

## **MICROSATELLITE ANALYSIS OF THE ANDEAN BEAR ACROSS ITS RANGE DISTRIBUTION**

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### **Microsatellite Evolution in Andean bear populations**

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### **Abstract**

DNA samples of 155 Andean bear (*Tremarctos ornatus*) from the five Andean countries where this species lives (Venezuela, Colombia, Ecuador, Peru and Bolivia) were analyzed for nine DNA-microsatellite markers. Seven of them were polymorphic which led us to investigate several population genetics parameters. Private alleles and differential gene frequencies were found between the populations studied, which demonstrated enough time of separation among some of these Andean bear populations. The gene diversity levels measured with these microsatellites were rather modest in this species. Hardy-Weinberg disequilibrium was especially found for the overall and the Ecuadorian samples which could be a manifestation of Wahlund effect or consanguinity. Significant genetic heterogeneity was mainly obtained among the Colombian and the Ecuadorian populations. Bayesian simulations clearly showed that two different gene pools were present, one conformed by the Venezuelan-Colombian bears and other by the Ecuadorian ones.

**Key words:** Andean bears, DNA microsatellites, Molecular Population Genetics, Venezuela, Colombia, Ecuador, Perú, Bolivia.

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The Andean or spectacled bear (*Tremarctos ornatus*) jointly with the mountain lion (*Puma concolor*) are the two most important predators of the Andean mountains at South America.

Its conservation status is problematic. IUCN classified this bear in the vulnerable category (A2bc) whereas CITES listed it in the Appendix I, as an endangered species.

Any way, spectacled bears are threatened by habitat degradation, hunting, conflict with cattle ranches, road building, and illegal trade. A few data are enough to illustrate the dangerous situation of the Andean bear:

1) In occasions, Andean bears kill cattle or predate crops, especially maize or grazing pastures, which induce colonists and indigenes to kill them. There is strong evidence that bears reduce both their habitat use and communication with each other following the introduction of cattle in wilderness areas. Yerena (1998) has claimed that this problem is perceived to be as great a problem as habitat destruction. 2) Furthermore, the destruction and fragmentation of its habitat is a relevant threat. Colonization contributes to a massive conversion of forests into pastures for agriculture and cattle grazing. About 73.000 Km<sup>2</sup> are involved in this process in the Andean and Piedmont regions (Peyton et al. 1998). At least 20% of the spectacled bear's range is occupied by landless peasants who are involved in the production and trafficking of narcotics, underdeveloped subsistence farming, mining and road building (Peyton et al. 1998). Moreover, the rate of colonization and habitat clearing is increasing at an exponential level. 3) Additionally, rivers from the Andes have frequently traces of gold and precious minerals, which provokes intensive exploitation of areas with heavy machinery.

The two first threats in Colombia result in the death of 50 bears and the loss of 300-500 Km<sup>2</sup> of potential bear habitat annually. Sport hunting also accounts for a small number of bear deaths (10 deaths/year) (Orejuela and Jorgenson 1996). If these threats are not enough, some people used bear meat seasonally for consuming and its fat for cooking. The poaching and illegal international trade of bear parts, such as claws, teeth and gall bladders, supply the demand for traditional east Asian medicine, creating new threat, especially in countries like Ecuador.

Only a few handle of ecological studies have been carried out with this emblematic species regard to its food habits and distribution (Peyton 1980, 1981, Rodriguez et al. 1986;

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Yerena 1994). Their works pointed out that this is a key and umbrella species in the Andean ecosystems, which needs urgent protection measures in all the countries where this species is distributed (Venezuela, Colombia, Ecuador, Perú and Bolivia).

The most outstanding benefits of Andean bear conservation agree quite well with watershed maintenance (the loss of watershed products due to the destruction of bear habitat imperils up to three quarters of the people in the five Andean nations which live in highland areas close to spectacled bears), biodiversity benefits (Andean bear range occupied only 3.2% of land area in South America which, however, contains 76% of the mammal species of this continent; Mares 1992), and cultural and spiritual reasons of indigene tribes, all highly important to Andean residents (Peyton et al. 1998).

From a phylogenetic perspective this species is also unique. Nash and O'Brien (1987), Goldman et al. (1989) and Waits et al. (1999) demonstrated at a karyological and at a molecular (allozymes and mtDNA genes) levels that this species conformed a single separated branch from the Panda bear and from the six *Ursus* species with an antiquity around 12-15 million years.

Even though in the last decade, the DNA microsatellite markers (STRPs, Short Tandem Repeat Polymorphism) have shown an amazing power to reconstruct the genetic structure, migrations and natural history of three bear species (*Ursus americanus*, Paetkau and Strobeck 1994, 1995; *Ursus arctos*, Craighead et al. 1995; Paetkau et al. 1998 a,b; Taberlet et al. 1995, 1997; Waits et al. 2000; *Ursus maritimus*, Paetkau et al. 1995, 1999), no molecular studies have been published up now with a Third World bear species, with the exception of a preliminary approach to the Andean bear molecular composition by Ruiz-Garcia (2001a). In that study 82 DNA samples were analyzed for four polymorphic microsatellites distributed basically in three of the five countries where this species lives (Venezuela, Colombia and Ecuador).

In the present study, we bring new DNA microsatellite data for 9 STRPs for 155 Andean bear located in the five Andean countries where this species is present, being the main aims of this work as follows: 1- To determine the possible existence of private alleles and differential allele frequencies among the different populations studied. 2- To determine the gene diversity levels of this species within and among populations. 3- To analyze the Hardy-Weinberg equilibrium within the populations and at a global level. 4- To estimate

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the molecular genetic heterogeneity among the diverse Andean bear populations studied and to offer indirect gene flow estimates among these populations, and 5- To analyze the correct geographical assignment of the individuals studied to determinate how many gene pools are present in the current distribution of this species.

### **METHODS**

Several brief considerations about the samples and the molecular markers used are shown.

A total of 155 Andean bear DNA were analyzed having the following origins: 50 Colombian animals from eight different geographical points, 78 Ecuadorian specimens representing the two Andean cordilleras in that country and, at least, six different geographical points, 12 animals from Venezuela, 5 animals from diverse areas of Peru, with, at least, 3 geographical points represented and 10 samples from Bolivia. In Fig. 1, the geographic origins of the animals sampled are shown.

These samples were composed by 5 ml of blood preserved in EDTA disodic, in the case of samples obtained from captured animals with known geographical origins, by rooted hairs directly recorded at field, pieces of skins from animals killed by hunters and some teeth, bones and muscle tissues generously provided by hunters and collections.

Two different methods were used to extract DNA from the blood samples (Phenol-Chlorophorm and Chelex resin). For muscle tissues, pieces of skins, teeth and bones, the phenol-chlorophorm procedure was employed with several modifications on the most standard techniques. The DNA extraction from the hairs with follicle roots was carried out by using 10-20 % Chelex resin, with several modifications on the Walsh (1991) procedure. The DNA concentration extracted from the blood samples ranged from 8 to 1213 ng/ $\mu$ l.

The PCR reactions were undertaken as follows. The final PCR volume reaction, when the DNA was obtained by the phenol-chlorophorm and DTAB-CTAB procedures, was 25  $\mu$ l with 2.5  $\mu$ l of MgCl<sub>2</sub> 3 mM, 2.5  $\mu$ l of Buffer 10x, 2  $\mu$ l of dNTPs 0.04 mM, 10 pmol of each primer (forward and reverse), 13  $\mu$ l of H<sub>2</sub>O, 2  $\mu$ l of DNA and 0.5 of Taq Polymerase units. For the PCR with DNA extracted form hairs, by using 10-20% Chelex resin, the overall volume was 50  $\mu$ l, with 20  $\mu$ l of DNA and twofold amounts of all other reactives.

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The primers employed belonged to nine microsatellites, G1A, G1D, G10B, G10C, G10L, G10M, G10P, G10X and UarMU59, originally developed for the black bear (*Ursus americanus*) by Paetkau and Strobeck (1994) and Paetkau et al. (1995), the first eight meanwhile the last one was designed by Taberlet et al. (1997). The PCR reactions were carried out in a Geneamp PCR System 9600 thermocycler of Perkin Elmer. The temperatures employed were 95 °C for 5 minutes, 35 cycles of 1 minute at 95 °C, 1 minute at the most accurate annealing temperatures, 52-55-60 °C depending on the microsatellite, and 1 minute at 72 °C. After cycles, 5 minutes at 72 °C. The amplification products were kept at 4 °C until the moment that they were used. The PCR amplification products were run in denaturant 6% polyacrilamide gels in a Hoefer SQ3 sequencer vertical camera and were stained with silver nitrate. This method provides resolution up to one nucleotide base difference.

### *Population genetic analysis*

The levels of gene diversity of the spectacled bear populations studied were measured by means of the expected heterozygosity (Nei 1978).

The Hardy-Weinberg equilibrium (H-W E) was studied by means of the Weir and Cockerham (1984)'s F (W-C F) statistic. To measure the exact probabilities of this statistic, the Markov chain method was employed as it was implemented by Raymond and Rousset (1995) in the Genepop v. 3.1 program. The H-W equilibrium was analyzed by locus, by population and, simultaneously, by loci and populations employing the Fisher's method.

The genetic heterogeneity among the Andean bear populations of the five countries was obtained employing diverse strategies. Exact probabilities with Markov chains applying 5000 dememorization, 500 batches and 5000 iterations per batch were used. Furthermore, the hierarchical Wright F statistics were performed by means of the procedure of Michalakis and Excoffier (1996). In addition, the standard deviations and the 95% and 99% confidence intervals of the F statistics were performed using jackknifing over populations and over loci as well as bootstrapping over loci, with 10000 jackknife and 10000 bootstrap permutations.

Throughout the genetic heterogeneity statistics, indirect theoretical gene flow estimates (Nm) among the Ecuadorian and the Colombian bear populations, as well as between all

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the samples studied were obtained by using the infinite island and the n-dimensional models (Ruiz-Garcia 1993, 1998, Ruiz-Garcia and Alvarez 2000). The gene flow was also calculated using the private allele model (Slatkin 1985, Barton and Slatkin 1986). Slatkin and Barton (1989) demonstrated that these procedures are extremely robust to obtain accurate values of  $Nm$  independently of the geometric position of the populations, deviations from gene drift-mutation equilibrium, existence of several types of selection and presence of mutation.

To analyze how many different gene pools there are in the Andean bear distribution surveyed and to detect possible hybrid individuals among these gene pools, the theory described by Pritchard et al. (2000), and developed in the STRUCTURE program, was employed. This method, which employs Markov chain Monte Carlo (MCMC) procedures and the Gibbs sampler, uses multilocus genotypes to infer population structure and simultaneously individuals are assigned to specific populations. The model considers  $K$  populations, where  $K$  may be unknown, and the individuals are assigned probabilistically to one population or jointly to two or more populations if their genotypes are considered as admixed. The original theory considers H-E equilibrium and linkage equilibrium in a real population. Departures from these assumptions lead the overall sample to be split into different gene pools to which individuals are assigned. The posterior  $K$  probabilities are calculated assuming uniform prior values on  $K$ , in our case, between 1 and 6 (USEPOPINFO = 0). The presence of most probable number of gene pools within the data considered is revealed by the increasing likelihood. Once the most likely number of populations is found, the analysis was repeated but introducing the model with prior geographic population information (USEPOPINFO = 1). The analysis presented herein was carried out with one million iterations, following a burn-period of 30000 iterations. The analysis was performed twice, offering totally convergent results. An advantage of this procedure is that no H-W E in the data is needed “a priori” to carry out this analysis.

## RESULTS

### *Alleles found*

*Total sample.* Out of nine microsatellites studied, seven were clearly polymorphic in the Andean bear (Fig. 2) and the other two were monomorphic (G1A and UarMU59). G1D

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presented four alleles, with one of them predominant (178 bp, 0.859). Six alleles were determined at the G10B locus, being the 160 bp allele that with the highest frequency (0.462). G10C showed five alleles with one allele clearly dominant (105 bp, 0.866). G10M, jointly with G10P, yielded the highest number of alleles, nine, with two of them with the more important frequencies, 210 bp (0.324) and 216 bp (0.309), whereas G10P only showed a single allele with predominant frequency, 141 bp (0.500). G10X presented eight alleles with two alleles having the greatest frequencies, 125 bp (0.474) and 133 bp (0.237). Finally, G10L yielded seven alleles, with no one showing elevated frequencies.

*Samples by each country.* It is interesting to note the existence of some private alleles in the Andean bear samples of each country studied. The most outstanding findings were as follows: For G1D, a 180 bp allele was exclusive from Colombia as well as a 184 bp allele was exclusive from Ecuador. Perhaps the most noteworthy finding for G10B was the absence of a 154 bp allele from Colombia. G10C presented an exclusive allele of 109 bp for Venezuela and other of 101 bp for Bolivia, meanwhile Ecuador did not show the alleles of 109 and 115 bp typically found at Colombia and Venezuela. The Colombian sample did not contain the 212, 214, 218, 220 and 222 bp alleles found at the Ecuadorian sample for G10M. For G10P, the Colombian sample yielded an allele of 137 bp no detected at Ecuador whereas two alleles of 149 bp and 153 bp, respectively, were found in Ecuador but not in Colombia or Venezuela. G10X presented in Ecuador alleles of 123 bp and 137 bp no detected in Colombia or Venezuela meanwhile two characteristic alleles of Colombia and Venezuela such as 129 bp and 133 bp were not detected at Ecuador. Venezuela showed a private allele of 135 bp as well as Bolivia showed other of 121 bp. Lastly, Ecuador did not show the alleles of 127 bp, 129 bp and 137 bp, which characterized the Colombian Andean bears for G10L. Otherwise, the 139 bp, 141 bp and 147 bp typically found at Ecuador were not detected in the Colombian sample.

### *Levels of genetic diversity*

*Total sample.* The average number of alleles per locus (ANAP) was  $6.86 \pm 1.96$  when excluding the monomorphic loci and  $5.67 \pm 3.00$  when including them. The overall expected heterozygosity was  $0.56 \pm 0.32$ .

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*Individual country samples.* The ANAP for Ecuador including and excluding monomorphic loci were  $5.14 \pm 2.61$  and  $4 \pm 3.20$ , respectively. In the case of Colombia, the respective values were  $4.14 \pm 1.46$  and  $3.22 \pm 2.22$  allele. There were no significant differences among both countries. For the other country samples, the ANAP values, only for polymorphic loci, were lower than for Ecuador and Colombia but it has maybe not a biological significance because the sample sizes for these countries were considerably lower than for Ecuador and Colombia. These values were as follows: Venezuela  $2.67 \pm 1.03$ , Bolivia  $1.8 \pm 0.84$  and Peru  $1.3 \pm 0.58$ .

The average expected heterozygosity for each country sample including the nine microsatellites genotyped yielded the following picture. Venezuela presented the highest value ( $H = 0.571 \pm 0.421$ ), followed by Colombia ( $H = 0.432 \pm 0.341$ ), Ecuador ( $H = 0.403 \pm 0.325$ ) and Peru-Bolivia ( $H = 0.4 \pm 0.343$ ). There were really no significant differences between these gene diversities although the bears of Venezuela and Colombia seem to have slightly a higher level.

### *Hardy-Weinberg equilibrium*

*Total sample.* For the overall sample, the unbiased estimates of H-W exact P-values by Markov chain method for all polymorphic microsatellites taken together (test multi-locus) was  $0.0000 \pm 0.0000$  which put forward an extremely high excess of homozygous at a global level. Also the overall significance of the seven microsatellites with the Fisher's method revealed the no-existence of global H-W equilibrium ( $\chi^2 = \text{infinite}$ , 14 df,  $p = 0.000000$ ).

The individual analysis of each microsatellite showed that all them presented positive values of F (W-C)(Table 1), but the significant ones were G1D, G10B, G10M, G10X and G10L. The highest levels of homozygous excess were for G10X, G1D and G10L. If the Bonferroni's correction is applied ( $\alpha' = 0.00714$ ), G1D, G10M, G10X and G10L continue being significant. Thus only G10C and G10P did not significantly introduce homozygous excess.

*Samples separated by countries.* The independent H-W analyses by locus and by countries revealed some interesting traits (Table 2). For example, in Ecuador all the F values were positive with the exception of G10C, meanwhile four loci showed negative amounts (G1D,

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G10B, G10C and G10M) in Colombia. Even G10M yielded a remarkable heterozygous excess reaching a significant threshold in this country. In Ecuador, G1D, G10X and G10M showed a H-W bias by homozygous excess at a 0.05 level. In the same situation, the Colombian sample only presented a single microsatellite with significant homozygous excess, G10X. If the Bonferroni's correction was applied, this last signification disappeared. Therefore, while the Colombian population is near to H-W E, the Ecuadorian population significantly deviated by homozygous excess of H-W E. When H-W equilibrium was simultaneously measured for both countries (with Bonferroni's correction), G10M and G10X significantly deviated from H-W equilibrium, revealing that the Wahlund effect (subdivision effect) among the bear populations of these two countries is mainly provoked by these two loci. The test for all loci taken together and for both samples taken simultaneously showed an evident homozygous excess ( $\chi^2 = \text{infinite}$ , 20 df,  $p = 0.00000$ ), which highlights the existence of more than one gene pool for the Andean bears from Ecuador and Colombia taken together. Otherwise, the test by individual populations revealed a striking homozygous excess bias for the Ecuadorian sample ( $\chi^2 = \text{infinite}$ , 14 df,  $p = 0.00000$ ), whereas no significant departure from H-W equilibrium was revealed for the overall Colombian sample ( $\chi^2 = 18.3$ , 14 df,  $p = 0.193$ ). Even the H-W trends among these both samples were opposite for several microsatellites. This was the case of G1D and G10M, especially. The case of the Venezuelan sample resembled to that found in Colombia. Several loci showed high positive values of F, being the cases of G1D, G10B and G10X, although no statistical significance was reached by the low sample value of specimens from Venezuela, meanwhile G10C and G10P presented negative values (heterozygous excess), although without reaching statistical significance. For the Bolivian and the Peruvian cases, only a few loci could be studied for the minute number of DNA samples analyzed. G10C and G10X yielded positive values for Bolivia but no significant cases were reached. For Peru, the single locus studied for this analysis, G10P, also showed positive F values but no statistically significant. Therefore, the Colombian population showed the more limited H-W bias ( $F_{IS} = 0.099$ ), with Venezuela ( $F_{IS} = 0.396$ ), Ecuador ( $F_{IS} = 0.394$ ) and Peru-Bolivia ( $F_{IS} = 0.688$ ) offering higher amounts, although only the Ecuadorian value is significant. Therefore, there is not any relationship among the levels of homozygous excess and the samples sizes employed in each country.

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### *Genetic heterogeneity between the samples*

First we comment the genic differentiation by means of Markov chains among the Colombian and the Ecuadorian samples. G1D, G10C and G10P did not significantly offer differences among both samples. Nevertheless, conspicuous significant differences were arisen for G10B ( $p = 0.00001$ ), G10M ( $p = 0.00094$ ), G10X ( $p = 0.00000$ ) and G10L ( $p = 0.0019$ ). Applying Bonferroni's correction, all these values were also significant. The overall genic differentiation was straightforwardly significant ( $\chi^2 = \text{infinite}$ , 14 df,  $p = 0.000001$ ). Both populations are therefore representing at least two different gene pools. The gene flow estimate by means of the private allele method was  $Nm = 0.2727$ , which points out a practically absent gene flow (or very limited) among both Andean bear populations.

Secondly, we give some insights about the genic differentiation among the five country samples simultaneously taken. G1D and G10P did not detect significant heterogeneity throughout all the Andean bear range. G10C showed significant heterogeneity in this case ( $p = 0.00412$ ) regard to the Colombia-Ecuador comparison. The other loci were extremely significant as well, G10B ( $p = 0.00000$ ), G10M ( $p = 0.01823$ ), G10X ( $p = 0.00000$ ) and G10L ( $p = 0.00163$ ). The significance of G10M disappeared when the Bonferroni's correction was employed. G10B and G10X were the markers which discriminated more accurately between all these Andean bear samples. The overall genic differentiation taken in account all loci studied was noteworthy significant ( $\chi^2 = \text{infinite}$ , 14 df,  $p = 0.00000$ ). The gene flow estimate throughout private allele method again revealed an extreme low value ( $Nm = 0.64989$ ). This value was slightly superior than that determined among Colombia and Ecuador, although inside of a scenario of very limited gene flow. This slight higher  $Nm$  value, for the five countries taken together, than that detected only for the Ecuador-Colombia pair, means that among other country pairs, such as Ecuador-Perú, Ecuador-Bolivia, Ecuador-Perú and Colombia-Venezuela, the gene flow is superior than among the populations from Colombia and Ecuador.

The probability values for genic differentiation for each population pair locus-by-locus could be seen in Table 3. The only significant pair for G1D was among Colombia and Venezuela, although no significant heterogeneity was maintained when Bonferroni's

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correction was employed. G10B showed high significant differences between Venezuela, Colombia and Ecuador. The Ecuadorian-Venezuelan pair was the unique significant for G10C as well as the Ecuadorian-Colombian pair was the unique significant for G10M. For G10P the differentiation is highly limited. Only the Ecuadorian-Venezuelan pair is significant at 0.05 level, which disappeared with the Bonferroni's criteria. Contrarily, G10X was the locus yielding the highest degree of differentiation between all the samples analyzed. Venezuela and Colombia were outstandingly differentiated from Ecuador as well as Colombia was differentiated from Bolivia and Peru, and Venezuela from Peru. It is straightforward to note the significant differences among Ecuador and Bolivia and in turn among Bolivia and Peru. G10L registered significant differences among Colombia and Ecuador, and this last sample with Bolivia.

### *Hierarchical F-statistics*

F hierarchical estimates pointed out the existence of a significant structure for the Andean bear (Table 4). The overall values of  $F_{IT}$  (= 0.386),  $F_{IS}$  (= 0.254),  $F_{ST}$  (= 0.176) clearly revealed this fact. G10C and G10P did not add relevant amounts to this structure; G1D showed important deviations from H-W at the total and at the subpopulation levels, meanwhile all the other polymorphic microsatellites yielded strong H-W deviations at both hierarchical levels as well as high heterogeneity levels. Especially relevant was the apportionment effectuated by G10X to this structure. Jackknifing and bootstrapping over loci offered confidence intervals which clearly put in evidence the signification of the F values obtained. Table 4 shows by means of randomizing alleles and genotypes the significance of the F statistics. With the Bonferroni's criteria, G1D and G10X were the microsatellites, which clearly showed the higher significant  $F_{IS}$  values; G1D, G10B, G10M, G10X and G10L and all loci yielded significant  $F_{IT}$  values. Assuming random mating within samples, G10B, G10C, G10M, G10X, G10L and all loci together presented significant  $F_{ST}$  values, meanwhile not assuming random mating within samples only G10B, G10X and G10C together all loci showed significant  $F_{ST}$  amounts.

The gene flow estimates with these statistics were low but slightly higher than those obtained with the private allele method. The average  $F_{ST}$  value offered a  $Nm = 1.170$  for the infinite island model and  $Nm = 0.658$  for the n-dimensional model.

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### *Genetic admixture analysis and how many different Andean bear gene pools are detectable*

Two approaches were employed for this analysis. First, uninformative priors on all  $K$  were assumed, which means that before applying the model to the data, all samples were assigned to a hypothetical single population ( $USEPOPINFO = 0$ ). The probability of the number of populations ( $K$ ) for the pooled data was estimated, without using prior population information, by fixing prior values of  $K = 1-6$  and comparing the  $\ln$  likelihood values for each one of the  $K$  values. If the hypothetical single population is admixed and includes more than one population within itself, the likelihood values will be increased with  $K$ . Once the value of  $K$  has been determined, the second modeling approach is applied. Then we assume that samples should belong to one of the  $K$  populations predefined and asked the program to assign the individuals and infer the ancestry of possible hybrids by means of the option  $USEPOPINFO = 1$ . We analyzed different cases: Colombia and Ecuador, only Colombia, only Ecuador, and finally Venezuela, Colombia and Ecuador, all them pooled. In this analysis, the Bolivian and the Peruvian data were excluded because of their little sample sizes. When the Colombia-Ecuador group as well as the Venezuela-Colombia-Ecuador data were analyzed, the most optimal  $\ln$  likelihood value corresponded to  $K = 2$  (Table 5). Therefore it seems outstanding clear that two Andean bear gene pools are placed across all the northern Andean area. When the analysis was extended only for Colombia and only for Ecuador, the highest  $\ln$  likelihood value corresponded to  $K = 1$ . Hence, inside each one of these countries a single gene pool was found, being the Venezuelan bears a slight modified extension of the Colombian population.

The analysis extensively commented herein is that corresponding to the Venezuela-Colombia-Ecuador group. When  $USEPOPINF = 0$  was employed, the probability ( $q$ ) of each genotype to belong to each of the two clusters detected was 0.761 (Ecuadorian pool) and 0.239 (Colombian pool) for the Ecuadorian individuals, and 0.692 (Colombian pool) and 0.308 (Ecuadorian pool) for the Colombian specimens. The  $q$  values for the individuals is noteworthy to be explained (Table 6). Several Ecuadorian exemplars yielded higher  $q$  probabilities to belong to the Colombian-Venezuelan cluster than to its own cluster. All these animals were from the Cosanga region (Ecuadorian Eastern Andes) and from the Alto Chocó region (Ecuadorian Western Andes) placed at the north of Ecuador.

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Some exemplars (“Jav” and “Azv”) presented similar  $q$  probability values (0.443-0.557 and 0.496-0.504, respectively), which suggests that they are hybrids of both gene pools or direct descendents of these hybrids. Also, several Colombian exemplars showed higher  $q$  probability values for the Ecuadorian cluster than for the Venezuelan-Colombian group. Most of these Colombian bears are from the Nariño region, which, as it was previously aforementioned, is limiting with Ecuador. A few individuals are probably hybrids or descendent of these hybrids among these both gene pools (“Yad”, “Tin”, “Pep”, “Chcl” and “Azab”). One individual (“Don”) clearly belonged to the Ecuadorian cluster (0.953 vs 0.047) although it was sampled within Colombia. Four Venezuelan bears with known geographical origins were included in this analysis. Three of them were clearly assigned together to the Colombian animals, whereas one exemplar (“Man”) was more doubtless to belong to the Colombian cluster.

When USEPOPINF = 1 was employed, the posterior probability ( $q$ ) of each genotype to belong to each of the two clusters detected was 0.982 (Ecuadorian pool) and 0.018 (Colombian pool) for the Ecuadorian individuals, and 0.944 (Colombian pool) and 0.056 (Ecuadorian pool) for the Colombian specimens meanwhile the probability of the Venezuelan animals were 0.658 to belong to the Colombian set and 0.342 to belong to the Ecuadorian cluster. With this procedure, the assignment process was practically perfect. The single exception was a Colombian individual (“Don”) showing a higher probability to belong to the Ecuadorian cluster than to the Colombian-Venezuelan group.

## DISCUSSION

### *Limited gene diversity and private alleles*

The gene diversity levels of the Andean bear populations analyzed by countries is limited and lower, in general, than that reported for other neotropical carnivores using microsatellite DNA markers as well. The Venezuelan estimate was slightly higher ( $H = 0.57$ ) than the values found for the other country sets, while the levels of Colombia (0.43), Ecuador (0.4) and Bolivia-Peru (0.4) were practically identical. For instance, these amounts were approximately only a half than the gene diversity levels determined for jaguars ( $H = 0.83$ ), pumas ( $H = 0.75$ ), *Leopardus pardalis* ( $H = 0.84$ ), *L. wiedii* ( $H = 0.85$ )

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in Colombia for a similar number of microsatellites studied (Ruiz-Garcia 2001, Ruiz-Garcia et al. 2002). Even some neotropical primate species, such as *Lagothrix lagotricha* ( $H = 0.61$ ) which presented strong evidence of crossing a recent bottleneck (Ruiz-Garcia 2003), also yielded higher gene diversity levels than the Andean bear. Somebody could argue the existence of “ascertainment bias” (Ellegren et al. 1995, 1997, Amos and Rubinsztein 1996) in the case of the DNA-microsatellites employed herein for the Andean bear, because they were designed for the American black bear. Some studies have revealed that the split among the *Ursus* and the *Tremarctos* lineages was among 12-15 millions of years ago (Waits et al. 1999) and this could be the motif of the low gene diversity level found in the Andean bear. Nevertheless, the microsatellites employed for the Neotropical wild cats, which includes two different feline lineages (the ocelot and the Pantherine lineages), were constructed for the domestic cat (Menotti-Raymond and O’Brien 1995). This species diverged from the Ocelot lineage about 6-7 millions of years ago, and from the Pantherine lineage 4-5 millions of years ago and 10-12 millions of years have elapsed among the Ocelot and the Pantherine lineages (Johnson and O’Brien 1997). The microsatellites applied to the primate *Lagothrix lagotricha* were designed for *Alouatta palliata* (divergence about 8 millions of years) and for humans (divergence about 30-40 millions of years ago) (Ellsworth and Hoelzel 1998). Therefore, the same “ascertainment bias” could be invoked for these other Neotropical mammal species. However, no one of these species showed levels of gene diversity of the little magnitude as the Andean bear.

The gene diversity levels found for other bear species resulted higher than those reported herein for the Andean bear. Waits et al. (2000) showed a list of genetic diversity values for several brown bear studies in North America and Scandinavia with the same markers employed here. These studies determined heterozygosity levels that ranged from 0.61 to 0.78. The population of brown bear with the lowest genetic diversity reported up to now was that of the Kodiak Island ( $H = 0.265$ ) (Paetkau et al. 1998b). In identical sense, two black bear populations surveyed in two continental Canadian areas showed a genetic diversity level around 0.8, whereas an insular Newfoundland population had an average heterozygosity value of 0.41 (Paetkau and Strobeck 1994), similar to the gene diversity of the Andean bear populations reported herein.

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Ruiz-Garcia (2002) analyzed a little sample of 82 Andean bears, 8 individuals from Venezuela and 32 individuals from Colombia (from four different areas), and both populations showed the highest levels of gene diversity. In the current work with greater sample sizes, the most northern populations (again Venezuela and Colombia) also yielded a slight higher gene diversity amounts than the most southern Andean bear populations studied. Hence, this fact seems independent of the sample size employed and it is not a statistical artifact. This could reflect the original colonization process of this species across South America, with the most northern populations being the original ones (and showing the highest levels of gene diversity) meanwhile the southern populations were conformed by little propagules coming from the northern populations and, thus, losing gene diversity by reiterative founder effects. However, more southern samples must be studied before definitively affirming that the most southern an Andean bear population is, the lower its gene diversity is.

A potential utility of the presence of private alleles and others with highly differentiated frequencies among Ecuador and Colombia as well as the existence of some private alleles detected in Venezuela or Bolivia is the exact geographical determination of exemplars in the zoos or from decommissions. One example seems enough. All the European zoo Andean bears proceeded from a single pair of unknown origin. Nowadays, we have the potential tools and results to determine the geographical origins of these animals.

The detection of private alleles in the Venezuelan and in the Bolivian samples, both of small sizes, could be an indication that more new alleles could be found within these populations and the discrimination power could noteworthy increase.

### *Hardy-Weinberg equilibrium: what is happening within the Andean bear populations ?*

Several possible explanations for the heterozygous deficiencies found at the global level and in the Ecuador samples are (Rooney et al. 1999, Spong et al. 2000): population subdivision (Wahlund effect), strong genetic drift and consanguinity, hitchhiking, null alleles, synteny or natural selection in favor of homozygous. The most plausible explanation for our case could be population subdivision by the existence of diverse gene pools due to current habitat fragmentation or more possibly by ancestral population fragmentation during the colonization of the continent by this species. Strong gene drift and

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elevated consanguinity could be not totally discarded since the genetic diversity levels of the Andean bear populations seem low for the molecular markers employed. Nevertheless, elevated consanguinity should affect all loci studied in the same sense at the lowest sampling levels and this was not apparent in the samples studied. Hitchhiking and synteny are discarded since the loci studied were deliberately distributed in different chromosomes, and the possibility of all loci being affected is remote. The same explanation could be used to discard natural selection in favor of homozygous. Null alleles also seem unlikely to produce similar levels of homozygous excess, simultaneously for all the loci studied. As previously cited, the possible levels of null allele frequencies were low in the current data.

The fact that in each country sample the microsatellites which showed significant homozygous excess were different (for instance, G1D, G10M and the overall for Ecuador and Colombia) jointly with the differential allele behavior within each locus for G1D, G10M, G10X in each population is an indication that these H-W deviations are not the product of uniform selective constrictions on these microsatellites; rather they are more dependent of the population dynamics within each population considered.

### *What tell us the genetic heterogeneity found among Andean bear populations ?*

We have shown that the major fraction of significant genetic heterogeneity pairs are among the Ecuadorian sample and those from Colombia and Venezuela. It could be taken as the first strong evidence in favor that two differentiated gene pools are detected in these countries. The Peruvian and the Bolivian samples were relatively similar to the Ecuadorian one, although several findings could indicate also subdivision between them (G10X and G10L), but more DNA samples from Bolivia and Peru should be studied to determine if they constitute gene pools different to that registered at Ecuador.

Recall that Wright (1943, 1951) showed that if  $Nm > 1$  (in an infinite island model) or  $Nm > 4$  (in a stepping-stone model), the gene flow is enough to attenuate the genetic differentiation between populations balanced for migration and gene drift. According to the infinite island model, if  $1 < Nm < 0.5$ , genetic differentiation among populations could be small but important in a stepping-stone model. Whether  $Nm < 0.5$ , the populations are largely unconnected under any model of gene flow. Our estimates revealed that the Colombian and the Ecuadorian samples proceed from two populations which have

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independently evolved long time ago, but certain little gene flow could have occurred among them. Interestingly, the gene flow could have been higher among populations of central and northern Colombia and the Venezuelan ones, and among the Ecuadorian population and the surrounding Peruvian and Bolivian ones. It is interesting to note that all the gene flow estimates obtained, with different procedures, were extremely similar and that Slatkin and Barton (1989) showed that the methods employed herein are extremely robust independently of the geographical position of the populations, the no existence of gene drift-gene flow equilibrium or, even, under the presence of natural selection affecting some marker.

*Assignment and admixture genetic analyses: How many different gene pools are present and where they are located*

The high levels of correct assignment with and without prior geographic information, especially among the Ecuadorian and the Colombian samples, showed what was incipiently detected by the previous analyses: basically the Ecuadorian and the Colombian-Venezuelan populations constituted two highly differentiated gene pools. Identically, when the number of potential different gene pools were analyzed within Ecuador and Colombia, only one population was determined inside of these countries. The STRUCTURE results with incorporated geographical information practically differentiated all animals except one (1.7%). Having in mind that more microsatellites and other molecular traits should be analyzed (mitochondrial and Y-chromosome sequences), it is probably important to consider each one of these gene pools as separate management units (MUs) (Moritz 1994) for the moment. If comparative forthcoming results with mtDNA and Y-chromosome markers could demonstrate strong phylogeographic structure, even the category of evolutionary significant units (ESUs) could be assigned for these different Andean bear populations.

It is also remarkable the fact that some Ecuadorian exemplars from the middle northern part of Ecuador (Cosanga region at the Eastern Andean cordillera and Alto Chocó at the Western Andean cordillera) were more related to the Colombian group than to the Ecuadorian one when no “a priori” geographical information is provided. Two explanations are possible: 1- It is a simple spurious result or 2- they represent immigrants, or descendent

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of immigrant, from central Colombia, posterior to the formation of the Ecuadorian gene pool, which did not hybridize with the oldest Ecuadorian pool. If so, within the Ecuadorian pool we could find animals representing lineages which have typically Venezuelan-Colombian traits and that migrated within these areas more recently and did not mix with individuals from the most ancestral Ecuadorian pool. **This explanation agrees quite well with the significant homozygous excess detected within Ecuador**

Some of the future analyses that we want to undertake with the Andean bear from a molecular population genetics point of view are as follows: 1) To augment the number of microsatellites employed by means of other black and brown bear markers and by means of other markers specifically constructed for the Giant Panda bear (Lu et al. 2001)(Ame-μ). As this last species represents another different bear lineage, it is interesting to observe whether the gene diversity is also low for the Andean bear, which could demonstrate if this species really suffer of an important gene diversity depression. 2) To sequence several mtDNA genes to determine the possible females lineages and the migration routes of the females and 3) To augment the sample size, especially for the populations in Peruvian and Bolivian lands, to determine exactly how many different gene pools are present in those countries.

These new forthcoming results will led to construct a correct program for the conservation of the Andean bear.

## **Acknowledgments**

The authors are extremely grateful to the WSPA (World Society for the Protection of Animals) at its Latin America office at Costa Rica (Mr. Huertas) and to the Academic Vicerrectory of the Pontificia Universidad Javeriana at Bogotá, Colombia, for providing monetary resources to carry out this investigation. Also thanks go to a lot of researchers whom contributed for obtaining samples of Andean bears. They are Andres Eloy Bracho (Venezuela), Sergio Sandoval, Fernando Nassar, Jorge Gardeazabal, Luz Mercedes Borrero, Marcela Ramirez, Ricardo Botero, Luis Carrillo, Daniel Rodriguez, Jhon Poveda, Haidy Monsalve and Pedro Moreno (Colombia), Heinz Pflenge, Hugo Galvez and Judith Figueroa, “Pocahontas” (Peru) and Robert Wallace (WCS), Susan Paisley and Alvaro Gaitan as director of the Coleccion Boliviana de Fauna (CBF) at the Natural History

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Museum of La Paz (Bolivia), Huascar Azurday and the Noel Kempff Natural History Museum at Santa Cruz (Bolivia). Finally, the authors are indebted to Dr. Diana Alvarez by her help with tables and figures.

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Table 1. Exact tests and F of Weir and Cockerham (1984) applied to the analysis of the Hardy-Weinberg equilibrium at the overall level for the Andean bear. P-val = Probability value; S.E. = Standard deviation. D. f. = degree of freedom. \* Significant values,  $P < 0.05$ .

Total	P-val	S.E.	F Weir & Cockerham
G1D	0.0013*	/	0.499
G10B	0.0161*	0.0018	0.231
G10C	0.1221	0.0078	0.118
G10M	0.0000*	0.0000	0.294
G10P	0.0854	0.0111	0.170
G10X	0.0000*	0.0000	0.706
G10L	0.0012*	0.0004	0.325
(Fisher's method)			
$\chi^2$	Infinity*		
D.f.	14		
Prob	0.000001		

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Table 2. Exact tests and F of Weir and Cockerham (1984) applied to the analysis of the Hardy-Weinberg equilibrium at the Andean bear populations of each country studied. P-val = Probability value; S.E. = Standard deviation. D. f. = degree of freedom. Fisher's method was applied to all the loci pooled for each country population. \* Significant values,  $P < 0.05$ .

Populations	Loci	P-val	S.E.	F Weir & Cockerham	(Fisher's method)	
<b>Ecuador</b>	G1D	0.0080*	/	0.634	$\chi^2$	Infinity*
	G10B	0.2191	0.0117	0.109	D.f.	14
	G10C	1.0000	/	-0.030	Prob	0.000001
	G10M	0.0000*	0.0000	0.366		
	G10P	0.1421	0.0142	0.130		
	G10X	0.0094*	/	0.532		
	G10L	0.1076	/	0.250		
<b>Colombia</b>	G1D	1.0000	/	-0.016	$\chi^2$	23.1
	G10B	0.9279	0.0022	-0.015	D.f.	14
	G10C	1.0000	/	-0.083	Prob	0.0586
	G10M	0.0357*	/	-0.239		
	G10P	0.3448	0.0145	0.074		
	G10X	0.0027*	/	0.692		
	G10L	0.3143	/	0.100		
<b>Venezuela</b>	G1D	0.3333	/	1.000	$\chi^2$	10.8
	G10B	0.0667	/	0.667	D.f.	10
	G10C	1.0000	/	-0.286	Prob	0.3707
	G10M	-				
	G10P	1.0000	/	-0.333		
	G10X	0.2000	/	0.500		
	G10L	0.3333	/	0.500		
<b>Bolivia</b>	G1D	-			$\chi^2$	6.6
	G10B	-			D.f.	4
	G10C	0.1111	/	1.000	Prob	0.1591
	G10M	-				
	G10P	0.3333	/	0.821		
	G10X	0.3333	/	0.500		
	G10L	-				
<b>Peru</b>	G1D	0.3333	/	0.610	$\chi^2$	6.1
	G10B	-			D. f.	4
	G10C	-			Prob	0.1895
	G10M	0.2911	/	0.500		
	G10P	0.2000	/	1.000		
	G10X	0.3712	/	0.342		
	G10L	-				
<b>(Fisher's method):</b>						
<b>Chi2</b>	Infinity*					
<b>Df</b>	32					
<b>Prob</b>	0.000001					

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Table 3. All possible population pair comparisons for each one of the microsatellites studied. \*Significant genetic heterogeneity:  $P < 0.05$ . S.E. = Standard error.

<b>Locus</b>	<b>Populations compared</b>		<b>Probability</b>	<b>S.E.</b>
<b>G1D</b>	Ecuador	& Colombia	0.18053	0.00238
	Ecuador	& Venezuela	0.17304	0.00271
	Ecuador	& Bolivia	0.55538	0.00273
	Colombia	& Venezuela	0.04588*	0.00144
	Colombia	& Bolivia	0.28854	0.00318
	Venezuela	& Bolivia	1.00000	0.00000
<b>G10B</b>	Ecuador	& Colombia	0.00000*	0.00000
	Ecuador	& Venezuela	0.00748*	0.00075
	Colombia	& Venezuela	0.00582*	0.00058
<b>G10C</b>	Ecuador	& Colombia	0.35079	0.00222
	Ecuador	& Venezuela	0.00808*	0.00056
	Ecuador	& Bolivia	0.08270	0.00158
	Colombia	& Venezuela	0.07307	0.00218
	Colombia	& Bolivia	0.18272	0.00283
	Venezuela	& Bolivia	0.11468	0.00180
<b>G10M</b>	Ecuador	& Colombia	0.00140*	0.00027
	Ecuador	& Venezuela	0.38104	0.00584
	Ecuador	& Peru	1.00000	0.00000
	Colombia	& Venezuela	1.00000	0.00000
	Colombia	& Peru	0.25862	0.00368
	Venezuela	& Peru	0.33280	0.00147
<b>G10P</b>	Ecuador	& Colombia	0.81942	0.00412
	Ecuador	& Venezuela	0.04326*	0.00258
	Ecuador	& Bolivia	1.00000	0.00000
	Ecuador	& Peru	0.59261	0.00643
	Colombia	& Venezuela	0.06713	0.00256
	Colombia	& Bolivia	1.00000	0.00000
	Colombia	& Peru	0.66544	0.00459
	Venezuela	& Bolivia	0.19906	0.00173
	Venezuela	& Peru	0.08819	0.00146
Bolivia	& Peru	0.46589	0.00105	
<b>G10X</b>	Ecuador	& Colombia	0.00000*	0.00000
	Ecuador	& Venezuela	0.00000*	0.00000
	Ecuador	& Bolivia	0.00021*	0.00010
	Ecuador	& Peru	1.00000	0.00000
	Colombia	& Venezuela	0.29118	0.00349
	Colombia	& Bolivia	0.00116*	0.00018
	Colombia	& Peru	0.00321*	0.00031
	Venezuela	& Bolivia	0.07558	0.00167
	Venezuela	& Peru	0.00841*	0.00049
	Bolivia	& Peru	0.02889*	0.00103
<b>G10L</b>	Ecuador	& Colombia	0.00237*	0.00026
	Ecuador	& Bolivia	0.00792*	0.00064
	Colombia	& Bolivia	1.00000	0.00000

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Table 4. A) Hierarchical F-statistics of the Andean bear populations studied. B) Significance probabilities of  $F_{IS}$ ,  $F_{IT}$  and  $F_{ST}$  statistics by means of 10000 randomisations of alleles within samples ( $F_{IS}$ ), of alleles over samples ( $F_{IT}$ ), assuming random mating within samples with exact G-tests ( $F_{ST}$ ), and no assuming random mating within samples with log-likelihood G tests ( $F_{ST}$ ). \*Significant probabilities.

Loci	$F_{IT}$	$F_{ST}$	$F_{IS}$
G1D	0.510	0.058	0.480
G10B	0.306	0.228	0.101
G10C	0.138	0.071	0.071
G10M	0.338	0.135	0.234
G10P	0.179	0.029	0.155
G10X	0.744	0.359	0.601
G10L	0.375	0.184	0.234
Over all loci	0.386	0.176	0.254
Jackknifing over loci			
	0.387±0.083	0.179±0.047	0.251±0.065
Bootstrapping over Loci			
95% Confidence Interval			
	0.257	0.090	0.155
	0.541	0.258	0.391
99% Confidence Interval			
	0.226	0.059	0.134
	0.595	0.280	0.443

## B

$F_{IS}$	probability range
G1D	[0.00270 - 0.00020]*
G10B	[0.16290 - 0.07800]
G10C	[0.34970 - 0.12270]
G10M	[0.02460 - 0.00780]*
G10P	[0.08180 - 0.03240]
G10X	< 0.00010*
G10L	[0.21870 - 0.07520]
All Loci	< 0.00010*
$F_{IT}$	probability range
G1D	[0.00030 - 0.00030]*
G10B	[0.00020 - 0.00020]*
G10C	[0.06540 - 0.06540]
G10M	< 0.00010*
G10P	[0.02180 - 0.02180]*
G10X	< 0.00010*
G10L	[0.00340 - 0.00340]*
All Loci	< 0.00010*

## Microsatellite Evolution in Andean bear populations

### **G-test**

G1D	[0.09480 - 0.09110]
G10B	< 0.00010*
G10C	[0.00210 - 0.00200]*
G10M	[0.00470 - 0.00470]*
G10P	[0.25790 - 0.25770]
G10X	< 0.00010*
G10L	[0.00060 - 0.00060]*
All Loci	< 0.00010*

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### **log-likelihood G**

G1D	0.42350 - 0.42350
G10B	0.00140 - 0.00140*
G10C	0.00720 - 0.00720*
G10M	0.22570 - 0.22560
G10P	0.42430 - 0.42430
G10X	< 0.00010*
G10L	0.08630 - 0.08560
All Loci	< 0.00010*

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## Microsatellite Evolution in Andean bear populations

Table 5. Determination of the most probable number of gene pools conforming the Andean bear populations of Venezuela, Colombia and Ecuador (Ven/Col/Ecu), of Ecuador and Colombia (Ecu/Col) taken together, Ecuador and Colombia individually. We assumed uninformative priors on all K as each sample belonged to a hypothetical single population (USEPOPINFO = 0). The highest Ln likelihood value indicates the most probably number of different gene pools within these samples. Two gene pools were detected for the Venezuela-Colombia-Ecuador sample and for the Colombia-Ecuador sample, whereas only one gene pool was detected within Ecuador and within Colombia, respectively.

K	Ln likelihood			
	Ven/Col/Ecu	Ecu/ Col	Ecuador	Colombia
1	-570.7	-510.2	<b>-272.2</b>	<b>-169.8</b>
2	<b>-521.5</b>	<b>-468.5</b>	-301.0	-174.9
3	-525.3	-472.9	-321.3	-181.1
4	-532.9	-481.3	-293.9	-182.0
5	-537.2	-487.3	-293.2	-196.9
6	-545.8	-490.0	-287.7	-211.2

## Microsatellite Evolution in Andean bear populations

Table 6. Bayesian assignment and admixture analysis with prior geographic information (USEPOPINFO = 1). Two clusters (= gene pools) were determined. Cluster 1 characterized the Ecuadorian gene pool and Cluster 2 characterized the Colombian gene pool. Numbers are the probability to belong to each one of these two clusters. Only one bear from Colombia showed to have more probabilities to belong to the Ecuadorian cluster. For this task, the STRUCTURE program was employed.

population	Individuals	cluster 1- probability	cluster 2- probability
<b>Ecuador</b>	1	0.998	0.001
	2	0.998	0.002
	3	0.999	0.001
	4	1.000	0.000
	5	0.994	0.007
	6	1.000	0.000
	7	0.991	0.009
	8	1.000	0.000
	9	0.997	0.003
	10	0.999	0.001
	11	0.997	0.003
	12	0.992	0.009
	13	0.993	0.007
	14	0.891	0.108
	15	0.998	0.002
	16	0.976	0.024
	17	0.988	0.012
	18	0.999	0.001
	19	0.999	0.001
	20	0.999	0.001
	21	0.906	0.094
	22	0.995	0.005
	23	0.927	0.073
	24	0.942	0.058
	25	0.951	0.048
	26	0.951	0.048
	27	0.951	0.048
	28	0.967	0.032
	29	0.818	0.183
<b>Colombia</b>	1	0.044	0.956
	2	0.000	1.000
	3	0.046	0.954
	4	0.001	0.999
	5	0.131	0.870
	6	0.000	1.000
	7	0.000	1.000
	8	0.001	0.999
	9	0.000	1.000

## Microsatellite Evolution in Andean bear populations

10	0.008	0.992
11	0.061	0.939
12	0.092	0.908
13	0.082	0.918
14	0.001	0.999
15	0.177	0.823
16	0.002	0.998
17	0.011	0.989
18	0.022	0.979
19	0.008	0.991
20	0.014	0.987
21	0.044	0.956
22	0.044	0.956
23	0.017	0.984
24	0.118	0.882
25**	<b>0.810</b>	<b>0.190</b>
<b>Venezuela</b>		
1	0.291	0.709
2	0.279	0.721
3	0.337	0.663
4	0.462	0.538

## Microsatellite Evolution in Andean bear populations

### Figure Captions

**Fig. 1.** Map of the five Andean countries where Andean bear lives and points where samples were obtained.

**Fig. 2.** Histograms for microsatellite allele frequencies determined in the Andean bear for the global sample and for the samples by country.