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SHORT COMMUNICATION

Phylogeography and spatial structure of the lowland tapir (*Tapirus terrestris*, Perissodactyla: Tapiridae) in South America

Manuel Ruiz-García[1], Catalina Vásquez[1], Sergio Sandoval[2], Franz Kaston[3], Kelly Luengas-Villamil[1], and Joseph Mark Shostell[4]

[1]*Laboratorio de Genética de Poblaciones Molecular y Biología Evolutiva, Departamento de Biología, Facultad de Ciencias, Unidad de Genética, Pontificia Universidad Javeriana, Bogotá DC, Colombia,* [2]*Tapir Preservation Fund, Bogotá DC, Colombia,* [3]*Fundación Nativa, Cartagena, Colombia, and* [4]*Department of Biology, Penn State University-Fayette, Uniontown, Pennsylvania, USA*

Abstract

We sequenced the mitochondrial cytochrome b gene of 141 lowland tapirs (*Tapirus terrestris*) – representing the largest geographical distribution sample of this species studied across of South America to date. We compare our new data regard to two previous works on population structure and molecular systematics of *T. terrestris*. Our data agree with the Thoisy et al.'s work in (1) the Northern Western Amazon basin was the area with the highest gene diversity levels in *T. terrestris*, being probably the area of initial diversification; (2) there was no clear association between haplogroups and specific geographical areas; (3) there were clear population decreases during the last glacial maximum for the different haplogroups detected, followed by population expansions during the Holocene; and (4) our temporal splits among different *T. terrestris* haplogroups coincided with the first molecular clock approach carried out by these authors (fossil calibration). Nevertheless, our study disagreed regard to other aspects of the Thoisy et al.'s claims: (1) meanwhile, they detected four relevant clades in their data, we put forward six different relevant clades; (2) the Amazon River was not a strong barrier for haplotype dispersion in *T. terrestris*; and (3) we found reciprocal monophyly between *T. terrestris* and *T. pinchaque*. Additionally, we sequenced 42 individuals (*T. terrestris, T. pinchaque, T. bairdii*, and the alleged "new species", *T. kabomani*) for three concatenated mitochondrial genes (*Cyt-b, COI,* and *COII*) agreeing quite well with the view of Voss et al., and against of the claims of Cozzuol et al. *Tapirus kabomani* should be not considered as a full species with the results obtained throughout the mitochondrial sequences.

Keywords

Mitochondrial haplogroups, pleistocene diversification, population expansions, phylogeography, spatial autocorrelation, *Tapirus terrestris*, *T. kabomani*

History

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Introduction

In the Neotropics, only a few large mammalian species have been intensively studied in regard to their respective genetics and phylogeographic relationships (jaguar, *Panthera onca*; Eizirik et al., 2001; Ruiz-García, 2001; Ruiz-Garcia et al., 2003, 2006, 2007, 2013; Andean bear, *Tremarctos ornatus*; Ruiz-Garcia, 2003, 2007, 2013; Ruiz-Garcia et al., 2003, 2005; the pink river dolphin, *Inia geoffrensis* and *I. boliviensis*, Banguera-Hinestroza et al., 2002; Martínez-Aguero et al., 2006, 2010; Ruiz-García, 2010a,b; Ruiz-García et al., 2008). Similarly, the lowland tapir (*Tapirus terrestris*), the largest terrestrial herbivore in the South America, deserves to be studied more deeply of what have been studied so far from a molecular population genetics point of view. The aim of our study is to fill this gap in data.

The Tapiridae family (Gray 1821), within the order Perissodactyla, is currently composed of one unique genus, *Tapirus* (Brisson 1762). Around 20 different *Tapirus* species are recognized for North America, Europa, and Asia in the fossil record (Hulbert, 2010). Currently, there are four species of *Tapirus*; two exclusively present in South America (*T. terrestris* and *T. pinchaque*), one from Southern Mexico, Central America, and the Pacific coasts of Colombia (*T. bairdii*) and another in Asia (*T. indicus*).

From a molecular genetics point of view, only a few works have been published with this genus. Ashley et al. (1996) and Norman & Ashley (2000) analyzed the genetic relationships among the *Tapirus* species by using mitochondrial Cytochrome Oxidase subunit II (*COII*) and 12S rRNA gene sequences with a few individuals. More recently, the first molecular phylogeographic study of *T. terrestris* analyzed 45 specimens at the mitochondrial cytochrome b gene (*Cyt-b*) (Thoisy et al., 2010). Ruiz-García et al. (2012) showed that *T. pinchaque* did not show any significant spatial structure whereas *T. bairdii* showed a very significant spatial structure, due to isolation by distance. Cozzuol et al. (2013) claimed the existence of a new tapir species to compare *Cyt-b* gene sequences of four Amazon tapir individuals (two Brazilian animals and two Colombian specimens sampled by the first author of the present study) with regard to the 45 *Cyt-b* gene sequences published by Thoisy et al. (2010). Voss et al. (2014), however, put in doubt the validity of *T. kabomani* as a different species from *T. terrestris*.

Correspondence: Manuel Ruiz-García, Laboratorio de Genética de Poblaciones Molecular y Biología Evolutiva, Departamento de Biología, Facultad de Ciencias, Unidad de Genética, Pontificia Universidad Javeriana, Bogotá DC, Colombia. E-mail: mruizgar@yahoo.es, mruiz@javeriana.edu.co

We used sequences of the *Cyt-b* gene, which is commonly used among the molecular markers relevant for phylogeography, biosystematics, and genetic structure studies in mammal populations (Patton et al., 2000). We sequenced 141 individuals of *T. terrestris*, including more geographical areas and individuals than any previous study (Cozzuol et al., 2013; Thoisy et al., 2010) to determine levels of gene diversity, spatial genetic structure, and demographic changes of *T. terrestris* in South America. Additionally, 42 tapir individuals from *T. terrestris*, *T. pinchaque*, *T. bairdii*, and the alleged new species, *T. kabomani* were sequenced at the *Cyt-b*, *COI*, and *COII* genes to compare with the tree showed by Cozzuol et al. (2013) and to show evidence in favor or against of the alleged ''new species'' *T. kabomani*.

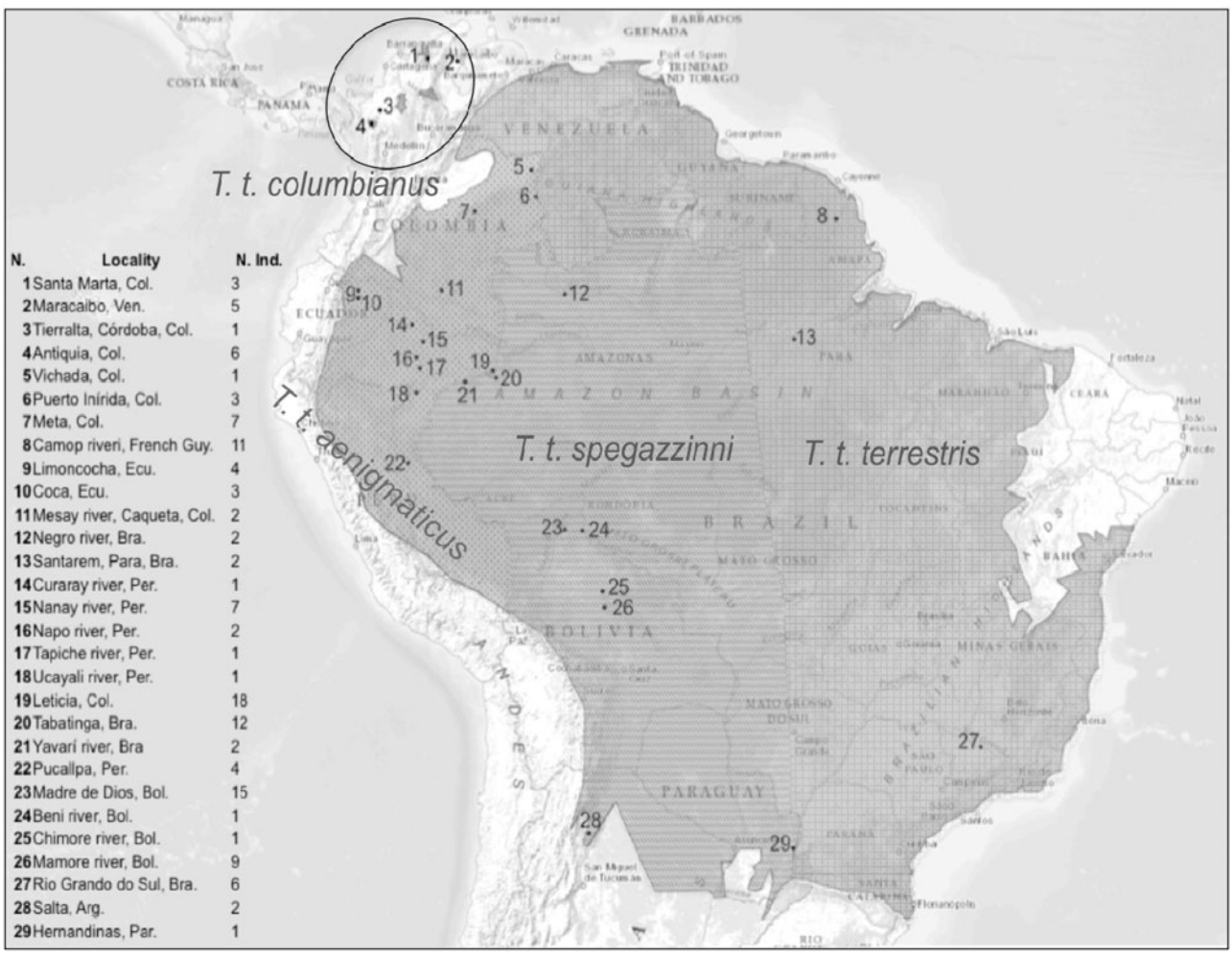
Methods

Samples

We sequenced a total of 141 individuals of *T. terrestris* at the *Cyt-b* gene from nine South American countries including Colombia (42 animals), Venezuela (six individuals), French Guiana (11 individuals), Ecuador (seven animals), Peru (30 animals), Bolivia (11 animals), Brazil (24 animals), Paraguay (one animal), and Argentina (two animals) (Figure 1). Additionally, one animal from the Barcelona Zoo (Spain), five animals from the Cincinnati Zoo (Cincinnati, OH) and one animal of unknown origin were also analyzed. These last seven animals were not included in those analyses where the origin of the animals was necessary. Thirty *T. pinchaque* (12 haplotypes), 30 *T. bairdii* (six haplotypes), and one *T. indicus* samples obtained by the first author were also employed. For the question of *T. kabomani*, 42 individuals were analyzed: 17 *T. terrestris* (one from Argentina, two from Bolivia, three from Brazil, five from Ecuador, three from Peru and three from the Cincinnati Zoo), five ''*T. kabomani*'' (the two Brazilian individuals reported by Cozzuol et al., 2013, and three ''classical morphological'' *T. terrestris* sampled by us but with haplotypes related to *T. kabomani*; one from San Martín de Amacayacu, Amazon River, Colombia, one from the Mazan River, a tributary from the Napo River, in the Peruvian Amazon and one from near to Tena, upper Napo River, at the Ecuadorian Amazon), 12 *T. pinchaque* (three Colombian and nine Ecuadorian individuals), and nine *T. bairdii* (all them from the Darien area in Panama). All these 42 individuals were sequenced for *Cyt-b* (1140 base pairs, bp), *COI* (640 bp), and *COII* (726 bp) genes summing up 2506 bp.

Molecular analyses

DNA from teeth, bones, muscle, and skins were obtained with the phenol–chloroform procedure (Sambrock et al., 1989), whereas DNA samples from hair and blood were obtained with 10% Chelex® 100 resin (Walsh et al., 1991). Amplifications for *Cyt-b* gene were achieved using primers designed for perissodactyls (Tougard et al., 2001). The amplification conditions employed followed Ruiz-García et al. (2012). For the *COI* and *COII* genes, the amplification conditions followed Ashley et al. (1996) and Hebert et al. (2003, 2004). All amplifications, including positive



N.	Locality	N. Ind.
1	Santa Marta, Col.	3
2	Maracaibo, Ven.	5
3	Tierralta, Córdoba, Col.	1
4	Antiquia, Col.	6
5	Vichada, Col.	1
6	Puerto Inírida, Col.	3
7	Meta, Col.	7
8	Camop riveri, French Guy.	11
9	Limoncocha, Ecu.	4
10	Coca, Ecu.	3
11	Mesay river, Caqueta, Col.	2
12	Negro river, Bra.	2
13	Santarem, Para, Bra.	2
14	Curaray river, Per.	1
15	Nanay river, Per.	7
16	Napo river, Per.	2
17	Tapiche river, Per.	1
18	Ucayali river, Per.	1
19	Leticia, Col.	18
20	Tabatinga, Bra.	12
21	Yavarí river, Bra	2
22	Pucallpa, Per.	4
23	Madre de Dios, Bol.	15
24	Beni river, Bol.	1
25	Chimore river, Bol.	1
26	Mamore river, Bol.	9
27	Rio Grando do Sul, Bra.	6
28	Salta, Arg.	2
29	Hernandinas, Par.	1

Figure 1. Map of the geographical localities where 141 lowland tapirs were sampled to sequence the *Cyt-b* gene.

and negative controls, were checked in 2% agarose gels, employing the molecular weight marker ϕX174 DNA digested with *Hind* III, *Hinf* I genes, and HyperLadder IV. Those samples that amplified were purified using membrane-binding spin columns (Qiagen, Valencia, CA). The double-stranded DNA was directly sequenced in a 377A (ABI, Vernon, CA) automated DNA sequencer. The samples were sequenced in both directions and all the samples were repeated to ensure sequence accuracy. The sequences were deposited in Genbank (accession nos. GQ 259910-1 and GQ 259954-1).

Data analyses

Genetic diversity and heterogeneity analyses

The sequences were edited and aligned with BioEdit Sequence Alignment Editor (Hall, 2004) and DNA Alignment (Fluxus Technology Ltd, Charlotte, NC).

The statistics employed to determine the genetic diversity at the *Cyt-b* gene for *T. terrestris* 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 the different *T. terrestris* groups, were considered. These tests were those of Hudson et al. (1992) (H_{ST}, K_{ST}, K_{ST}*, Z, and Z*), Snn test, and the Chi-square test on the haplotypic frequencies with permutation tests using 10,000 replicates. We also estimated the G_{ST} statistic from the haplotypic frequencies and the γ_{ST}, N_{ST}, and F_{ST} statistics (Hudson et al., 1992) from the nucleotide sequences. All these statistics were obtained by using the software DNAsp 5.10 (Florida Institute of Technology, Vero Beach, FL) (Librado & Rozas, 2009).

Phylogenetic analyses

To determine phylogenetic relationships and temporal splits among the different haplotype clades within *T. terrestris* at the *Cyt-b* gene, a Bayesian tree was performed using a GTR (General Time Reversible) model of nucleotide substitution. We used the BEAST v. 1.6.2 program (Viacom International Inc., New York, NY) for this analysis, assuming a Yule speciation model and a relaxed molecular clock with an uncorrelated log-normal rate of distribution (Drummond et al., 2006). Results from the two independent runs (40,000,000 generations with the first 4,000,000 discarded as burn-in) were combined with LogCombiner v1.6.2 software (Gene Code Corporation, Ann. Arbor, MI) (Viacom International Inc., New York, NY) (Drummond & Rambaut, 2007). Posterior probability values of each node were estimated, which provided an assessment of the degree of support of each node on the tree. We employed a calibration point of 18 ± 1 millions of year ago (MYA) between the ancestor of *T. indicus* and the ancestor of the Neotropical tapirs and the calibration point between the ancestor of *T. bairdii* and the ancestor of the two South American species, *T. terrestris* + *T. pinchaque* was taken as 9.5 ± 0.5 MYA following the paleontological and molecular results of different authors (Ashley et al., 1996; Colbert, 2005; Hulbert, 1995, 2010; Hulbert & Wallace, 2005; Hulbert et al., 2009; Norman & Ashley, 2000; Ruiz-García et al., 2012). No temporal split priors, or monophylia, between *T. terrestris* and *T. pinchaque*, were imposed by the authors, being the temporal split between these taxa estimated by the software.

To obtain proofs in favor or against of *T. kabomani*, we obtained a maximum likelihood tree (Felsenstein, 1981) for the three mitochondrial genes concatenated to compare with the same tree from Cozzuol et al. (2013).

Demographic changes

A Bayesian skyline plot (BSP) was obtained by means of the BEAST v. 1.6.2 and Tracer v1.5 software (Viacom International Inc., New York, NY). The Coalescent–Bayesian skyline option in the tree priors was selected with five steps and a piecewise-constant skyline model with 40,000,000 generations (the first 4,000,000 discarded as burn-in). A stepwise (constant) Bayesian skyline variant was selected with the maximum time as the upper 95% higher posterior density (HPD).

Spatial genetic analysis on *T. terrestris*

A spatial autocorrelation analysis (Sokal & Oden, 1978a,b; Sokal & Wartenberg, 1983) was applied. Coordinates were given to each one of the individuals sequenced and the AIDA procedure implemented by Bertorelle and Barbujani (1995) was applied. We calculated the autocorrelation coefficient *II* and their respective correlograms (equivalent to the traditional Moran's I index; Moran, 1950). To connect the individuals sequenced within each distance class, the Gabriel-Sokal network (Gabriel & Sokal, 1969; Matula & Sokal, 1980) was employed.

Results and discussion

The 141 individuals of *T. terrestris* we analyzed showed high levels of genetic diversity. These gene diversity statistics were also calculated in three different geographical levels: within the haplogroups found, by geographical regions and by being north or south of the Amazon River (Table 1). All the nucleotide diversities are similar within each haplogroup, with both the North and the Amazon III haplogroups, the lowest. By geographical region, the Northern Western Amazon yielded the highest nucleotide diversity, whereas the French Guyana and the Southern South America region were those which presented the lowest nucleotide diversities. If we analyze all the individuals sampled to the north and south of the Amazon River, the north set showed a higher nucleotide diversity.

Our results showed similar levels of gene diversity to those estimated by Thoisy et al. (2010) ($H_d = 0.988$ and $\pi = 0.0093 \pm 0.0004$), although in our sample (141 versus 45 individuals), we detected a considerable higher number of haplotypes (80 versus 35). The highest levels of gene diversity were located in the lowland tapir population from the Western northern Amazon basin. This agrees quite well with the fact that the most ancestral and original *T. terrestris* population was this one. This result ratifies findings by Thoisy et al. (2010).The genetic heterogeneity among different tapir sets can be seen at Table 2. The classification of haplogroups is based on the highest levels of genetic heterogeneity among the different tapir sets.

The seven genetic heterogeneity tests indicated significant findings. Additionally, the F_{ST} statistic was considerably large ($F_{ST} = 0.716$). The seven heterogeneity tests were significantly different by geographical regions, but the values of F_{ST} were considerably smaller than in the previous case ($F_{ST} = 0.279$). This is a consequence of different haplotypes belonging to different haplogroups and being intermixed within the same geographical area. Finally, we compared the genetic heterogeneity between the animals sampled north and south of the Amazon River. Although the seven genetic heterogeneity tests were significant, the value of F_{ST} ($= 0.045$) was small, which could support that the Amazon River has not been a complete geographical barrier to the dispersion of the tapir.

Thoisy et al. (2010) affirmed that the Amazon River acted as a barrier to gene flow. However, our results showed that the Amazon River was only a partial barrier for haplotype dispersion in *T. terrestris* (Nm = 11–33).

Table 1. Gene diversity statistics for *Tapirus terrestris* by mitochondrial lineages, geographic regions, and different Amazon River banks at the *Cyt-b* gene.

	S	NH	H_d	π	*K*	θ per sequence
Total sample studied of *Tapirus terrestris*	107	80	0.984 ± 0.003	0.0114 ± 0.0003	10.335 ± 4.743	19.376 ± 4.739
Mitochondrial lineages						
Amazon I	14	10	0.949 ± 0.051	0.0038 ± 0.0019	3.436 ± 1.876	4.51 ± 2.024
Amazon II	40	20	0.937 ± 0.020	0.0039 ± 0.0005	3.563 ± 1.849	6.583 ± 2.232
Amazon III	30	20	0.926 ± 0.025	0.0043 ± 0.0006	3.937 ± 2.017	7.186 ± 2.442
North	27	18	0.921 ± 0.034	0.0051 ± 0.0005	4.065 ± 2.082	6.704 ± 2.362
South	20	10	0.867 ± 0.079	0.0040 ± 0.0008	3.642 ± 1.946	6.027 ± 2.479
Geographical regions						
Northern Colombia and Venezuela	27	14	0.948 ± 0.031	0.0065 ± 0.0002	5.943 ± 2.953	7.505 ± 2.835
Guyana region	10	4	0.600 ± 0.154	0.0033 ± 0.0011	2.982 ± 1.685	3.414 ± 1.664
Upper Northern Amazon	69	40	0.973 ± 0.009	0.0129 ± 0.0001	11.779 ± 5.402	14.545 ± 4.140
Southern Amazon (Southern Peru and Bolivia)	33	15	0.910 ± 0.044	0.0091 ± 0.0002	8.263 ± 3.962	8.740 ± 3.131
Southern South America	11	5	0.806 ± 0.120	0.0034 ± 0.0002	3.779 ± 1.779	4.545 ± 2.010
Amazon River						
Northern Amazon river bank	86	58	0.980 ± 0.005	0.0121 ± 0.0002	10.969 ± 5.029	16.643 ± 4.361
Southern Amazon river bank	51	24	0.945 ± 0.022	0.0089 ± 0.0004	8.154 ± 3.863	11.990 ± 3.794

The statistics estimated were the number of polymorphic sites (S), the number of haplotypes (NH), the haplotypic diversity (H_d), the nucleotide diversity (π), the average number of nucleotide differences (*K*), and the θ statistic by sequence.

Table 2. Genetic heterogeneity statistics for *Tapirus terrestris* by mitochondrial lineages, geographic regions, and by the Amazon River banks at the *Cyt-b* gene.

Genetic differentiation estimated	*p*	Gene flow	
Mitochondrial lineages			
$\chi^2 = 696.063$, d$f = 390$	0.0000*	$G_{ST} = 0.071$	Nm = 6.54
$H_{ST} = 0.0609$	0.0000*	$\gamma_{ST} = 0.6384$*	Nm = 0.28
$K_{ST} = 0.6264$	0.0000*	$N_{ST} = 0.7177$*	Nm = 0.20
K_{ST}* = 0.3752	0.0000*	$F_{ST} = 0.7160$*	Nm = 0.20
$Z_S = 1403.773$	0.0000*		
Z_S* = 6.8447	0.0000*		
$S_{nn} = 0.9857$	0.0000*		
Geographical regions			
$\chi^2 = 457.206$, d$f = 280$	0.0000*	$G_{ST} = 0.0732$	Nm = 6.33
$H_{ST} = 0.0641$	0.0000*	$\gamma_{ST} = 0.1509$*	Nm = 2.81
$K_{ST} = 0.1333$	0.0000*	$N_{ST} = 0.2789$*	Nm = 1.29
K_{ST}* = 0.1215	0.0000*	$F_{ST} = 0.2794$*	Nm = 1.29
$Z_S = 3896.878$	0.0000*		
Z_S* = 7.6261	0.0000*		
$S_{nn} = 0.7661$	0.0000*		
Amazon River			
$\chi^2 = 120.256$ df = 77	0.0000*	$G_{ST} = 0.0148$	Nm = 33.30
$H_{ST} = 0.0131$	0.0000*	$\gamma_{ST} = 0.0243$	Nm = 20.09
$K_{ST} = 0.0180$	0.0000*	$N_{ST} = 0.0449$	Nm = 10.62
K_{ST}* = 0.0186	0.0000*	$F_{ST} = 0.0450$	Nm = 10.60
$Z_S = 4727.594$	0.0000*		
Z_S* = 8.1055	0.0000*		
$S_{nn} = 0.8377$	0.0000*		

*Significant probability ($p < 0.0001$).

Six well-differentiated mitochondrial lineages in *T. terrestris*, with significant genetic heterogeneity among them, support the fact that different events occurred, which fragmented and isolated these lineages, in the past. However, after these isolation events, these different mitochondrial lineages again migrated, expanded, and mixed in sympatrical areas. It is for this reason that there are several mitochondrial lineages in the same geographical area today. For instance, six out of six mitochondrial haplogroups were found in the Colombian Amazon and four out of six were found in the Northern Peruvian Amazon.

We detected two new Amazon haplogroups which go unnoticed by Thoisy et al. (2010): the Amazon 0 (clade very relevant because coincides with that Cozzuol et al., 2013 claimed as a different species, *T. kabomani*) and another Amazonian haplogroup (Amazon III). It is very easy to determine the correspondence of our remainder haplogroups with the four clades of Thoisy et al. (2010) because we provide the major part of the samples analyzed in Thoisy et al. (2010), and some of them were also enclosed in this work: Amazon I is the clade II, Amazon II is the clade I, North is the clade III, and South is the clade IV.

Phylogeny and phylogeography of *Tapirus terrestris*

The Bayesian tree (Figure 2) showed six haplogroups within *T. terrestris* at the *Cyt-b* gene. The posterior probabilities (p) of these main haplogroups were elevated in the majority of the cases: Amazon 0 ($p = 1$), Amazon I ($p = 1$), Amazon II ($p = 1$), Amazon III ($p = 1$), North ($p = 0.84$), and South ($p = 0.23$). Amazon 0 and Amazon I were the first two clades in which ancestors first diverged inside *T. terrestris*. Amazon 0 was composed by two exemplars sampled at the Colombian Amazon (San Martín de Amacayacu) and at the Mazan River (a tributary of the Napo River) in the northern Peruvian Amazon. These two individuals showed haplotypes related to *T. kabomani* sensu Cozzuol et al. (2013). Amazon I was composed of 13 individuals (Colombian Amazon, Northwestern Brazilian Amazon, Northern Peruvian Amazon and Ecuadorian Amazon). Amazon II was composed of 40 individuals (three Colombian Amazon departments, Colombian Eastern Llanos, Northern Colombia, Northern and Southern Peruvian Amazon, Central Brazilian Amazon, Northwestern Brazilian Amazon, Bolivian Amazon and Ecuadorian Amazon). Amazon III was composed of 37 individuals (Colombian Amazon, Eastern Colombian Llanos, Northwestern Brazilian Amazon, Central Brazilian Amazon, Northern and Southern Peruvian Amazon, Bolivian Amazon, and French Guyana). South was composed of 16 individuals (Southern Brazil, Paraguay, Bolivia, and Central Brazilian Amazon). The last haplogroup was named North and it was composed of 32 individuals. It was constituted by individuals from (French Guyana, Eastern Colombian Llanos, Northern Colombia and Venezuela and some individuals from Argentina, Southern Peruvian Amazon, Colombian Amazon, and Northwestern Brazilian Amazon).

We estimated the temporal splits of the main nodes in the Bayesian tree. The temporal split between the ancestors of *T. indicus* and the Neotropical tapirs was around 18.29 MYA.

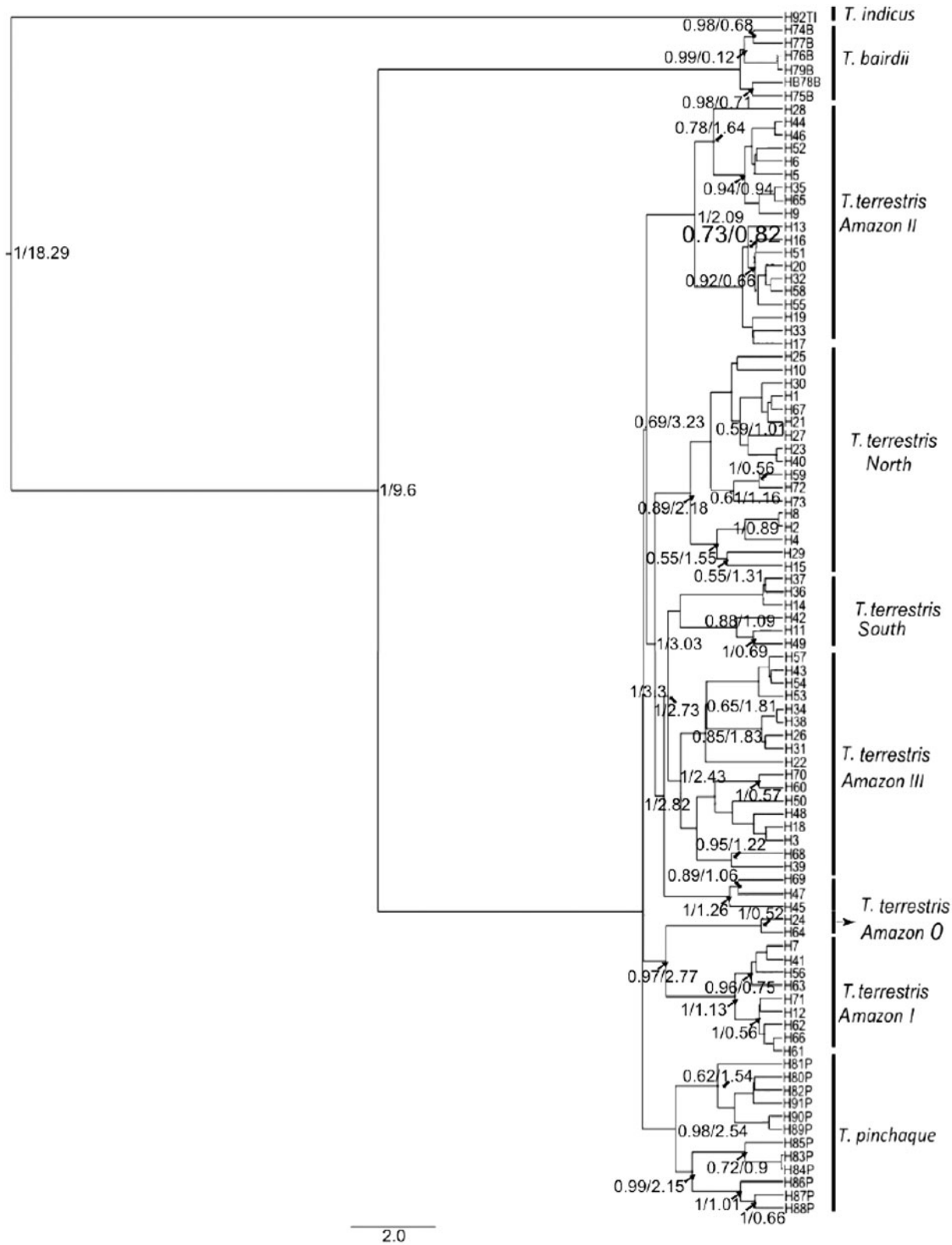


Figure 2. Bayesian tree (Beast program) with the haplotypes obtained from 141 *Tapirus terrestris* individuals sequenced at the *Cyt-b* gene. First numbers in the nodes are the posterior probabilities and second numbers are divergence time splits in millions of years ago. This Bayesian tree also encloses one haplotype (one individual) of *T. indicus* from Thailand, 12 haplotypes (30 individuals) of *T. pinchaque* from Colombia and Ecuador, and six haplotypes (30 individuals) of *T. bairdii* from Mexico, Guatemala, Costa Rica, and Panama.

The ancestor of *T. bairdii* diverged from the ancestor of *T. pinchaque* + *T. terrestris* around 9.6 MYA. The split between *T. pinchaque* and *T. terrestris* was estimated around 3.33 MYA with a haplotype diversification process within the first taxon around 2.54 MYA. The ancestors of the Amazon 0 + Amazon I haplogroups diverged from the ancestors of the other *T. terrestris* haplogroups around 2.77 MYA, whereas the diversifications within the other haplogroups occurred slightly more recently and

were similar to each other (Amazon III and South, 2.43 MYA; North, 2.18 MYA and Amazon II, 2.09 MYA). There are three periods of haplotype diversification within the Amazon I, II, and III haplogroups as well as in the North and South haplogroups. These three temporal splits occurred around 1.2–1.7 MYA, 0.6–0.9 MYA, and 0.25–0.14 MYA, respectively.

Two main results from a phylogenetic point of view were obtained by Thoisy et al. (2010). The first one is that their Bayesian analysis favored *T. pinchaque* paraphyly in relation to lowland tapir over reciprocal monophyly. Our tree did not agree with the Thoisy et al. (2010)' conclusion. Our Bayesian tree clearly showed that there was reciprocal monophyly between *T. terrestris* and *T. pinchaque*. No constriction was imposed in this tree to force this reciprocal monophyly. Second, Thoisy et al. (2010) employed two approaches to estimate temporal splits within the clades of *T. terrestris*. In the first approach, they used fossil data to calibrate the molecular clock with the timing of the Rhinocerontidae and Tapiridae split (46.7 MYA) and another split within Rhinocerontidae (17.1 MYA). In the second approach, they employed a direct mutation rate at the *Cyt-b* gene for Perissodactyls and they obtained a very recent mitochondrial diversification within *T. terrestris* in the mid to late Pleistocene (0.33 MYA). We employed a different approach to calibrate the molecular clock by using simultaneously mixed fossil and molecular data. Our split results agree considerably better with the first approach of Thoisy et al. (2010) than with the second one.

Demographic changes

Our analyses of BSP findings indicate several important points. For the total *T. terrestris* sample, there was a population contraction around 2.2–2.0 MYA. However, 1.9 MYA, there was a strong population expansion, which reached its ''plateau'' around 1.5 MYA. Since then, the overall tapir population has remained constant until today. By haplogroups, Amazon I began decreasing 5500 YA and continued to do so until around 1400 YA. Since then, this population has expanded up until the last 500 years when it was constant. Amazon II and III experienced steady decreasing population trends beginning 75,000 YA and ending 12,000 YA in the case of Amazon II and 14,000 YA in the case of Amazon III. Both haplogroups have since increased up until the last 500 years when their size became constant. North experienced a constant decreasing population trend that started around 14,000 YA and ended 3000 YA, after which the lineage population increased until today. South experienced a slight but constant decreasing population trend from 6000 YA until 2500 YA. This lineage has increased since that time until today.

Amazon I suffered a population declination 5500 YA coinciding with one of the dry periods in the Holocene after the Optimum Climaticum (OP). This dry period was detected in the Amazon, Caqueta and lower Magdalena River basins as well as in Andean lagoons in Colombia and Peru (Thompson et al., 1995; Van der Hammen, 2001; Van der Hammen & Cleef, 1992). Amazon II and III revealed a population decrease 75,000 YA coinciding with the ending of the Eemiense inter-glacial period and the beginning of the upper Pleniglacial period (Van der Hammen, 1992). These two Amazonian haplogroups began to again expand around 12,000 and 14,000 YA after LGM. In contrast, North decreased 14,000 YA, agreeing with the massive extinction of mammals across the Earth including South America during LGM. This corresponds with the Younger Dryas (Dryas III), typical of Northern Europe and Scandinavia (Clapperton, 1993). Finally, South suffered a population declination around 6000 YA coinciding with the drier period between 7000 and 5500 YA that we commented on above.

Spatial genetic structure

The AIDA analyses generated different correlograms. This analysis, applied to the total tapir population, indicated that the individuals separated by 300 km or less (first distance class, 1 DC) were significantly more related than expected by random, whereas the animals separated by 700 and 1300 km showed a significant negative spatial autocorrelation (3 and 4 DC). After 1300 km, the genetic relationships among the individuals were more randomly distributed following a ''crazy quilt'' pattern following the suggestion of Sokal & Oden (1978a). The 7 DC (1650–2150 km) was positively significant (which suggests genetic flow among animals separated by this distance interval), whereas between 2150 and 2600 km, the autocorrelation was significantly negative (which suggests some obstacle or some geographic barrier at this distance interval). Thus, this AIDA analysis showed genetics patches with a diameter of around 1300 km. The AIDA analysis – taking all the individuals sampled in Colombia and Venezuela, showed a correlogram typically representing an isolation by distance pattern. The individuals within the 1 DC (less than 350 km) showed significant genetic resemblance. However, animals separated by 900–1800 km (3 and 4 DC) were significantly different. The AIDA analysis applied to the Western Amazon showed a unique distance class that was significantly positive (4 DC, 490–690 km). Therefore, migration events seem to have occurred at these distances. This particular area of the Amazon seems to have less spatial genetic structure compared to other geographical areas analyzed. Finally, an AIDA analysis was applied to those tapirs sampled at the southern Amazon. The correlogram showed two significant distance classes. The first was 1 DC (0–150 km), where the autocorrelation was positive. For the second one, 4 DC (780–900 km), the autocorrelation was negative. Thus in this Southern Amazonian area, the presence of a monotonic cline was detected.

There was no clear association between haplogroups and specific geographical areas – revealing a complex system of migration and colonization by the lowland tapir, which coincides with the Thoisy et al.'s (2010) claim to reject the null hypothesis of allopatric divergence. However, we provide much more robust evidence of this complex system of migration and colonization than the Thoisy et al.'s (2010) work throughout the autocorrelation analysis. The application of AIDA revealed the existence of genetics patches of a diameter around 1300 km for the overall tapir population, but with repetitive events of gene flow at long distances (up until 5000 km). These genetics patches agree quite well with the existence of the Pleistocene Refugia (Haffer, 1969, 1997, 2008). The AIDA analysis applied to the Upper Western Amazon basin indicated the smallest degree of spatial structure. This could be related with the fact that this was the population with less evidence of population expansion, and with the highest gene diversity levels because this was probably the original and more demographically stable lowland tapir population in South America. This agree quite well with the Napo Refugium (or Napo island) both claimed by the Pleistocene Refugia hypothesis (Whithmore & Prance, 1987) and the Recent Amazon lake hypothesis (Nores, 1999, 2004).

The question of ''*T. kabomani*''

Figure 3 shows the maximum likelihood tree for the three mitochondrial genes concatenated. There is no evidence that *T. kabomani* should be a full species, being only a haplogroup (clade) within *T. terrestris*. Clearly, to augment the number of *T. pinchaque* individuals sequenced with reference to the work of Cozzuol et al. (2013), *T. pinchaque* is more differentiated from *T. terrestris* than *T. kabomani* is.

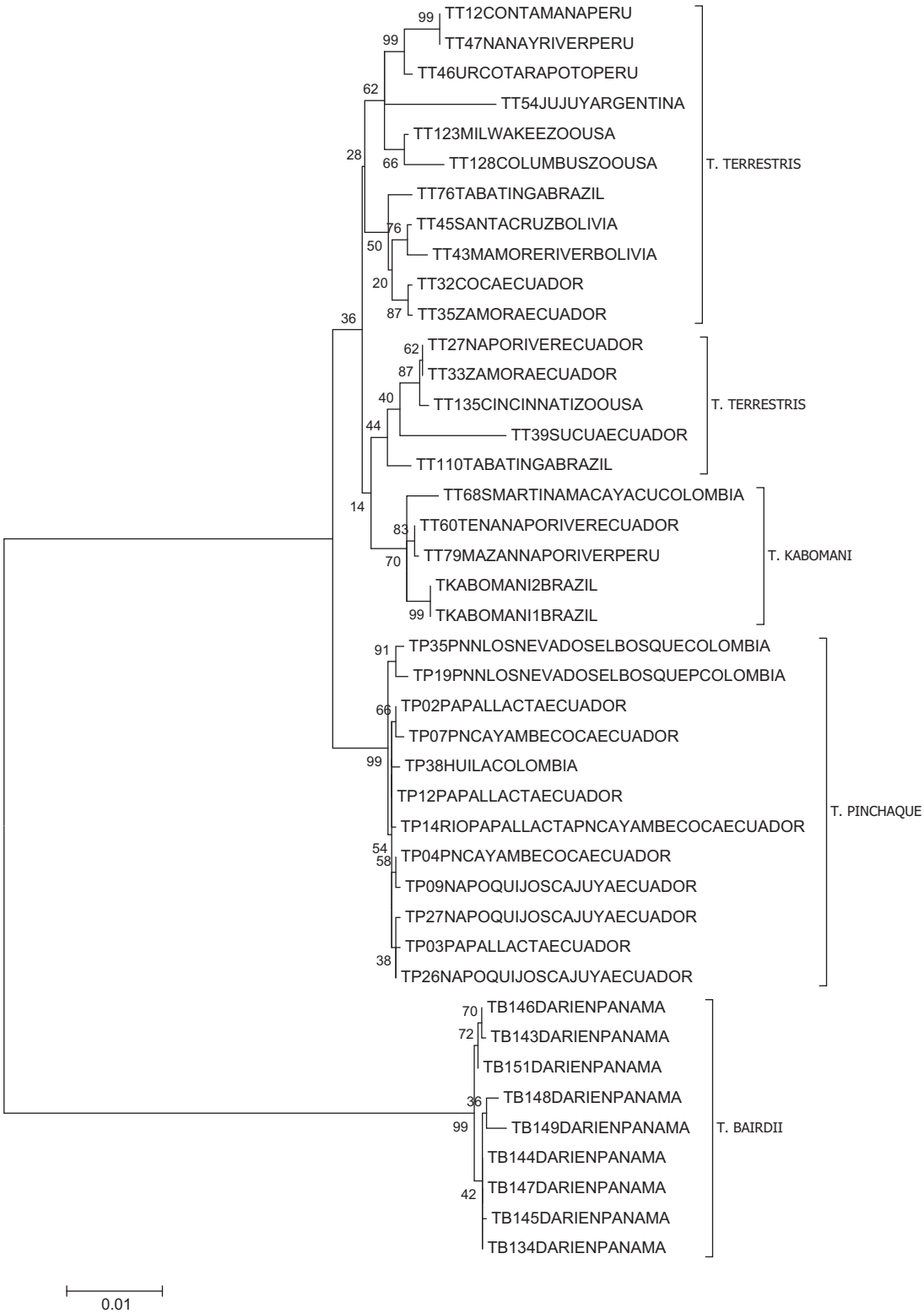


Figure 3. Maximum likelihood tree with three concatenated mitochondrial genes (*Cyt-b* + *COI* + *COII*) for 42 tapirs, including *T. terrestris*, *T. kabomani*, *T. pinchaque*, and *T. bairdii*. *Tapirus kabomani* is a clade within *T. terrestris*. There was reciprocal monophyly between *T. terrestris* and *T. pinchaque*.

The question of the alleged new species, *T. kabomani*, is relevant for the knowledge of the phylogeography of *T. terrestris* because we provide proofs that *T. kabomani* is a haplogroup within *T. terrestris*. Cozzuol et al. (2013) affirmed the existence of *T. kabomani* because four sequences of two Colombian and two Brazilian tapirs were outside of the *T. terrestris* + *T. pinchaque* clade. However, our maximum likelihood tree showed that *T. kabomani* is more related to *T. terrestris* than *T. pinchaque* is. The inaccurate result obtained by Cozzuol et al. (2013) was probably related with the fact that these authors only analyzed five samples of *T. pinchaque* at the *Cyt-b* gene and only one sample at the *Cyt-b* + *COI* + *COII* genes. The very small *T. pinchaque* sample employed by Cozzuol et al. (2013) did not probably represent all the mitochondrial gene diversity of *T. pinchaque* and,

as the genetic differences between both *T. terrestris* and *T. pinchaque* are very small, by chance the no representative mitochondrial gene diversity of the *T. pinchaque* of the small sample of Cozzuol et al. (2013) was nested within the gene diversity of *T. terrestris*. Nevertheless, at the moment that we enlarged the *T. pinchaque* sample, this phenomenon disappeared. Henceforth, our tree, which contained the highest number of individuals of *T. terrestris*, *T. kabomani*, and *T. pinchaque* analyzed until date for the three concatenated mitochondrial genes employed by Cozzuol et al. (2013), agrees extremely well with the point of view of Voss et al. (2014) and against Cozzuol et al.'s (2013) claims.

We believe more prudent to consider *T. kabomani* as a *T. terrestris* haplogroup until new data, employing nuclear and *MHC* gene sequences, have been generated for this taxon. Especially important in our view is to obtain karyotypes of this taxon to determine, or no, possible stasipatric speciation (due to the inexistence of geographical barriers between *T. terrestris* and the alleged *T. kabomani*; White, 1968, 1978).

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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