Genetic differentiation and diversity of the Bolivian endemic titi monkeys, *Plecturocebus modestus* and *Plecturocebus olallae*

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Abstract

The genetic variability of New World primates is still poorly documented. We present the first genetic study on two threatened endemic titi monkey species in northern Bolivia (*Plecturocebus modestus* and *Plecturocebus olallae*) using six microsatellite markers to investigate genetic structure and variability of 54 individuals from two wild populations. A low level of genetic diversity was found (34 alleles in the total sampled population). Locus 1118 presented the greatest number of alleles. The mean number of alleles per locus in the total population was 5.6 and the average heterozygosity was 0.38 (range: 0.12–0.88). The F_{IS} value for the total population using all microsatellite loci shows a statistically significant heterozygote deficit. The inbreeding coefficients (F_{IS}) were positive and significantly different from zero (0.064 for *P. olallae* and 0.213 for *P. modestus*). The genetic differentiation between populations (F_{ST}) was moderate with a pair-wise F_{ST} estimate of 0.14. Population structure analyses assigned the two populations to two differentiated clusters (K=2). These results suggest that these two species with very close distributional ranges arose from a single population, and that they remain in a process of genetic differentiation and speciation. This study further underlines the urgent need for conservation actions for both endemic primate species.

Key words: Olalla´s titi monkey; Beni titi monkey; microsatellites; genetic diversity; conservation

1. Introduction

The study of genetic variation and population structure is critical to better understand how species evolve, adapt and co-exist, as well as to propose rational conservation and management strategies (Eguiarte, 1990). Molecular markers provide an estimate of genetic diversity, with multiple alleles being useful to detect heterozygosity and polymorphism levels in natural populations, determine inter and intra-population relations, characterize genetic population structure, and analyse genetic distances for evolutionary studies (Avise, 2000). As such, genetic studies are increasingly relevant to taxonomy, biodiversity conservation and environmental monitoring programs to ensure adequate management of species and populations (Groom, 2006).

The Olalla brothers' titi monkey (*Plecturocebus olallae*) and the Beni titi monkey (*Plecturocebus modestus*) are two endemic primate species in Bolivia, which were described by Lonnberg in 1939. Although their type localities are less than 65 km apart (Figure 1), the respective specimens were consistently considered as belonging to two different species in a series of taxonomic assessments for the entire Callicebinae group (Hershkovitz, 1990; Kobayashi, 1995; Van Roosmalen *et al*., 2002; Wallace *et al*., 2013). Studies on the distribution of both species have confirmed the presence of two similar, but phenotypically distinct forms that correspond to the original descriptions with distributional ranges quite close to each other (Figure 1; Felton *et al.*, 2006; Martínez & Wallace, 2007, 2010, 2013, Martinez et al., 2013). The two Bolivian titi monkey species were considered Endangered by the IUCN (Veiga *et al*., 2008[a], 2008[b]), however in the most recent assessment *P. olallae* was recognized as Critically Endangered (Martinez & Wallace, 2016). These categorizations stem from the extremely restricted distributions

(especially *P. olallae*), habitat fragmentation, as well as low population density estimates (Felton *et al.*, 2006; Martínez & Wallace, 2007, 2010, 2013; López-Strauss & Wallace, 2015).

A new taxonomic arrangement for titi monkey species was recently published in which the original genus *Callicebus* was split up in three genera (*Cheracebus*, *Plecturocebus*, and *Callicebus*). This decision was based on a molecular assessment of species relationships and supported by biogeographical information that together explained the species radiation process of titi monkeys in the Neotropics (Byrne et al., 2016; 2018). We follow this latest taxonomy for the two Bolivian endemic titi monkey species. Despite substantial advances in taxonomical knowledge, there remains a lack of information for species of the "donacophilus" group of titi monkeys which includes *P. olallae* and *P. modestus*. Here, using molecular microsatellite markers, we present the results of an analysis of the inter and intra-species genetic variation of the two Bolivian endemic titi monkey species *P. olallae* and *P. modestus*.

2. Methods

Study area

We conducted our study in two localities. La Asunta is a cattle ranch found 2 km northwest of the upper Yacuma River and 22 km from the original type locality of *P. olallae*. The *P. modestus* study site was the Aguaizal community, approximately 45 km from the original type locality (Figure 1). Both localities were known as titi monkey sites from previous distributional studies (Martinez & Wallace 2017). The two localities were found in central portions of the known distributional ranges of the titi monkey species and separated by around 45 km.

Preliminary assessment of faecal samples as DNA source

We were committed to a non-invasive method to obtain DNA samples especially given the risks that failures in procedures of live capture and release of individuals could have on these threatened species. We identified faecal samples as a potential DNA source. To assess the quality of faecal DNA samples we conducted a PCR amplification control quality comparing faecal and blood samples from an individual of the geographically neighbouring species, *Plecturocebus donacophilus*, held at the Zoologico de Fauna Sudamericana, Santa Cruz de la Sierra, Bolivia. Extraction of DNA from blood tissue was conducted using conventional CTAB-chloroform protocols (Doyle & Doyle, 1987; Woodward *et al*., 1994), modified and adapted at the Institute of Molecular Biology and Biotechnology laboratory. The PCR amplification of all 12 microsatellite loci exhibited a 100% allelic correspondence across faecal and blood samples, highlighting the potential of faecal samples as a DNA source.

As an additional confirmation, we found similar results from a comparison between faecal samples obtained from our fieldwork (see details below) against muscular and hairs samples from collected specimens of *P. modesuts* and *P. olallae* (Martinez *et al*., 2013), respectively.

Faecal sample collection

We collected faecal samples from multiple individuals of titi monkeys belonging to 8 groups in La Asunta and 9 groups in Aguaizal. Titi monkeys are territorial primates which emit early morning territorial calls (Kinzey 1988, Bicca Marquez & Heymann, 2013). We approached titi monkey groups while they were emitting these calls to ensure

all members were concentrated in a relatively small area thereby avoiding erroneous assignation of individuals to other groups. At each encounter with a group we collected faecal samples from 1-3 individuals by waiting for animals to defecate and immediately collecting samples. This usually happened a few seconds after they noticed our presence. In order to avoid multiple samples from the same individual, care was taken to ensure that multiple sampled individuals from the same group were clearly distinguishable using age-sex classes. Faecal samples were stored at ambient temperature in plastic bottles with silica gel until transported to the laboratory.

DNA isolation and genotyping

Extraction of DNA was conducted using QIAamp DNA Stool Mini Kit (50) commercial kit according to manufacturer specifications from collected primate faecal samples: 23 for *P. olallae* and 31 for *P. modestus*. Given the lack of specific microsatellite markers for *P. olallae* and *P. modestus*, we used seven heterologous markers developed for *Lagotrhix lagotricha* (1110, 1118, 1115, 311, 312, 157, 113) (Di Fiore, 2004) and five markers developed for *Alouatta palliata* (Ap 6, Ap 20, Ap 40, Ap 68, Ap 74) (Ellsworth, 1998). Polymerase chain reactions (PCR) was conducted under standard conditions, using 40 ng DNA in 1X of PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 0.5 – 0.8 mM of each primer, 0.2 µg/ml of BSA and 0.05 U/µl –of Promega™ GoTaq™ DNA Polymerase and additional ddH_2O for a final volume of 30 µL. The reaction cycle started at 95º C for 5 min; followed by 36 cycles of 35 sec at 94º C for denaturation, 30 sec at 50º C for primer annealing (Di Fiore *et al.*, 2004), and 25 sec at 72º C for extension; and a final step of 10 min at 72º C. Each amplification reaction was performed four times for each sample. The amplified fragments were separated in 10% polyacrylamide gel (ratio 19:1) and further

stained with silver nitrate. Allele sizes were estimated using a 25bp DNA Step Ladder, and genotypes were checked four times. Quality control in genotype assignment was performed with two examiners. Discrepancies identified through a custom-made database were double-checked and resolved.

Data analyses

Genetic variability was evaluated according to the mean number of alleles per locus (A), the mean number of effective alleles per locus (ne), and the observed (Ho) and expected (He) heterozygosity under the Hardy–Weinberg equilibrium, F_{IS} and their respective means. These parameters were calculated using the GENETIX v. 4.05 32 software (Belkhir *et al*., 2004). Deviations from the Hardy–Weinberg equilibrium were tested using the Markov chain method (100 batches of 1,000 interactions with a 1,000-step dememorization process), applied to calculate unbiased estimates of exact probabilities (P values). These tests were run on GENEPOP v.3.1c software (Raymond & Rousset, 1995). Microsatellite loci were removed according to the type of data analysis as long as they didn't have high frequencies of null alleles and strong deviations from Hardy-Weinberg Equilibrium in both species. Estimations of null alleles and PIC (*Polymorphism Information Content*) were performed with CERVUS 3.0.3 software. For this work, any marker was removed. We determined differences between populations with the F_{ST} statistic following Weir and Cockerham (1984) and genetic distance following Nei (1972). Estimates of gene flow (Nm) were generated with the F_{ST} statistic. Genetic distances, and factorial analyses of correspondence were conducted with GENETIX v. 4.05.

Individuals were assigned to populations using STRUCTURE v.2.0 (Pritchard *et al*., 2000b). This method allows inferences about genetic population structure using genotypes from a group of loci, as well as determining the probability that an individual belongs to a given population. We used an algorithm that infers that the populations were mixed. The value for the K parameter used in the analysis varied from 1 to 3 and the number of iterations for each K was 50,000, with 10 replicate runs for each K using correlated allele frequencies and an admixture model. Graphic representation of the indicators of posterior probability of K cluster (Ln Pr (X/K) and Δ(K)) were obtained with the web-based STRUCTURE HARVESTER software v.0.6.94 (Earl & VonHoldt, 2012).

3. Results

Genetic diversity

Six of the 12 microsatellite loci analysed were polymorphic (1118, 311, Ap40, 1115, 312, Ap74), one was monomorphic (Ap68) and five resulted in poor amplifications (Ap6, Ap20, 1110, 157, 113). Subsequent statistical analyses for both species were limited to the six polymorphic microsatellite loci.

A total of 34 different alleles were observed across the 54 individuals of *P. olallae* and *P. modestus* analysed, with a mean number of alleles of 5.6. The locus that presented the greatest polymorphism was 1118 with 12 alleles, while the lowest amount of alleles was for locus AP74 with 2 alleles (Table 1). The highest heterozygosity was observed for locus 1118 (Ho = 0.90), whereas the lowest occurred on marker 312 (Ho = 0.09). The locus showing the highest expected heterozygosity was 1118 (He = 0.86), whereas locus 312 had the lowest value for this parameter (He = 0.15). Three out of the six loci analysed

(311, 312, Ap 74) were identified to be in Hardy–Weinberg (HW) disequilibrium in the global population (see Table 1). We found a statistically significant heterozygote deficit (according to inbreeding F_{IS} value) for all the loci analysed except 1118 and Ap40 which showed heterozygote excess, although not at a significant level (Table 1). The population analysis showed only one marker in HW disequilibrium in both populations, therefore the information of all markers was considered in subsequent genetic differentiation analyses. The frequency of null alleles estimated ranged from 0 to 0.3849 while the polymorphic information content (PIC) ranged from 0.15 to 0.84 (Table 1).

Both species showed the greatest number of alleles in the locus 1118 microsatellite marker (Table 1). The mean number of alleles per locus was higher in *P. modestus* than in *P. olallae* (Table 2). Seven exclusive or private alleles were identified for *P. olallae* and nine for *P. modestus*, with different frequencies in the main markers (Table 2). The observed and expected heterozygosity by species showed the average Ho ranged from 0.3146 (*P. olallae*) to 0.3656 (*P. modestus*), whereas the average He varied between 0.3283 (*P. ollalae*) and 0.4555 (*P. modestus*) (Table 2). In both species the heterozygote deficit was significant (Table 2).

We found a low and nonsignificant heterozygote deficit (F_{IS},) for *P. olallae*, but it was higher and statistically significant for *P. modestus*, indicating that the populations assessed were not in a panmictic status (Table 2). No significant recent population bottleneck was identified for either species.

Genetic differentiation

We found a low but significant genetic population structuring between the samples from *P. olallae* and *P. modestus* (F_{ST}=0.137, $p<0.01$). Based on this result, the gene flow

between *P. modestus* and *P. olallae* indicates a theoretic migration of two individuals between the two titi monkey species per generation (Nm=1.57).

According to the factorial analysis of correspondence, the first component explained 100% of the total variation and separated *P olallae* and *P. modestus* individuals, showing a clear tendency for separation of both species, forming two groups in each inertia axis, with some overlapping individuals (Figure 2).

The results of the Bayesian analysis to assign individuals to groups indicated a clear structure between the two titi monkey species. The highest likelihood was obtained when K was set to two, with the distribution of L (K) showing a clear peak for K = 2. Using the method of correction of Evanno, the maximal K occurred at K = 2. Based on this and the biological significance of the results, K = 2 was chosen as the final estimated number of populations. The graphical representation of the clustering outcomes for K = 2 is shown in Figure 3 with a few *P. modestus* individuals showing a high genetic relation with *P. olallae*. According to these findings, the proportion of individuals correctly assigned to each cluster was 90.4% for *P. olallae* and 79.2% for *P. modestus*.

4. Discussion

Genetic variability

This is the first study on the genetic variability, inter-specific genetic differentiation and relationships between the Bolivian *P. olallae* and *P. modestus* whose restricted distributional ranges are proximal (Martinez & Wallace, 2010, Wallace et al., 2013). We obtained a larger number of alleles compared to previous titi monkey studies (Di Fiore, 2004), probably because of the larger number of sampled individuals (n=54). Di Fiore (2004) reports failure to amplify the locus 312 for titi monkeys, but we amplified this

229 locus for both Bolivian *Plecturocebus* endemics showing better sequence compatibility.
230 For the primers which had not previously been tested for titi monkeys, we obtained few
231 amplified alleles for two of the loci (1 for Ap 68, 2 for Ap 74), and 5 alleles for Ap 40.

232 Our analysis based on the six DNA microsatellite loci revealed low allelic variability for
233 the two Bolivian titi monkeys (5.6 alleles for the entire population, 4.2 for *P. olallae* and
234 4.5 for *P. modestus*). The results described herein reveal low levels of genetic variability
235 comparable with previous studies performed on other species of monkey populations
236 using similar markers (Ellsworth, 1998, Di Fiore & Fleischer, 2004, Ruíz, 2007). This
237 may reflect the fact that the primers used are not specific to these species. Nevertheless,
238 the lack of correlation observed between the HWE deviation and the occurrence of null
239 alleles, suggest the existence of population subdivision (Wahlund effect), probably due to
240 a reduced effective population size within the studied species.

241 The differences in the number of identified private alleles (7 for *P. olallae* and 9 for *P.*
242 *modestus*) were especially useful for genetic differentiation between species, as their
243 presence suggests independent evolution. The total studied population had a mean
244 heterozygosity of 0.3709, while *P. olallae* presented a mean heterozygosity observed of
245 0.3146 and *P. modestus* 0.3656, values that are similar to the 0.33 mean reported for
246 *Plecturocebus moloch* (Alcantarino et al., 2009). Heterozygosity values of 0.5 or more
247 were reported for other threatened Neotropical primates such as *Alouatta pigra* (0.5;
248 García et al., 2005), *Lagothrix lagotricha* (0.8; Di Fiore & Fleischer, 2004). Therefore,
249 the values for the Bolivian titi monkeys suggest that their genetic variability might be
250 under ecological pressures.

Both *P. modestus* and *P. olallae* showed consanguinity, relatively low and not significant for *P. olallae* (F_{IS} = 0.064), but higher and statistically significant for *P. modestus* (F_{IS} = 0.231), indicating that factors such as consanguinity, isolation, genetic drift or selection may be acting on this species. Moreover, these results suggest a biased mating in the *P. modestus* population between individuals with similar alleles. Population health can decline due to consanguinity occurring in a few generations, a scenario that is an especially serious conservation problem in small populations (Gilpin & Soule, 1986). The consanguinity levels found may be a direct consequence of forest fragmentation present across the entire distribution range of both endemic titi monkey species, with *P. modestus* occupying a larger area, but in a drier forest, than *P. olallae* (Felton et al., 2006; Martinez & Wallace, 2007, 2010, 2013). Drier habitat may be more sensitive to forest fragmentation processes (Onderdonk & Chapman, 2000) promoting the spatial isolation of *P. modestus* groups, thereby affecting their genetic variability.

Moreover, our sampling site for *P. modestus* is not as fragmented as the overall distribution range of this species (Martinez & Wallace, 2010, Wallace et al., 2013) and higher consanguinity levels could be expected for this species across its range. This finding highlights the importance of population genetics information in the design of conservation efforts, as combined with distribution and demographic information it provides more details of a species conservation status.

Given the lack of significant evidence for recent bottlenecks in the studied populations, the significant F_{IS} values identified in both studied populations may be the result of high levels of inbreeding related to inbreeding depression and a reduction in the average phenotypic values.

Population structure

The locus 1118 presented the largest number of alleles and the largest number of private alleles for both species, with frequencies indicating a 68.75% possibility of finding one of these alleles in *P. modestus*. The locus 1118 is therefore a good diagnostic marker for *P. modestus*. The locus 311 presented 2 private alleles for *P. olallae*, with a 23.62% possibility of finding them in individuals of *P. olallae*. The loci 1115 and 312 also had low frequency private alleles for *P. modestus* (Table 3).

According to the genetic population structure that we found between the two species (F_{ST}=0.137), 86% of the variation in allele frequencies is found within populations, whereas 14% of the variance is attributable to differences between the populations. This level of genetic differentiation suggests *P. olallae* and *P. modestus* could be recognized as either subspecies or species (Holsinger et al., 2009). Additionally, although our results show that a theoretical gene flow might exist between the two species and that divergence due to genetic drift is low, the two populations do not behave as a single population, which is a parameter that suggest a recent process of speciation (Chikhi et al., 2004).

The factor analysis of correspondence showed noticeable clusters for both endemic *Plecturocebus* species. These results suggest that only a few individuals tended to separate out from their population and lean towards the opposing species. The fact that sampling localities are separated by around 45 km makes individual movements unfeasible based on the knowledge about distribution, territoriality, and dispersion patterns of the study species (Martinez & Wallace, 2010). Rather, this analysis supports the hypothesis of a recent speciation event between the two titi monkey populations.

296 Genetic structure supports the previous statement, showing a clear distribution of the
297 population into two clusters when assigning two populations (K=2), with a reduced
298 number of shared genes, and a small number of migrants. When K was set as one (K=1),
299 thereby considering both species as a larger single population, individuals were assigned
300 in the same manner, forming two separate clusters with some individuals sharing genetic
301 characters. In addition, the alpha index value of 0.1213 indicates moderate genetic
302 population structuring, forming two separate populations, although certain individuals
303 still share alleles.

304 The patterns of genetic differentiation found in our study indicate that *P. modestus* and *P.*
305 *olallae* show genetic differences through characteristic private alleles, a distinguishable
306 distribution in a factorial analysis of correspondence and moderate but significant genetic
307 population structuring for the population. Nevertheless, we also found a number of
308 theoretic migrants (2) that still contribute to gene flow between the two species. Taken
309 together this suggests that these two species with very close distributional ranges arise
310 from a single population and that they are probably still in a process of genetic
311 differentiation and speciation.

312 The distribution range of *P. olallae* is found in the middle of the *P. modestus* range with
313 narrow but effective landscape scale barrier, in the form of seasonally flooded savannah,
314 impeding migration of individuals between populations (Martinez & Wallace, 2007;
315 2010, Wallace et al., 2013). This barrier might be relatively recent in evolutionary time-
316 scale, isolating a small population of titi monkeys, that later gave rise to *P. olallae*, in the
317 midst of a larger ancestral population that today we recognize as *P. modestus*. Genetic
318 parameters show these populations of *Plecturocebus* as clearly different at a subspecies

level, but the evidence also suggests an apparent recent speciation process, which when combined with already identified morphological and phenotypic distinctions, suggests that *P. olallae* and *P. modestus* are two very close species still undergoing genetic divergence.

We found a higher level of consanguinity for *P. modestus*, which may signify a higher extinction risk than for *P. olallae*. Given that *P. modestus* has a larger distributional range and population size than *P. olallae* (Martinez & Wallace, 2007, 2010; Lopez-Strauss & Wallace, 2015), these observations were unexpected. However, larger populations do not necessarily have higher genetic variability and less consanguinity (Lacy, 1987). The forests are fragmented across the range of both species, and our results may simply reflect the population dynamics on each specific study site, although the *P. modestus* locality had larger and more continuous forest coverage than the *P. olallae* site. From a genetic perspective, our preliminary results suggest that *P. modestus* may be in a critical situation as compared to *P. olallae*. Future studies should consider including mitochondrial markers and expanding sampling sites across the distribution ranges of each species.

The IUCN previously classified both *P. olallae* and *P. modestus* as Endangered due to small distribution ranges, habitat fragmentation and habitat loss risks, as well as population decline (Martinez & Wallace, 2007, 2010, 2013; Veiga et al., 2008a, 2008b; Wallace et al., 2013; López-Strauss & Wallace, 2015). More recently, P. *olallae* was re-categorized as Critically Endangered due to an extremely limited distribution (Martinez & Wallace, 2016). From a genetic perspective, *P. modestus* faces a greater risk of loss in genetic quality than *P. olallae*, which should be considered in further conservation actions.

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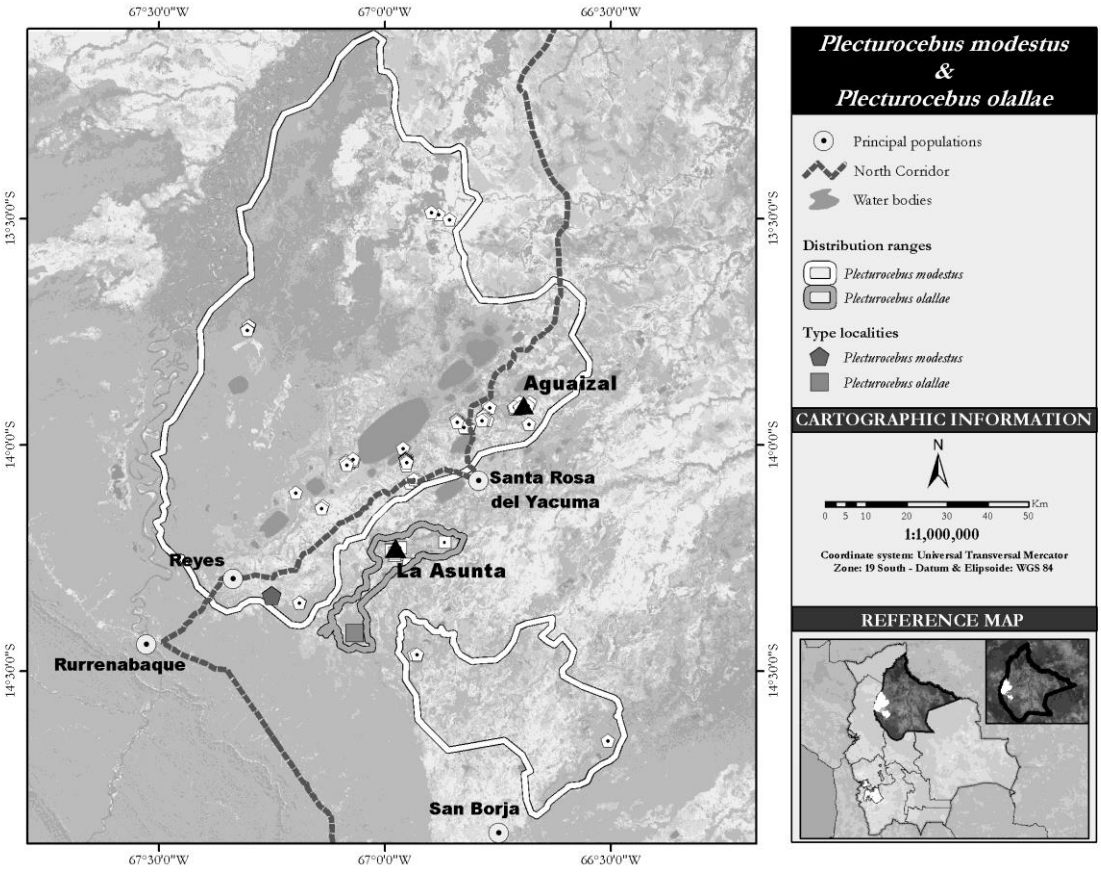
Figures

Fig. 1 Map showing known distribution of *Plecturocebus modestus* and *Plecturocebus olallae*, original type localities and study samples localities.


67°30'0"W
67°0'0"W
66°30'0"W
13°30'0"S
14°0'0"S
14°30'0"S
Aguaizal
Santa Rosa
del Yacuma
Reyes
La Asunta
Rurrenabaque
San Borja
Plecturocebus modestus
&
Plecturocebus olallae
Principal populations
North Corridor
Water bodies
Distribution ranges
Plecturocebus modestus
Plecturocebus olallae
Type localities
Plecturocebus modestus
Plecturocebus olallae
CARTOGRAPHIC INFORMATION
N
0 5 10 20 30 40 50 Km
1:1,000,000
Coordinate system: Universal Transversal Mercator
Zone: 19 South - Datum & Elipsoide: WGS 84
REFERENCE MAP

468

Fig.2 Positioning of individuals within the Cartesian plane constructed from factorial analysis of correspondence of *Plecturocebus olallae* (dark gray) and *Plecturocebus modestus* (light grey)

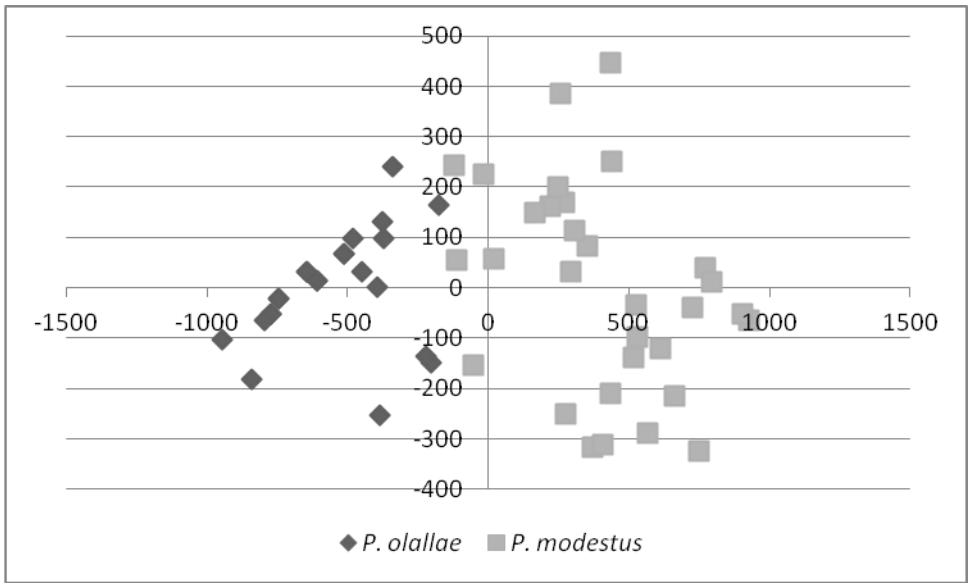


Fig.3. Graphical representation of the clustering outcomes suggested by the Bayesian analysis performed to assess the structure of the studied populations at K = 2. Each color represents one cluster, and the length of the colored segment shows the individual's estimated proportion of membership in that cluster. Black lines separate the individuals of the different populations**.**

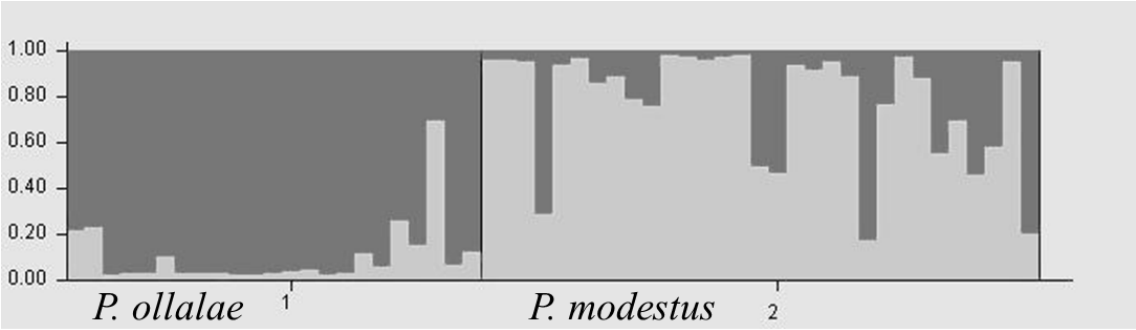


Table 1. Genetic diversity parameters estimated for the 6 microsatellite markers analyzed in the two *Plecturocebus* species considered in this study

Marker	**A[a]**	**Ho[b]**	**He[c]**	**HWE[d]**	**Fis[e]**	**F (null)[f]**	**PIC[g]**
1118	12	0.9057	0.8610	ns	-0.0423^{ns}	0.0335	0.847
311	7	0.4151	0.7577	***	0.4597***	0.2220	0.717
Ap 40	5	0.2778	0.2476	ns	-0.1126^{ns}	0.0000	0.231
1115	4	0.1852	0.2368	ns	0.2268*	0.0000	0.228
312	4	0.0926	0.1562	*	0.4150**	0.1089	0.150
Ap 74	2	0.1852	0.3656	***	0.5004**	0.3849	0.299
Total	34						

[a] A: number of alleles per locus.
[b] Ho: average observed heterozygosity.
[c] He: average expected heterozygosity.
[d] HWE: significant deviation from the Hardy–Weinberg equilibrium (P<0.001).
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*P<0.05.
** P<0.01.
*** P<0.001.
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Table 2. Genetic variability parameters estimated for two species of *Plecturocebus* based on the analysis of 6 microsatellite markers

Species	**n[a]**	**Ho[b]**	**He[c]**	**HEW[d]**	**MNA[e]**	**pA[f]**	**Fis[g]**
P. ollalae	23	0.3146	0.3283	*	4,2	7	0.06484
P. modestus	31	0.3656	0.4555	**	4,5	9	0.21311***

[a]n: sample size.
[b]Ho: average observed heterozygosity.
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Table 3 Frequency of private alleles per locus for each species

Species	Locus	Allele	Frequency
P. modestus	1118	162	0.3875
		174	0.1375
		178	0.1625
	1115	226	0.0875
	312	162	0.0750
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Figures

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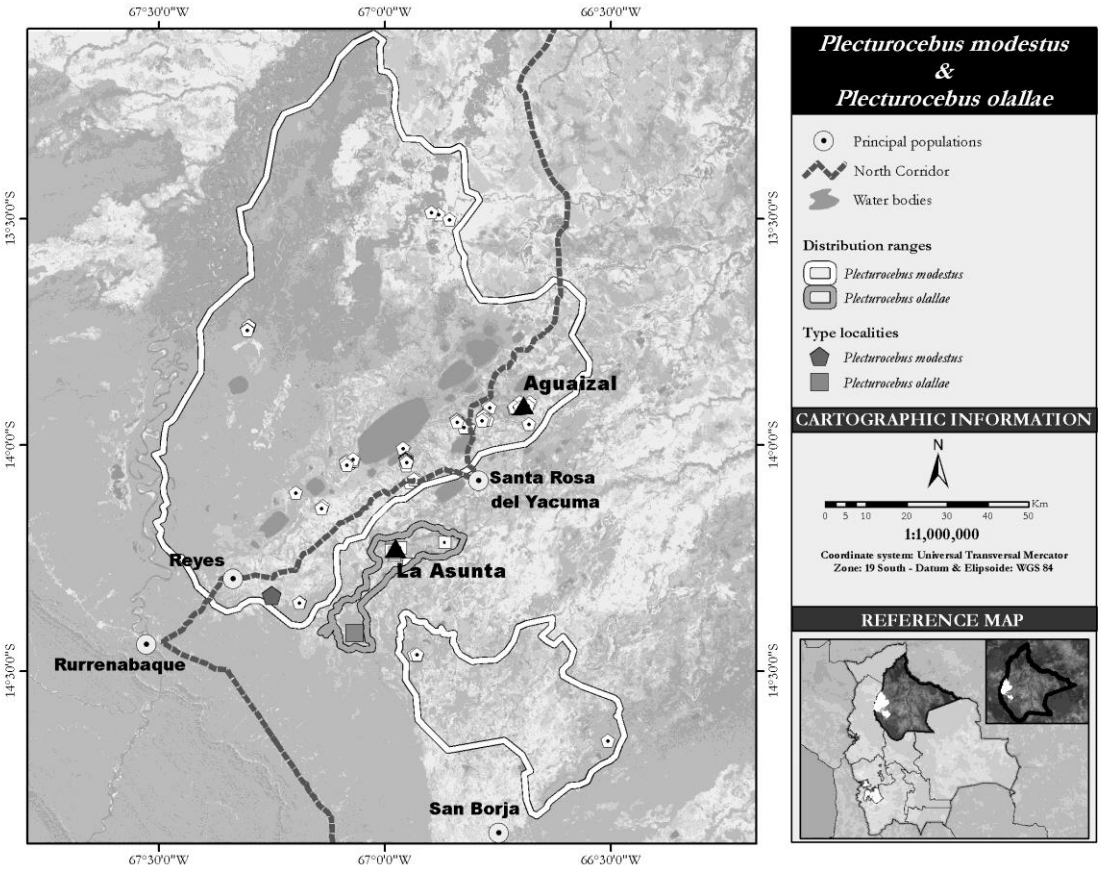

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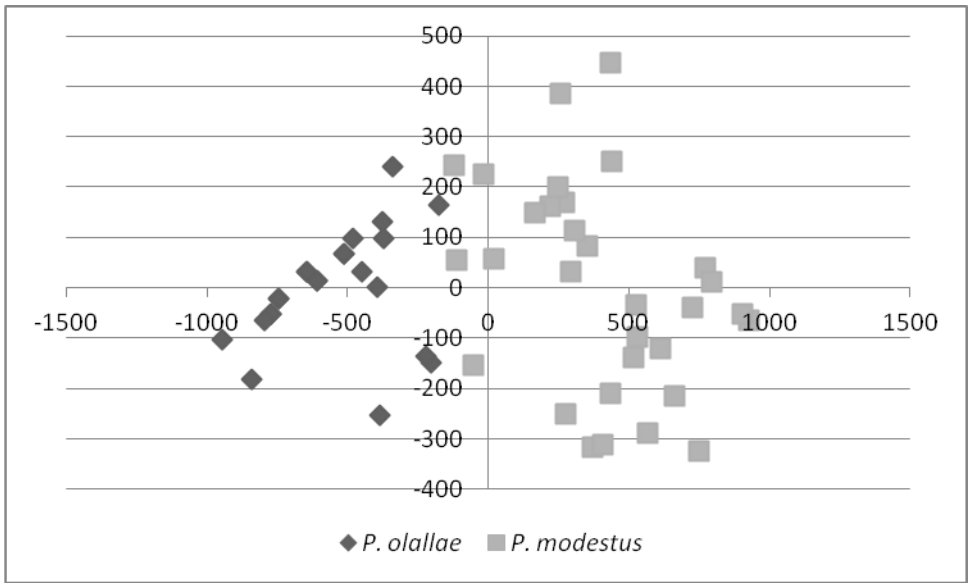

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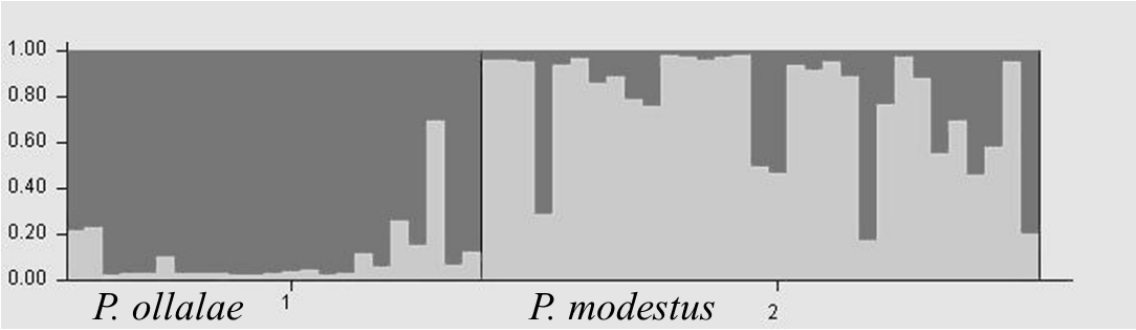

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Table 2. Genetic variability parameters estimated for two species of *Plecturocebus* based on the analysis of 6 microsatellite markers

Species	**n**[a]	**Ho**[b]	**He**[c]	**HEW**[d]	**MNA**[e]	**pA**[f]	**Fis**[g]
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