometric analyses indicated two new species: a more robust form than the current *T. terrestris* (the Acre specimens) as well as a more gracile form (the Rondonia specimen). These fossils were dated to the middle Pleniglacial period, around 30,000-45,000 years ago. Thus, many different *Tapirus* species have existed in South America during the Pleistocene.

However, the discovery of 75 individuals of *T. polkensis* in the Gray Fossil Site in eastern Tennessee showed that a unique species was present but it had considerable intraspecific variation including development of the sagittal crest, outline shape of the nasals and the number and relative strength of lingual cusps on the P1 (Hulbert et al. 2009). This means that some of the quoted fossils found in South-America should belong to *T. terrestris*. If ancient DNA could be extracted from some of these more recent tapir fossil remains, it could be conclusive to demonstrate if only one *Tapirus* migration wave arrived to South-America and if they were really different species from *T. terrestris*.

In contrast, the morphometrics of the current *T. bairdii* seems to be more related with the North-American fossil tapirs (Ferrero and Noriega 2007) [*T. johnsoni, T. simpsoni, T. polkenis* and *T. webbi* for the Miocene; *T. merriami, T. haysii* from the Pliocene and these last two species plus *T. veroensis* for the Pleistocene; Hulbert 1995, 1999; Hulbert and Wallace 2005; Spassov and Ginsburg 1999; Tong 2005; Hulbert et al. 2009]. This agrees quite well with the molecular data showed here, which showed *T. bairdii* as belonging to a different lineage. However, our molecular study contradicts the morphometrics study of Hulbert (1995), which considered *T. terrestris* and *T. bairdii* to be the closest sister taxa. However, Ferrero and Noriega (2007) showed a close relationship among *T. bairdii* and *T. haysii* and *T. veroensis*. Thus, the molecular data agree quite well with the fossil knowledge that we have for the American tapirs. The first North American tapir was *T. johnsoni* from the Miocene of Nebraska with a divergence about 9-11 MYA (Colbert 2005). This temporal split agrees with the estimates obtained with the molecular data for the divergence between the lineage of *T. bairdii* and the lineage of *T. terrestris-T. pinchaque* (10.9 MYA for the Bayesian hypothesis tree and 9.6 MYA for the haplotype MJ network).

Future works should utilize nuclear sequences (introns, HLA loci, Chromosome Y) and microsatellites to study the phylogenetic relationships among the four living *Tapirus* species as well as the genetic structure for each one of the *Tapirus* species.

5. Acknowledgment

6. References

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