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DIVERSITY AND GENE FLOW IN FOUR COLOMBIAN TURTLE POPULATION OF *Podocnemis vogli* (Testudines: Podocnemididae)

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To my parents and my brother for supporting and guiding me.

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Resumen

Diversidad y flujo genético en cuatro poblaciones colombianas de la tortuga *Podocnemis vogli* (Testudines: Podocnemididae)

La tortuga Sabanera *Podocnemis vogli* es una especie restringida a las sábanas de la cuenca del Orinoco en Colombia y Venezuela. Sus poblaciones han disminuido y desaparecido en algunos lugares debido a la explotación ilegal y la alteración y destrucción de su hábitat a causa de actividades humanas. Esta especie no ha sido evaluada a nivel global por la Unión Internacional para la Conservación de la Naturaleza (IUCN), pero se encuentra categorizada en preocupación menor (LC) a nivel regional y está regulada bajo el apéndice II de la convención CITES. Con el objetivo de analizar su estado de conservación usando herramientas moleculares por primera vez, se evaluaron 19 loci de microsatélites mediante la técnica de amplificación cruzada, así como secuencias del gen mitocondrial (mtADN) Cyt *b,* en cinco poblaciones localizadas en Puerto Carreño (Vichada), Paz de Ariporo (Casanare), Puerto López (Meta), Unillanos (Villavicencio, Meta), San Martin (Meta) and 26 individuos mantenidos en cautiverio en la Estación de Biología Tropical Roberto Franco (EBTRF). Los datos obtenidos permitieron analizar para la especie su diversidad y estructura genética, así como declives poblacionales (*bottlenecks*) y flujo genético. Como resultado, se identificaron diez marcadores polimórficos adecuados para llevar a cabo el estudio de genética de poblaciones, los cuales fueron útiles para revelar una estructura genética compuesta por los cinco grupos correspondientes a las localidades de muestreo. Estos grupos genéticos poblaciones corresponden a Unidades de Manejo Independiente (IMU *sensu* Moritz 1994). Sin embargo, los análisis indicaron presencia de cuellos de botella recientes en las poblaciones localizadas en Paz de Ariporo y Puerto Carreño, siendo evidencia de disminución poblacional debido muy probablemente a actividades antrópicas. Adicionalmente, estas poblaciones presentaron flujo genético bajo y asimétrico entre ellos, con un flujo mayor hacia Paz de Ariporo desde las otras poblaciones genéticas a causa posiblemente de actividades antrópicas. Con respecto al análisis de la secuencia parcial del Cyt *b*, la red de haplotipos revelo una diferenciación débil, pero con presencia de haplotipos únicos en las poblaciones localizadas en Puerto Carreño, Paz de Ariporo, EBTRF y una secuencia de *genbank* proveniente de San Martín (Meta) y un haplotipo compartido identificado en Puerto López, Puerto Carreño, Paz de Ariporo, Unillanos y la EBTRF. Los resultados de esta investigación nos permiten proponer lineamientos de manejo y conservación para estas poblaciones en Colombia.

Palabras clave: Conservación genética, microsatélites, Cyt *b*, variabilidad genética, flujo génico, amplificación cruzada.

Abstract

Diversity and gene flow in four Colombian turtle populations of *Podocnemis vogli* (Testudines: Podocnemididae)

The Savannah side-necked turtle *Podocnemis vogli* turtle is a species restricted to the savannas of the Orinoco basin in Colombia and Venezuela. Its populations have decreased and disappeared in some places due to illegal exploitation, as well as modification and destruction of their habitat due to human activities. This species has not been evaluated globally by the International Union for Conservation of Nature (IUCN), but it is categorized as Least Concern (LC) at the regional level and is regulated under Appendix II of the CITES convention. To analyze their conservation status using molecular tools for the first time, 19 microsatellite loci were evaluated using the cross-amplification technique, as well as sequences of the mitochondrial gene (mtDNA) Cyt *b* in four populations located in Puerto Carreño (Vichada), Paz de Ariporo (Casanare), Puerto López (Meta), Unillanos (Villavicencio, Meta), San martin (Meta) and 26 individuals kept at the Roberto Franco Tropical Biology Station (EBTRF). The data obtained allowed us to analyze for the species its diversity and genetic structure, as well as population declines (*bottlenecks*) and gene flow. As a result, ten suitable polymorphic markers were identified to carry out the population genetics study. Those markers were useful to reveal a genetic structure composed of the five groups corresponding to the sampling locations, as well as to detect high levels of genetic diversity in them. These five populations correspond to four Independent Management Units (IMU *sensu* Moritz 1994). However, the analyzes also indicated the presence of recent bottlenecks in the populations located in Paz de Ariporo and Puerto Carreño, being evidence of a population decline most likely due to anthropic activities. Additionally, the five populations presented low and asymmetric genetic flow between them. Regarding the analysis of the sequence of Cyt *b*, the haplotype network revealed weak differentiation but with the presence of unique haplotypes in the populations located in Puerto Carreño, Paz de Ariporo, EBTRF, a *genbank* sequence from San Martín and a shared haplotype identified in Puerto López, Puerto Carreño, Paz de Ariporo, Unillanos and the EBTRF. The results of this research allow us to propose management and conservation guidelines for these populations in Colombia.

Keywords: Conservation, microsatellites, Cyt *b*, cross-amplification test, genetic diversity, and gene flow**.**

Content

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Introduction

Turtles are very ancient animals with an origin estimated around 228 million years ago, in the Triassic period (Li et al., 2018). These vertebrates are anapsids, amniotes, ectodermal and oviparous (Páez et al., 2012a; Rueda-Almonacid et al., 2007), which are characterized by having a shell of bony dermal plates that fuse with the ribs and the trunk, have no teeth, and instead have a corneal case similar to the beak of birds. Their shell, late sexual maturity, extended reproductive life, and longevity allowed these vertebrates to successfully adapt in many terrestrial, freshwater, and marine ecosystems (Stanford et al., 2020).

The tortoises, freshwater, and marine turtles are the most threatened vertebrates, with an extreme generalized declination of their population (Stanford et al., 2020). Their main affectation is the consequence of human activity such as habitat loss and degradation, food consumption of the individuals and their eggs, pet trade and traditional medicinal use, invasive species, and climate change (Stanford et al., 2020). Furthermore, they are also vulnerable due to the lack of prioritization in species based conservation programs, as well as the conservation of biodiversity hotspots that harbor the highest turtle species richness (Lovich et al., 2018; Stanford et al., 2020).

Turtles are of great interest due to their ecological role, economic and cultural value (Mancera Rodríguez & Reyes García, 2008). They are an important part of the Earth's ecosystems, which provide services and processes such as bioturbators of soils, seed dispersal and germinators, mineral cyclers, and miners (Lovich et al., 2018). Additionally, they play a crucial role in the food chain and energy and nutrients flow of the water bodies and keep the water quality (Lovich et al., 2018; Páez et al., 2012a; Trujillo et al., 2008). Therefore, this ancient animal group must be conserved because those critical ecological roles are essential in the function and the structure of the ecosystems (Lovich et al., 2018).

Colombia contains approximately 10% of the species of the world due to its variety of ecosystems (Páez et al., 2012a). Furthermore, it ranks the third in the richness of reptiles in the world (SIB-Colombia, 2020) and second in the diversity of turtles in South America (Asociación Colombiana de Herpetología (ACHerpetología), 2011). The highest diversity of turtles in South America is presented in Brazil, followed by Colombia with 34 species (five marine and 29 continental) (Asociación Colombiana de Herpetología (ACHerpetología), 2011), including two new continental species described in recent studies: *Trachemys medemi* (Vargas-Ramírez et al., 2017) and *Chelus orinocensis* (Vargas-Ramírez et al., 2020). The families of the continental species in Colombia are Chelidae, Chelydridae, Emydidae, Geoemydidae, Kinosternidae, Testudinidae y Podocnemidae (Ministerio de Ambiente y Ambiente Sostenible, 2012). Particularly, Podocnemidae comprises eight freshwater species restricted to Madagascar and South America; the genus *Podocnemis* and *Peltocephalus* are both present in the last continent (Gaffney et al., 2011).

There is a decline in the reptile populations in Colombia with 9% classified as threatened due to anthropogenic activity such as exploitation, source of food and derived products, and transformation and degradation of their habitat (Morales-Betancourt, Lasso, et al., 2015). However, abundance and density studies are lacking for many reptile groups, generating inappropriate criteriums for classified species in any category of threat (Morales-Betancourt, Lasso, et al., 2015). Additionally, illegal trade of wildlife is a recurrent problem, being reptiles and particularly turtles, one of the most trafficked groups. Thus, based on seizure and confiscation records between 2005 and 2009, the most affected genus of turtles in Colombia were *Trachemys, Chelonoidis, Kinosternon, Podocnemis, and Rhinoclemmys* (Arroyave Bermudez et al., 2014), where the genus *Podocnemis* was the fifth more trafficked (Ministerio de Ambiente y Ambiente Sostenible, 2012).

Podocnemis vogli

Figure 1. *Podocnemis vogli*. Adult female at the Estación de Biología Tropical Roberto Franco EBTRF. Photography Juan Manuel Vargas Ramírez.

Distribution and ecology

The Savannah side-necked turtle *Podocnemis vogli* (Müller, 1935) is a species that belongs to the Pleurodira suborder, Podocnemididae family and it is known locally as “tortuga de río sabanera, sabanera, Gurruña, Galápago, Galápaga, galápago llanero, Galapaguita”. This species presents a restricted distribution along the savannas of the Orinoco basin of Colombia and Venezuela (Rhodin et al., 2021; Rueda-Almonacid et al., 2007). In Colombia, it occupies approximately an extension of 191.599 km^2 (Forero-Medina et al., 2014); from the north of the Eastern Cordillera up to the Orinoco, Guayabero and Guaviare rivers (Ministerio de Ambiente y Ambiente Sostenible, 2012) (Fig. 2). Specifically, it is found in Arauca, Casanare, Guaviare, Meta, Vichada (Rhodin et al., 2021).

This species is an aquatic and diurnal turtle found mainly in lentic ecosystems such as lakes, backwaters, or pools of water with little vegetation, small and shallow water bodies, and clay soil, but rarely in rivers (Alarcón-Pardo, 1969). However, there is evidence of the presence of individual in big water bodies such as the Orinoco and Guayabero rivers (IAvH, 2021). Individuals usually bask on the shores during the day but at night are mostly

in the water, usually buried in the mud (Alarcón-Pardo, 1969). In cleared savanna, they are inconspicuous because of their dark gray shell (Alarcón-Pardo, 1969; Fig. 1).

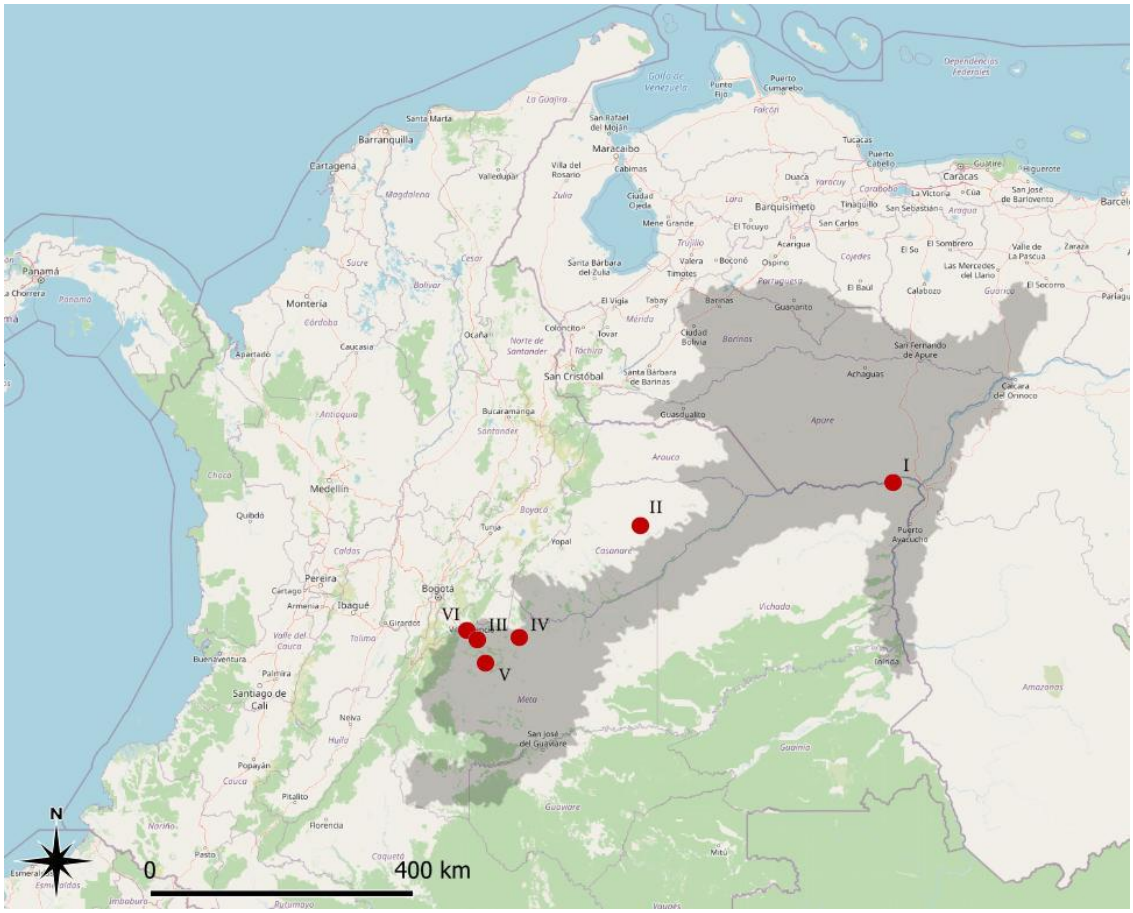


Figure 2. Distribution range of *Podocnemis vogli* in gray, according to Rhodin et al., 2021. Red dots indicate the sampled localities of this study: I. Puerto Carreño (Vichada); II. Paz de Ariporo (Casanare); III. Unillanos (Villavicencio, Meta); IV. Puerto López (Meta); V. San Martin (Meta) and VI. Estación de Biología Tropical Roberto Franco (EBTRF), (Villavicencio, Meta).

The Savannah side-necked turtle has sexual dimorphism regarding coloration and size. Males have a yellow stain on the muzzle (Páez et al., 2012a) with a long tail, twice longer than female's tails. Besides, the females are bigger than males with an average shell length of 23cm and a weight of 2000g, and 17cm and 860g, respectively (Rueda-Almonacid et al., 2007). The specie principal diet is vegetal matter such as stems, leaves and seeds (Alarcón-Pardo, 1969) but it also eats insects, mollusks, crustaceans, fish, and carrion (Ramo, 1982). In Colombia, there are no reproductive ecology studies in wild

population but there are few in Venezuela. The nesting season corresponds to the Orinoquía summer between the end of October to the beginning of January. Generally, females walk a long distance to lays their eggs in high places of clay soil, which lasts four months to hatch (Alarcón-Pardo, 1969). Each female lay between two and three positions annually, with an average of 13 to 15 eggs each (Ramo, 1982).

Threats to populations of *Podocnemis vogli*

Research on *P. vogli* focused on the ecology and evaluation of the threats status has been performed (see Morales-Betancourt, Lasso, et al., 2015; Ortiz Moreno & Rodríguez Pulido, 2017; Sepúlveda Seguro, 2018; Viloria & Forti, 2015). Therefore, *Podocnemis vogli* is classified as Low Concern (LC) for conservation at the regional level due to the apparent abundance of individuals (Morales-Betancourt, Lasso, et al., 2015). In contrast, the IUCN Red List of Threatened Species has not yet evaluated this specie (IUCN, 2021). However, the International Union for Conservation of Nature Tortoise and Freshwater Turtle Specialist Group (TFTSG) listed *P. vogli* as Vulnerable (VU) (Rhodin et al., 2018). In addition, this species is regulated under Appendix II of the Convention on International Trade for Endangered Species (CITES, 2019).

Thousands of individuals have been removed from their natural habitat and used as food and economic sources for human communities for a long time (Morales-Betancourt, Lasso, et al., 2015). Furthermore, juveniles are collected and sold as pets due to their bright colors (CORCAMARENA, 2019). Additionally, this turtle has been affected by indirect threats such as alteration and destruction of their habitat due to human activities such as agriculture, deforestation, livestock, illicit crops, oil activity, and contamination of water sources (Páez et al., 2012b). Moreover, several populations have been extirpated in vast sectors of Meta, Casanare and Arauca and, especially in the surroundings of urban centers because of exploitation and habitat destruction (Alarcón-Pardo, 1969).

There is evidence of an increase of the number of individuals of *Podocnemis vogli* seized or delivered to some regional corporation such as CORMACARENA and CORPORINOQUIA caused by its illegal exploitation (Table 1).

	Number of individuals of *Podocnemis vogli* seized or delivered											
	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	TOTAL
CORMACARENA	19	13	19	10	12	31	14	29	27	60	20	254
CORPORINOQUIA	3	4	19	*	*	3	69	35	20	62	16	231

Table 1. Number of seized or delivered individuals of *Podocnemis vogli* to the Corporación para el Desarrollo Sostenible del Área de Manejo Especial La Macarena (CORMACARENA) and Corporación Autónoma Regional de la Orinoquia (CORPORINOQUIA) from 2010 to April of 2020. * Data no available. Conservation initiatives have been carried out to reduce the threats against this species.

There are management plans aimed at protecting and managing *Podocemis vogli*; for instance, the management and conservation plan in Tuparro Biosphere Reserve (Trujillo et al., 2008), the Continental Turtle Conservation Plan of Colombia (Morales-Betancourt, Páez., et al., 2015), the Comprehensive Regional Climate Change Plan for the Orinoquía (PRICCO) (CIAT & CORMACARENA, 2017) and management plans for national natural parks. Furthermore, there are environmental groups restoring populations through the release and transfer of hundreds of individuals of *Podocnemis vogli* in other geographic locations (SuVersión, 2017; Violeta, 2020). However, these initiatives were done without any genetic studies to properly manage the populations. Therefore, genetics studies should be carried out in this species followed the fundamentals of conservation genetics and population genetics.

Genetic analysis to assess conservation status

Conservation genetics is a novel discipline that uses the theory and methods of genetics to identify, evaluate and conserve the genetic diversity in endangered species and provide information for their conservation and management (Frankham, 2015). Besides, one of their primary purposes is to maintain the species as dynamic individuals able to deal with environmental change (Frankham, 2010). Additionally, the conservation scheme applied for endangered species, aim at preventing the loss of genetic diversity, maintaining the natural gene flow, population size, and avoiding artificial selection (Ferrière et al., 2004;

Frankham, 2010). However, usually such measurements are taken too late and after other considerations (Attard et al., 2016). Furthermore, preserving biodiversity is essential because it provides economic resources, ecosystem services, aesthetic value, and ethical considerations such as the living rights of the individuals (Frankham, 2010).

For assessing the conservation status in species of concern, this discipline uses genetic markers such as microsatellites (nuclear DNA; nDNA) and mitochondrial DNA (mtDNA) to provide robust information for genetic analysis (Fienieg & Galbusera, 2013).

Nuclear DNA: Microsatellites

The genetic diversity assessed using microsatellites consists of the quantification of the variation of a DNA sequence in a locus of individuals, population or species (Ellegren & Galtier, 2016), due to mutations that generate new alleles (Ouborg et al., 2010). The neutral genetic study processes within a landscape include inbreeding, gene flow and bottleneck (Holderegger et al., 2006).

Mitochondrial DNA: Cytochrome b

The evolutionary relationship among individuals can be also analyzed by detecting the dissimilarities among their homologous sequences of DNA or protein; thereby, these differences reveal a genetic divergence due to the molecular evolutionary process (Patwardhan et al., 2014). The cytochrome b is a mitochondrial coding gene with approximately 1143 bp that includes conservative and more variable regions. Also, it is considered one of the most useful in bringing information about the phylogenetic relationships at several taxonomic levels (Patwardhan et al., 2014), as well as genetic diversity, genetic structure and phylogeographic differentiation (Castilla et al., 1998).

Since *Podocnemis vogli* is facing population decreases due to habitat modification and exploitations (Páez et al., 2012b), it is crucial to evaluate whether such declines have left a genetic signature, as well as the conservation status of its populations along its distribution range. A conservation genetics study for this species would contribute to increase the knowledge required for start delineating conservation measurements. This is the aim of this research project. Consequently, in the Chapter 1, we focused on assessing and characterizing a set of 19 microsatellite loci developed for close related species, through cross amplification test to determinate which of them are appropriate for performing a

genetic study of four populations of *Podocnemis vogli* located along several areas of the species distribution range in Colombia. In Chapter 2, we focused on quantifying the genetic variation and on identifying a possible differentiation within and between populations through the analysis of polymorphic microsatellite loci and Cyt *b* mtDNA sequences. Furthermore, we use the obtained dataset to estimate important demographic parameters such as presence of recent bottlenecks, gene flow and inbreeding, to provide the knowledge on the conservation state of those populations and to delineate measures for its preservation.

Chapter 1. Evaluating 19 loci of microsatellites in *Podocnemis vogli* using the cross-amplification test

1.1 Introduction

The assessment of patterns of the neutral genetic variation uses different neutral genetic markers such as Allozymes, RAPDs (random amplified polymorphic DNAs), AFLPs (amplified fragment length polymorphisms), single nucleotide polymorphism (SNP), and microsatellites (Holderegger et al., 2006). Allozymes, SNP, and microsatellites are co-dominant and show a high variation; therefore, they are frequently used in Conservation Genetics studies (Allendorf et al., 2010; Frankham, 2010). Their high variation makes them potent tools in differentiating between threatened and no-threatened species and discriminating the kinship (Frankham, 2010).

1.1.1 Microsatellites

The microsatellites are Mendelian inherited and co-dominant molecular markers found in all the genome, frequently in the non-coding DNA, and rare in protein-coding regions (Li et al., 2002). Microsatellites consist of tandem repeat sequences of DNA. The different allelic states (polymorphism) are detected through the size of the repeated motif and allow for each locus to differentiate as heterozygous or homozygous in diploid individuals (Ellegren & Galtier, 2016). The size of the repeated motif can extend from 5 to 40 pb in a locus (Selkoe & Toonen, 2006) and are generated due to mutations mechanism such as DNA replication errors (DNA slippage), DNA damage (Ellegren & Galtier, 2016) or in the process of DNA recombination (Y. Li et al., 2002). The microsatellite mutation rate oscillates between 10^{-2} to 10^{-6} events per locus per generation (Li et al., 2002), and it is independent for each microsatellite (Ellegren & Galtier, 2016).

Microsatellites are one of the more useful markers in studies of genetic diversity, bottleneck, population structure, kinship and paternity analysis (González, 2003) due to

their high polymorphism and high mutation rate (Allendorf et al., 2010; Frankham, 2010). However, it is important to assess each microsatellite under the assumptions of genetic studies through the analysis of: 1) Tendency to Hardy-Weinberg Equilibrium (HWE) for each locus which show the relationship between the alleles' frequency and genotype of the panmictic population in the lack of perturbing forces such as nonrandom mating, genetic drift, mutation, gene flow, and selection in the population (Frankham, 2010; Guo & Thompson, 1992), and 2) Linkage disequilibrium (LD) between pair of loci, which refers to a non-random allelic association at more than one locus in a population; therefore, those alleles are transmitted together and showed an independence of the data which are not useful in genetic analysis (Mueller, 2004).

- **Disadvantage**

The presence of null alleles must be analyzed for each microsatellite because caused error in genotypification of the individuals generate the overestimation in genetic distance and genetic differentiation (F_{ST}; Chapuis & Estoup, 2007). Null alleles are the alleles that do not amplify in the PCR and could be caused by: 1. amplification failure in the PCR (Dakin & Avise, 2004) attributable to the presence of mutations in the bidding primer-region (González, 2003; Pompanon et al., 2005; Selkoe & Toonen, 2006), 2. unspecific amplification due to the poor condition of the PCR and/or the reagents (Kantartzi, 2013; Pompanon et al., 2005), 3. polymerase slippage during DNA replication (Ellegren & Galtier, 2016), 4. human error, and 5. problems with detecting allele by the software (Waples & Allendorf, 2015).

There are some strategies to reduce the genotyping errors, including: (i) good maintenance and conservation of samples, (ii) use of negative control in the extraction and PCR procedure, (iii) following an automated protocol with a standard condition, (iv) have adequate knowledge of the marker, and perform a replicate amplification (Bonin et al., 2004). Furthermore, regarding the scoring of the markers, it is crucial to: (i) analyze carefully each allele, (ii) identify a good scoring before genotyping, (iii) use different software, and (iv) use reference samples (Bonin et al., 2004).

- **Advantages**

A key aspect of using microsatellites is that non-invasive biological samples as blood, stool, skin, hair and tissue can be used to amplify the markers (González, 2003; Taberlet et al., 1999). Furthermore, storing these samples for a long time is easy because it only needs ethanol and a freezing place (Selkoe & Toonen, 2006). Another significant advantage is the simple and rapid automation of the genotyping of individuals because of the PCR technique (Ellegren & Galtier, 2016). Moreover, the multiplex PCR technique permit to work with various primers in the same reaction, obtaining cost-effectively results in a short time (Ellegren & Galtier, 2016). The cross-amplification strategy has been extensively used for genetic research in the absence of microsatellite development for a specific species. This strategy uses primers designed for closely related species in a PCR, which lets to amplify specific genes or locus of the genome in a species of interest. The amplification is possible because the DNA adjacent to microsatellites is conserved in related species, especially in turtles with a highly conserved genomes (King & Julian, 2004).

1.1.2 Cross amplification in Podocnemidid turtles

Numerous microsatellites have been developed for turtles and tortoises and some of them have been already listed (see Engstrom et al., 2007). Polymorphic microsatellites have been identified and characterized in several species belonging to the genus *Podocnemis* such as in *Podocnemis unifilis* (Loci: Puni_1B2, Puni_1B11, Puni_1C3, Puni_1D9, Puni_1E1, Puni_1H9, Puni_2A9, Puni_1B11, Puni_1D12, Puni_1F10, Puni_2C11, Puni_2F6, Puni_1A5, Puni_1C9, Puni_1D11, Puni_2D9, Puni_2D10, Puni_2E7; Fantin et al., 2007) and in *Podocnemis expansa* (Loci: PE344, PE519, PE1075, Valenzuela, 2000; Pod1, Pod61, Pod79, Pod91, Pod128, Pod147, Sites Jr. et al., 1999). Furthermore, those loci have been tested by using the cross-amplification procedure in closely related species such as *Podocnemis lewyana* (Vargas-Ramírez et al., 2011), *Podocnemis sextuberculata, Podocnemis erythrocephala, Podocnemis expansa* and *Podocnemis vogli* (Fantin et al., 2007).

The research performed by Vargas-Ramírez et al. (2011) for *Podocnemis lewyana*, evaluated via cross-amplification tests 18 microsatellite loci developed for other species of the genus *Podocnemis*. As a result, they found ten loci unlinked and polymorphic; seven loci developed for *Podocnimis unifilis* (Puni_1B2, Puni_1B10, Puni_1B11, Puni_1F10,

Puni_2A9, Puni_2C11, Puni_2D9, Fantin et al., 2007) and three for *Podocnemis expansa* (Pod62, Pod128, Sites et al. 1999; PE519, Valenzuela, 2000). The results of this research showed a low level of genetic diversity, heterozygosity, and allelic richness in this species (Vargas-Ramírez et al., 2011)

In a study performed by Fantin et al. (2007), specific primers to amplify microsatellites in *Podocnemis unifilis* were developed. Additionally, they performed a cross-amplification test for a few samples (4-12) in different species, indicating for each locus if they were polymorphic, monomorphic, or lacked amplification (Fantin et al., 2007). Specifically, from eight samples of *Podocnemis vogli*, seven polymorphic loci were detected (Puni_1B2, Puni_1B11, Puni_1C3, Puni_1D9, Puni_1E1, Puni_1H9, Puni_2A9), five loci were monomorphic (Puni_1B11, Puni_1D12, Puni_1F10, Puni_2C11, Puni_2F6) and six loci did not amplify (Puni_1A5, Puni_1C9, Puni_1D11, Puni_2D9, Puni_2D10, Puni_2E7) (Fantin et al., 2007).

Considering the limited results obtained by Fantin et al., 2017 for *Podocnemis vogli*, this project aims at assessing many more loci via cross-amplification, to increase the number of usable loci that could allow performing a robust genetic analysis. For this, the goals of this research were: 1. To assess 19 additional microsatellites loci developed for other Podocnemidids in *Podocnemis vogli* : five primers designed for *Podocnemis expansa* (Fantin et al., 2007) and 14 for *Podocnemis unifilis* (Valenzuela, 2000), and 2. To characterize the polymorphic microsatellites to perform a genetic study including four populations of *P. vogli* in Colombia.

1.2 Materials and methods

1.2.1 Sampling

For this part of the research project, we used blood samples of *Podocnemis vogli* from five natural populations corresponding to Paz de Ariporo (Casanare) (22), Puerto López (Meta) (10), Unillanos (Villavicencio, Meta) (20), San Martin (Meta) (30) and Puerto Carreño (Vichada) (16) (Figure 2). Theses samples were collected from April of 2016 to February of 2022 and provided by the Banco de Tejidos de la Biodiversidad (BTBC) of the Instituto de Genética, Universidad Nacional de Colombia, Bogotá.

1.2.2 DNA extraction and quantification

The Genomic DNA was extracted from blood samples, following the Phenol-Chloroform protocol (Green & Sambrook, 2017). The quality and concentration of DNA samples were measured by Thermo Scientific™ NanoDrop 2000 Spectrophotometer, which calculates the light absorption of each sample.

1.2.3 PCR amplification

The cross-amplification test was performed using two samples for each locality to test the successful amplification of the chosen 19 microsatellites. We used five primers designed for *Podocnemis expansa* (Pod1, Pod62, Pod128, Sites Jr. et al., 1999; PE519, PE1075, Fantin et al., 2007) and 14 designed for *Podocnemis unifilis* (Puni_2A9, Puni_1B2, Puni_1B10, Puni_1B11, Puni_1C3, Puni_2C11, Puni_1D11, Puni_1D12, Puni_2F6, Puni_1E1, Puni_2E7, Puni_1F10, Puni_1H9, Puni_2D9, Valenzuela, 2000) (Table 2). The PCRs were run in Eppendorf master cycler, with a final volume of 10 µL, which contains: 5 µL of My Tag HS MIX (2X), 0,2 µM of reverse primer of each locus, 0,2 µM of forward primer of each locus, 200 ng of DNA and completed with ppH2O up to 10 µL.

The thermocycling conditions were the same reported for *P. unifilis* (Fantin et al., 2007). It followed the next steps: Initial denaturing for 1 min at 94°C, followed by 25 cycles with denaturing for 50 s at 94°C, annealing temperature for each locus during 50 s, and primer extension for 1 min at 72°C, followed by 20 cycles with denaturing 40 s at 92°C, annealing 50s at 50 °C, and primer extension for 40 s at 68 °C and a final elongation of 20 min at 72°C (Fantin et al., 2007). The PCR products of each microsatellite loci were verified through electrophoresis in a 2% agarose gel (30 ml of TBE and 0.6 mg of agarose) with a DNA ladder (Gene Ruler 100pb Thermo Scientific), which ran for one hour at 50 V. The gels contained four µl of the EZ-VISION™ DNA fluorescent dye to visualize the DNA bands in the Bio-Rad Molecular Imager UV transilluminator.

1.2.4 Data Analysis

To define which loci of microsatellites are useful for the population genetic analyzes using the cross-amplification procedure, it is important: 1. To confirm target microsatellite locus via sequencing of the repeat motif, 2. once the target locus has been confirmed, to

determinate the fragment length for each locus and 3. To evaluate for each microsatellite the tendency to Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD) and the presence of null alleles.

1. Repeat motif

To confirm the correct amplification of the target locus, a positive PCR of each locus was sequenced via sanger method, using the ABI 3500 genetic analyzer (Applied Biosystems) at the in SSiGMol (Servicio de Secuenciación y Análisis Molecular) of the Instituto de Genética, Universidad Nacional de Colombia, Bogotá. The microsatellite motifs were confirmed using the software Chromas v2.23 (Technelysium, Australia) and compared with the repeat motif reported in the literature.

2. Fragment length analyses

After confirming the presence of the microsatellite motif, the multiplex PCRs for the 98 individuals were performed by combining three and four molecular markers in one reaction (Table 9). The PCR products of each reaction followed the previous thermocycling condition reported but included the fluorochrome forward primers (FAM, HEX, PET, NED) for each reaction. The fragment length analysis was performed in the ABI 3500 genetic analyzer (Applied Biosystems) and the data were observed in the software OSIRIS v2.12 (National Center for Biotechnology Information) and GeneMapper (Applied biosystems) to genotype all individuals, with which it was possible to generate a genotypes database.

3. Evaluation for the microsatellite loci

Tendency to Hardy Weinberg Equilibrium (HWE), Linkage Disequilibrium (LD) and the presence of null alleles must be assessed to the loci of microsatellites to indicate if they are statistically and biologically significant for the population genetic analyzes. To eliminate any molecular market due to technical issues, or a biological problem and not because of a random event, is necessary to evaluate the frequency and the distribution of the significance values for these tests in two different scenarios: 1) the dataset was structured for each geographic sampling unit and 2) all individuals were assigned as one population.

The Hardy-Weinberg equilibrium evaluates if the locus followed the H-W proportion (HWP) for the genotype frequency. It was estimated through the exact test implemented in the software GENEPOP (Raymond & Rousset, 1995; Rousset, 2008), which ran with 1000 dememorization steps, 500 batches, and 1000 iterations per batch. Moreover, to determinate if the statistically significant values for the HWE test in a locus were the consequence of a random event, technical issues, or a biological problem, other complementary tests were evaluated to search paterns of the significant values. This test were the W&C test implemented in the software GENEPOP (Raymond & Rousset, 1995; Rousset, 2008) that indicate the values of F_{IS} (Weir & Cockerham's 1984) and the exact test to evaluate the heterozygosity excess and heterozygosity deficit for each microsatellite (Raymond & Rousset, 1995). Additionally, to assess the presence of technical problems such as null alleles, we used the test of heterozygosity deficit in the software GENEPOP (Raymond & Rousset, 1995; Rousset, 2008) and Frequency of null alleles using the software FREENA (Chapuis & Estoup, 2007). For the last tests a value higher than 0.05 indicates the presence of null alleles. Finally, the linkage disequilibrium evaluates the non-random allelic association between a pair of loci in the software ARLEQUIN (Excoffier et al., 2005) which implemented the likelihood-ratio test with a setting of 10000 random permuted samples. The multiple comparison test implemented in ARLEQUIN, increases the error type I when many tests are compared simultaneously with the same samples data, level of significance and the unique universal null hypothesis (Lee & Lee, 2018). There is a probability that in multiples comparison tests occur that at least one test will be significant by chance. Therefore, it is possible to adjust the significance level to reduce this type of error by the correction of Bonferroni which was manually implemented in each test.

4. Characterization for the microsatellite loci

Expected heterozygosity (H_e) and observed heterozygosity (H_o) were also estimated using the software ARLEQUIN (Excoffier et al., 2005), as well as the allelic range (Ar), Number of alleles (K) and the annealing temperature (Ta) for each microsatellite in *Podocnemis vogli*.

1.3 Results

It is important to determine and reduce the error in sampling, transcribing, and analyzing the genetic information. Therefore, this research was implemented with an excellent maintenance and conservation of the samples, the use of controls in each procedure, monitoring the condition for the PCR and reagents, as well as confirmation of the presence of each microsatellite by sequencing (as suggested by Bonin et al., 2004; Kantartzi, 2013; Pompanon et al., 2005). Furthermore, regarding the scoring of the markers, each allele was individually and carefully analyzed (Bonin et al., 2004), using two different software: OSIRIS v2.12 and GeneMapper.

1.3.1 Initial characterization of 19 microsatellites

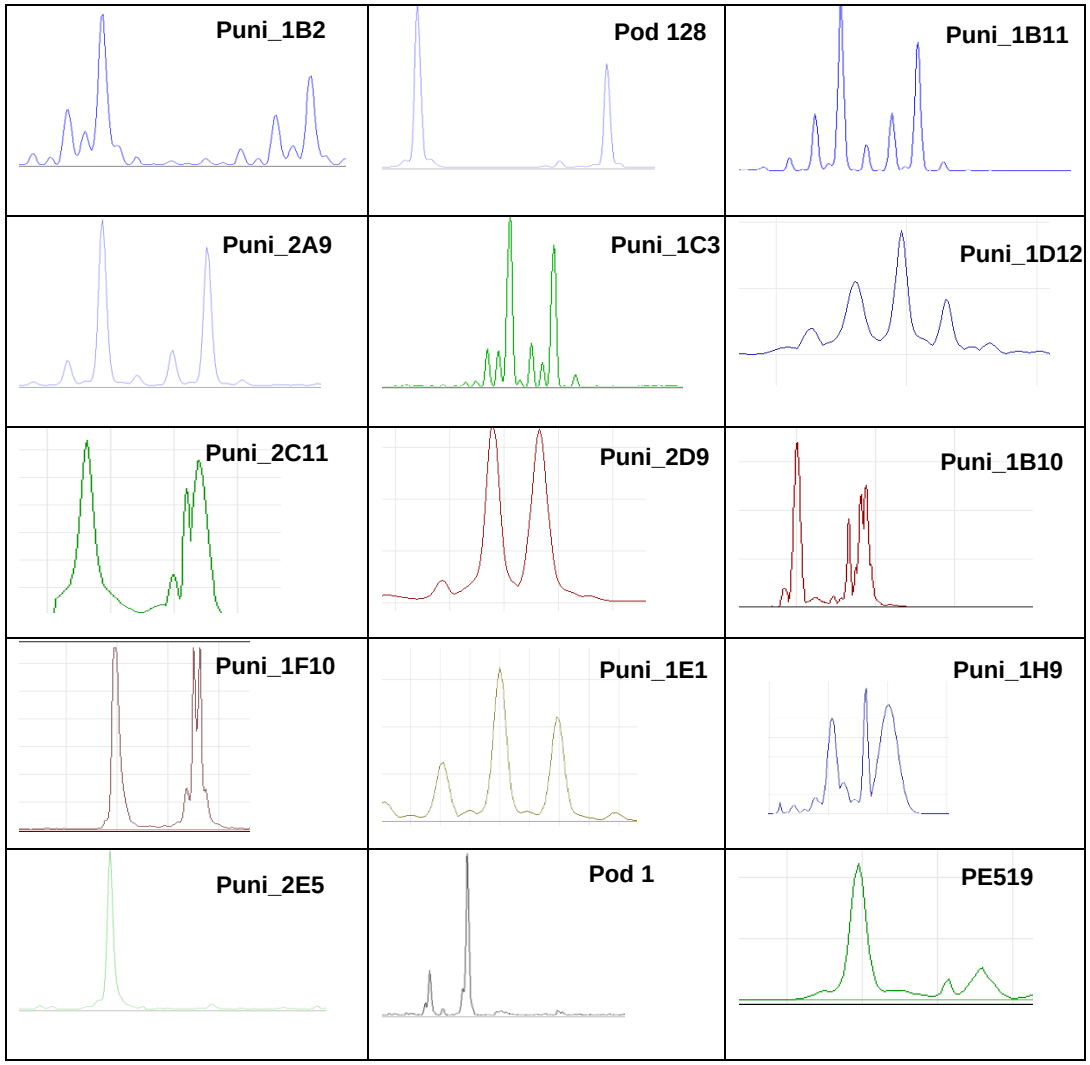
An excellent concentration and quality of DNA of the *P. vogli* individuals were obtained from the blood samples. All 19 microsatellites showed a successful amplification in a single reaction, and most of them were amplified using the same annealing temperature condition reported in the source literature (Puni_1B2, Puni_1B11, Puni_1H9, Puni_1B10, Puni_1F10, Puni_2D9, Puni_2E7, Puni_1E1). However, some microsatellites amplified with the following higher annealing temperatures: 1°C (Puni_1C3, Puni_1D12, Puni_2C11, Puni_1D9, Pod 1 and Pod 128), 3 °C (Puni_2F6) and 5°C (Puni_2A9) (Table 2). The repeat motifs in some loci of microsatellites in *Podocnemis vogli* differed from the source species (*P. expansa, and P. unifilis*). This behavior was observed in Puni_1B11, Puni_2D9 and Puni_1E1.

Finally, from 19 microsatellites for the 72 samples, four loci (Puni_2F6, Puni_1D9, PE1075, Pod 62) presented an unsuccessful amplification in the multiplex PCRs, 12 were polymorphic microsatellites loci and three monomorphic loci (Tables 2 and 3). Additionally, the size range of the length of the microsatellites amplified in *P. vogli* was closed to the size range reported in the source (Table 2; Fantin et al., 2007; Sites Jr. et al., 1999; Valenzuela, 2000)

Table 2. Characterization of 19 microsatellite loci in *Podocnemis vogli* using a cross-amplification test. * No reported

Source of the primer	Locus	Primer sequence	Ta (°C)		Repeat motif		Size range	
			P. vogli	Source	*P. vogli*	Source	*P. vogli*	source
P. unifilis	Puni_1B2	F-5'-GTAGTGGCACTGCGACAAAT-3' R-5'-CCACTGTCACATCTCCTGAAAC-3'	55-56	55	$(GA)_{19}$	$(GA)_{17}$	328-362	342-369
	Puni_1B11	F-5'-CCAGACCTCTCCTGTTTTGG-3' R-5'-GGTTCTGGGCTCCTTACACA-3'	60	60	$(GA)_{23}$	$(GA)_7gg(GA)_9$	247-287	265-287
	Puni_1C3	F-5'-CCCTACCGAAACAGCTTGAG-3' R-5'-ATCTGGCTTGGAGCTGTGTT-3'	63	62	$(GA)_5$	$(GA)_8$	114-136	182-218
	Puni_1H9	F-5'-GGGGCTACAGAGAAGGAGAA-3' R-5'-ATTTATATGGGCCCCCTACC-3'	60-63	60	$(GA)_5$	$(GA)_{12}$	140-158	133-141
	Puni_2A9	F-5'-CTGTTCCCAACAGCTGAGAG-3' R-5'-GGTCCTCAAGAAAGCCCAAA-3'	60	55	$(GA)_7$	$(GA)_{12}$	137-167	185-209
	Puni_1B10	F-5'-CCAAACTAGGTTCATGTCCAAA-3' R-5'-GAAGCGTCAGGAAGGAAAGA-3'	60	60	$(GA)_4$	$(GA)_8$	208-218	161-183
	Puni_1D12	F-5'-AGGAGCTGCAGGTGCAAAC-3' R-5'-GATCACCCAGATGCTGACCT-3'	56-60	55	$(GA)_7$	$(GA)_{10}$	150-154	203-215
	Puni_1F10	F-5'-GCCTGCaGCTCCTCTCATAA-3' R-5'-CCCAGGAACTTGAGAATAGTGG-3'	60	60	$(CT)_7$	$(CT)_8$	176-186	238
	Puni_2C11	F-5'-AAGGTGCCTGGAGAATAGGA-3' R-5'-TGCACCCTTCCATTTAAGC-3	56-60	55	$(CT)_{12}$	$(CT)_{17}$	231-241	240-246
	Puni_2F6	F-5'-CTGGTCCAACCCAATTTCTG-3' R-5'-CCTTGACCAGGACTGCACTT-3'	63	60	Did not amplify	$(CT)_6t(CT)_{10}$	Did not amplify	171-191
	Puni_2D9	F-5'-CAGCATTTTCTGACAGACAGC-3' R-5'-CCACAGCAACCATCTCAGC-3'	60	60	$(GA)_{20}$	$(GA)_7ca(GA)_6$	201-211	174-180
	Puni_1D9	F-5'-GCTGGGGAACTGACTACCT-3' R-5'-CACGAGGTAGGAATGCCTGT-3'	63	62	Did not amplify	(GA)12	Did not amplify	133-141
	Puni_2E7	F-5'-CTGGACCCATATGCAGTGAC-3' R-5'-CACTTGAGCTTCTGAGGGAGA-3'	56	56	$(GA)_4gc$	$(GA)_5gc(GA)_8$	232	260-262
	Puni_1E1	F-5'-GGCCTCTACTGTCTGAAAGTCC-3' R-5'-GAAGGAGAGCTCCAGGTGAA-3'	60- 63	60	$(CT)_{11}$	$(CT)_9tt(Ct)_7$	184-208	291-325
P. expansa	PE519	F-5'-GCTGAGCTAGACTAACATGC-3' R-5'-GTAAATTGCCATACTTGGA-3'	56-60	*	$(CT)_9(CA)_1$	$(CT)_7(CA)_8(CG)_2(CA)_8$	206	239-327
	PE1075	F-5'-ATGAGCCTGAAGAGTTGGAA-3' R-5'-AACTTAGGCTGCATGAGTTG-3'	56-60	*	$(AG)_{23}$	$(AC)_{11}$	Did not amplify	247-283
	Pod1	F-5'-GATCTTTCTTTACAGGTGCAGTTC-3' R-5'-CACAACTAAATTACAGCACTCCG-3'	56	55	$(CA)_3$	$(CA)_{32}$	144	154-204
	Pod62	F-5'-ATGAGTGTGGAATGAGAGGAAC-3' R-5'-CCCATCCACAGAAGCAAATTCC-3'	60	*	Did not amplify	$(GT)_{11}(TA)_5$	Did not amplify	140-209
	Pod128	R-5'- CCAGTAAAATTCACTACCAGCATG-3' F-5'-GTGTCAGGGCTACCATCAAGATTG -3'	56	55	$(AG)_2tt(AG)_6$	$(GT)_{27}(GC)_7$	112-134	182-214

Table 3. Example of amplified alleles of 15 microsatellites loci in *Podocnemis vogli.* The loci Puni_2E7, Pod 1 and PE519 were monomorphic and Puni_1B2, Pod 128, Puni_1B11, Puni_2A9, Puni_1C3, Puni_1D12, Puni_2C11, Puni_2D9, Puni_1B10, Puni_1F10, Puni_1E1 and Puni_1H9 were polymorphic.



1.3.2 Evaluation of microsatellites

The first step to evaluate whether the 12 polymorphic microsatellites can be used for the population genetics analyses, is to test the tendency to the Hardy-Weinberg Equilibrium (HWE) (Table 4). The test evaluates the null hypothesis as the presence of random union of two gametes where the locus follows the Hardy-Weinberg Proportion (HWP) (Excoffier et al., 2005). The results revealed that various loci were in deviation of HWP for the scenario, where all individuals were analyzed as one population ($P < 0.004$) (loci Pod128, Puni_1B2, Puni_1D12, Puni_1B11 and Puni_1B10), which may be explained due to the possible presence of structure in the population. In contrast, the test of tendency to the HWE, in the scenario where there is differentiation per each geographic sampling unit, showed a deviation of HWP for the loci Pod 128, Puni_1D12 and Puni_1B10 after Bonferroni correction (P-value < 0.0008). However, Puni_1D12 revealed a pattern of deviation of HWE in various geographic sampling units.

Table 4. P-values of Hardy-Weinberg equilibrium for each locus and each geographic population. Values in bold were statistically significant for each geographic population (P-value <0.0008) and all individuals as one population (P-value <0.004). * Data not reported.

LOCUS	PAZ DE ARIPORO	UNILLANOS	PUERTO LÓPEZ	SAN MARTIN	PUERTO CARREÑO	ALL INDIVIDUALS AS ONE POPULATION
Pod 128	0.8203	0.6226	1.0000	**0.0000**	**0.0001**	**0.0001**
Puni_1B2	0.0306	0.2051	0.7312	0.0280	0.2637	**0.0000**
Puni_1H9	0.4176	0.1060	0.2188	0.2629	1.0000	0.1787
Puni_1C3	0.6621	0.8960	0.5371	0.3405	0.7950	0.0195
Puni_1E1	0.9229	0.3770	0.9669	0.2434	0.3794	0.0033
Puni_1D12	**0.0000**	**0.0000**	0.0069	**0.0000**	**0.0001**	**0.0000**
Puni_1B11	0.1867	0.0299	0.1552	0.2168	0.0009	**0.0001**
Puni_2C11	1.0000	0.5490	1.0000	*	*	0.6356
Puni_2D9	0.5451	0.2779	0.0068	*	1.0000	0.0071
Puni_2A9	0.0422	0.2373	0.4540	0.7201	0.9516	0.0021
Puni_1F10	0.0156	0.0427	0.0115	1.0000	1.0000	0.0237
Puni_1B10	0.0383	0.0027	**0.0001**	0.0914	1.0000	**0.0000**

From above results, it is necessary to determine if the deviation of the HWP in three loci is explained by chance and not by a biological issue such as structuring, nonrandom mating, genetic drift, mutation, gene flow, and selection in the population (Frankham, 2010; Guo & Thompson, 1992). For that, we used the estimator of F_{IS} and the test of heterozygote excess (Table 5, 6) to verify if the pattern in the deviation of the HWP was due to an

excess of heterozygotes or a random event, as it was expected to have a low significance value.

Table 5. Fixation index for 12 microsatellites analyzed per each geographic population. Values in bold indicate locus with excess of heterozygosity * Data not reported.

LOCUS	PAZ DE ARIPORO	UNILLANOS	PUERTO LÓPEZ	SAN MARTIN	PUERTO CARREÑO	ALL INDIVIDUALS AS ONE POPULATION
Pod 128	0.0118	0.0324	**-0.1776**	**-0.7487**	**-1.0000**	**-0.3510**
Puni_1B2	0.3420	**-0.0494**	**-0.0208**	**-0.0166**	**-0.1439**	0.1437
Puni_1H9	**-0.2857**	0.0500	**-0.5000**	**-0.2872**	**-0.0345**	**-0.1431**
Puni_1C3	**-0.2934**	0.0256	**-0.3279**	**-0.1326**	**-0.0769**	**-0.0159**
Puni_1E1	**-0.0547**	0.1502	**-0.1250**	-0.1009	0.0314	0.0737
Puni_1D12	**-0.9130**	**-0.9048**	**-0.8182**	**-0.6992**	**-1.0000**	**-0.8399**
Puni_1B11	0.0516	0.0273	**-0.4173**	**-0.0985**	**-0.2000**	**-0.0019**
Puni_2C11	**-0.1351**	**-0.2258**	**-0.3012**	*	*	**-0.1127**
Puni_2D9	**-0.0791**	**-0.3103**	**-1.0000**	*****	**-0.1667**	**-0.0994**
Puni_2A9	**-0.1388**	0.0397	**-0.2587**	**-0.1018**	**-0.1136**	0.0526
Puni_1F10	**-0.5833**	**-0.5200**	**-0.6981**	**-0.0093**	**-0.2174**	**-0.2651**
Puni_1B10	**-0.3147**	**-0.5232**	**-1.0000**	**-0.6183**	**-0.1538**	**-0.3858**

Table 6. Test of heterozygote excess for 12 microsatellites. Values in bold were statistically significant for each geographic population (P-value <0.0008) and all individuals as one population (P-value <0.004). * Data not reported.

LOCUS	PAZ DE ARIPORO	UNILLANOS	PUERTO LÓPEZ	SAN MARTIN	PUERTO CARREÑO	ALL INDIVIDUALS AS ONE POPULATION
Pod 128	0.6487	0.6520	0.6544	**0.0000**	**0.0001**	**0.0000**
Puni_1B2	0.9663	0.3315	0.5844	0.4719	0.2163	0.8965
Puni_1H9	0.1254	0.9698	0.1972	0.0410	0.9680	0.6705
Puni_1C3	0.0096	0.6634	0.1203	0.1815	0.3312	0.3543
Puni_1E1	0.3127	0.9065	0.3847	0.0815	0.8536	0.8743
Puni_1D12	**0.0000**	**0.0000**	0.0055	**0.0000**	**0.0001**	**0.0000**
Puni_1B11	0.9817	0.6926	0.0344	0.1694	0.0442	0.7595
Puni_2C11	0.6764	0.4212	0.3479	*	*	0.2896
Puni_2D9	0.4366	0.2243	0.0054	*	0.6740	0.7418
Puni_2A9	0.6081	0.9231	0.0912	0.1500	0.1907	0.9950
Puni_1F10	0.0127	0.0274	0.0056	0.9821	0.0589	**0.0005**
Puni_1B10	0.0404	**0.0001**	0.0055	**0.0001**	0.6944	**0.0000**

The values of F_{IS} and the heterozygote excess test (P-value <0.0008) showed that the two loci Pod 128 and Puni_1B11 presented a deviation of HWP in some geographic localities due to heterozygosity excess (Table 5, Table 6), indicating a random event for the deviation of HWP in these localities. Contrary to the previous results, the locus Puni_1D12 showed heterozygotes excess in all geographic population through the F_{IS} values with a significative value for heterozygosity excess test (P-value <0.0008) in four of the localities (Table 6). These results suggested an indeterminate problem in this specific marker (i.e., biological and/or methodological) and therefore, it is inappropriate for the genetic population analysis and must be eliminated. Analyzing the individuals as one population revealed that there is a heterozygosity excess in most of the loci but was significant in Pod 128, Puni_1D12, Puni_1F10 and Puni_1B10 (P-value <0.004) (Table 6), indicating a possible random event.

Additionally, we use the heterozygosity deficit test and the frequency of null allele (FN) (Table 7) to evaluate the presence of technical problems as null alleles. The results showed no evidence of null alleles with the Heterozygote deficiency test (P-value <0.01) as well as in the null allele frequency with values lower than 0.05, which means no presence of null alleles.

Table 7. Test of heterozygote deficiency for 12 microsatellites evaluated with a significance level of 0.0008 per each geographic population and 0.004 for all individuals as once population and the estimation of null allele frequency (FN). * Data not reported.

LOCUS	PAZ DE ARIPORO	UNILLANOS	PUERTO LÓPEZ	SAN MARTIN	PUERTO CARREÑO	ALL INDIVIDUALS AS ONE POPULATION	FN
Pod 128	0.5605	0.5124	0.7962	1.0000	1.0000	1.0000	0.00001
Puni_1B2	0.0666	0.7854	0.7091	0.5512	1.0000	0.1035	0.03505
Puni_1H9	1.0000	0.0498	1.0000	1.0000	1.0000	0.3296	0.01615
Puni_1C3	0.9990	0.4013	0.9758	0.8492	0.7960	0.6470	0.00000
Puni_1E1	0.7603	0.1024	0.8953	0.9370	0.1551	0.1257	0.02576
Puni_1D12	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.00000
Puni_1B11	0.0209	0.3243	1.0000	0.8490	1.0000	0.2407	0.00002
Puni_2C11	1.0000	1.0000	1.0000	*	*	1.0000	0.00000
Puni_2D9	0.6082	1.0000	1.0000	*	1.0000	0.2585	0.00001
Puni_2A9	0.3971	0.0790	1.0000	0.8639	0.9362	0.0050	0.01338
Puni_1F10	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.00739
Puni_1B10	0.9598	1.0000	1.0000	1.0000	1.0000	1.0000	0.00000

Finally, the test of linkage disequilibrium was used to determine if the genotypes at one locus are independent of the other locus. As in the previous analysis, we examine the presence of a pattern of the significative value for each locus and geographic sampling. The loci Puni_1B10, Puni_1F10 and Puni_1D12 were repeatedly found in linkage disequilibrium (LD) with other loci and between them in three geographic population (P-value < 0.0008) and in all individuals analyzed as one population (P-value > 0.0001) (Table 8). The Puni_1B10 and Puni_1D12 showed significant values with other loci in both scenario while Puni_1F10 have an association only with Puni_2D9 in the scenario as one population. The significance level of Puni_2C11 & Puni_2D9 was identified in the analysis of all individuals as one population and Puni_2D9 in Puerto López, indicating a random event. Therefore, the loci Puni_1B10 and Puni_1D12 are linked and are inappropriate for genetic analysis and must be eliminated.

Table 8. Test of linkage disequilibrium for all pairs of loci with a significance level of 0.0008. Left: the analysis for each demographic unit and Right: all individuals as one population.

	Pair of loci	**P-value**			**Pair of loci**	**P-value**
Paz de Ariporo	Puni_1F10 & Puni_1B10	0.0002			Puni_2C11 & Puni_2D9	0.0000
Puerto López	Puni_2D9 & Puni_1B10	0.0002			Puni_2D9 & Puni_1F10	0.0000
Puerto Carreño	Pod 128 & Puni_1D12	0.0000		**One population**	Puni_1B2 & Puni_1B10	0.0004
					Puni_1D12 & Puni_1B10	0.0000
					Puni_1F10 & Puni_1B10	0.0000
San Martin	Pod 128 & Puni_1D12	0.0000			Puni_1H9 & Puni_1B10	0.0000
					Pumi_2D9 & Puni_1B10	0.0002

1.3.3 Characterization of ten unliked microsatellites with tendency to Hardy-Weinberg proportions

As a result of the evaluation of 12 polymorphic microsatellites, we determine that the loci Puni_1B10 and Puni_1D12 are not useful in the analysis of genetic population because are linked and the locus Puni_1D12 are in disequilibrium of the HWP. Using the remaining ten microsatellites, we performed a genetic characterization of them from 98 samples

which revealed 84 alleles in total, which range from three (Puni_2C11, Puni_2D9) to 16 (Puni_1B11) (Table 9). Additionally, the observed heterozygosity (H_o) ranged from 0.218 (Puni_2C11) to 0.816 (Puni_1B11), and the expected heterozygosity (H_e) varied from 0.196 (Puni_2C11) to 0.840 (Puni_1B2). The average of these parameters was 0.623±0.199 and 0.603 ± 0.231, respectively. However, the locus Puni_2C11 presents low variability with the lowest observed heterozygosity (Table 9).

Table 9. Multiplex PCR assays and characterization of 10 microsatellites. Ar: allelic range K: Number of alleles, H_o: observed heterozygosity, H_e: expected heterozygosity, Ta: annealing temperature.

Multiplex	Locus	Ta (°C)	Fluorescent dye	Ar	K	Ho	He
1	Pod 128	56	6-FAM	11	5	0.755	0.559
	Puni_1B2		6-FAM	14	15	0.722	0.840
2	Puni_1H9	64	6-FAM	9	5	0.469	0.410
	Puni_1C3		HEX	10	9	0.804	0.791
	Puni_1E1		PET	12	10	0.732	0.789
3	Puni_1B11	60	6-FAM	14	16	0.816	0.814
	Puni_2C11		HEX	5	3	0.218	0.196
	Puni_2D9		PET	5	3	0.402	0.366
4	Puni_2A9		6-FAM	15	14	0.814	0.858
	Puni_1F10		PET	7	4	0.505	0.400
	Mean	-	-	-	8.4	0.623	0.603
	s.d.		-	-	4.9	0.199	0.231

1.4 Discussion

Podocnemis vogli is an ancient continental turtle species under extreme pressure from human activities that have altered their natural habitats and therefore have reduced its populations sizes (Morales-Betancourt, Lasso, et al., 2015; Ortiz Moreno & Rodríguez Pulido, 2017). It is necessary to performance genetic studies for this species to know their genetic status and delineating conservation measurements to protect, recover, and maintain the species as dynamic individuals able to deal with the environment and avoid extinction (Frankham, 2010).

Microsatellites are useful markers in genetic diversity studies that give information of gene flow, bottleneck, population structure, kinship and paternity analysis (González, 2003). However, it is crucial to select the appropriate microsatellite loci for adequate inferences;

therefore, the evaluation of microsatellites must be supported based on both statistical and biological significance (Waples & Allendorf, 2015).

Firstly, the successful cross-amplification test was confirmed through sequencing and validation of the repeat motif of each microsatellite loci, which indicates the presence of the corresponding allele (Davy et al., 2012). Secondly, the evaluation of tendency to Hardy-Weinberg equilibrium is the primary evidence to select the adequate loci. To eliminate any molecular market is necessary to determine the frequency and the distribution of P-values in different scenarios where the level of significance was established based on the Bonferroni correction due to incorporation of multiple tests, which reduce the type I error (Lee & Lee, 2018). Therefore, it was expected to have a low significance value due to random and not for any perturbing forces such as nonrandom mating, genetic drift, mutation, gene flow, and selection in the population (Frankham, 2010; Guo & Thompson, 1992). Most of the loci did not show a deviation of HWP in the different localities except for the locus Puni_1D12 with a heterozygote excess, corroborating by the negative values of F_{IS} and the heterozygote excess test. Therefore, this locus was not appropriate for performing a population genetics analysis. Additionally, the analysis in all datasets as one population presented five loci with deviation in HWP, which give a hint of a Wahlund effect for the collecting distribution.

Thirdly, there is no evidence of null alleles using the evaluation of heterozygote deficiencies in the HWE test (Chapuis & Estoup, 2007), or by the frequency of null alleles (Chapuis & Estoup, 2007). In contrast, there is a high-level heterozygosity of excess in most markets revealed by H_o, H_e and negative value of F_{IS}, which did not cause departures of the HWP.

Finally, the linkage disequilibrium test showed that Puni_1B10, Puni_1F10 and Puni_1D12 have a significant association after Bonferroni correction in the different collected regions and all individuals analyzed as one population. However, the Puni_1F10 only presented association with Puni_1B10 and Puni_1D12 and the values of the observed and expected heterozygosity, are slightly higher than the other loci. Therefore, Puni_1B10 and Puni_1D12 should be excluded from further genetic population analyzes.

In the previous study performed by Fantin et al. (2007), the locus Puni_1H9 was characterized in *Podocnemis vogli* as monomorphic instead of polymorphic as our results

revealed (Table 1, Figure 3). Similarly, Puni_2A9, Puni_1D9, Puni_2C11, and Puni_1B10 did not amplify in their research, but in our study, these markers amplified and were polymorphic. This discrepancy may happen because they only used eight samples while we tested various individuals from different collected regions. Additionally, the locus Puni_1D12 in *Podocnemis unifilis* and *Podocnemis vogli* presented a heterozygosity excess and was in linkage disequilibrium with the loci Puni_1B2 and Puni_1B11 (Fantin et al., 2007). Regarding the loci Puni_1F10 and Puni_1B10, which are in Linkage disequilibrium in *Podocnemis vogli*, Vargas-Ramírez et al., 2011 revealed no association between them in *Podocnemis lewyana* and tended to HWP.

Some research in turtles have demonstrated the importance of the cross-amplification test for using microsatellite loci developed in closed related species, to understand the genetic situation of endangered populations (Davy et al., 2012; Vargas-Ramírez et al., 2011). The cross-amplification test for *Podocnemis vogli* revealed high genetic diversity, due to their high number of alleles and high heterozygosity values. Additionally, this study defined a powerful genetic marker system that will allow the evaluation of the genetic situation of this species. These results of this evaluation are presented in chapter 2.

Chapter 2. Genetic characterization of four colombian populations of the turtle *Podocnemis vogli* (Testunide:Podocnemidae): Begin proposing urgent strategies for the species conservation and management.

2.1 Introduction

It is crucial to increase research on species that are ecologically important and are part of the evolutionary process that maintains biodiversity. The loss of an old lineage means the loss of the accumulation of historical evolution and a vital representative of the world biodiversity (Mace et al., 2003; May, 1990).

One of the animals with these characteristics are turtles which are ancient vertebrate that play an important ecological role in the ecosystem as well as they present economic and cultural value (Mancera Rodríguez & Reyes García, 2008). Whitin this characteristics are the provision of services and processes such as bioturbators of soils, seed dispersal and germinators, water quality maintainers, mineral cyclers, miners and are part of the food chain, flowing the energy and nutrients (Lovich et al., 2018; Páez et al., 2012a; Trujillo et al., 2008).

There is evidence that the population of turtles are declining due to anthropogenic impacts (Stanford et al., 2020). Therefore, this ancient animal must be conserved because of an aesthetic value, ethical considerations and those critical ecological roles are essential in the function and the structure of the ecosystems (Lovich et al., 2018). Furthermore, preserving biodiversity is essential because it provides economic resources, ecosystem services, aesthetic value, and ethical considerations such as the living rights of the individuals (Frankham, 2010).

The conservation strategies in these species consist of: 1) expanding the information of management plans which must include ecology, demography, habitat management, genetic and change of external variables such as man-made affectations and climate change and 2) monitoring of the population for decades because those species are long-lived and present late sexual maturity and extended fertility (Stanford et al., 2020). Among the strategies for the conservation of population, the genetic analysis bring information for conservation and management of endangered species, in order to maintain the genetic diversity, natural gene flow, population size, and avoids artificial selection that will allow species to continue for a long time and to deal with environmental change (Ferrière et al., 2004; Frankham, 2010).

Particularly*, Podocnemis vogli* is essential as a conservation target because apart from the considerations previously described, it is part of an ancient group of turtles; the genus *Podocnemis*, which has survived from the Cretaceous period (Gaffney et al., 2011). Based on one of the families' phylogenyetic hypotheses, this species represents one of the most ancient podocnemidids, revealed as a basal lineage in the genus *Podocnemis* clade, together with *P. expansa* and *P. sextuberculata* (Vargas-Ramírez et al., 2008).

To date, the conservation status of populations of *Podocnemis vogli* is unknown due to the lack of knowledge in their accurate distribution, population structure, natural history (Páez et al., 2012a), as well as threat factors. Since, this species has been deeply affected by its habitat modification throughout its distribution range and there has been a dramatic increase in the extraction of individuals of all age classes from their habitats, due to the reduction of the other species of *Podocnemis* in the region (Morales-Betancourt, Lasso, et al., 2015), it is urgent to perform a genetic evaluation that could offer information on the conservation status of populations of the species and that will allow proposing measures for their management. Consequently, by analyzing ten polymorphic microsatellites (nDNA) and sequences of cytochrome b, this research has the following goals: (i) To quantify the genetic diversity and evaluate the presence of genetic structure in four populations of *Podocnemis vogli* located along a great portion of its distribution range in Colombia, (ii) To assess whether such populations have experienced size

reduction by evaluating the presence of bottlenecks, as well determining other crucial demographic parameters such as inbreeding and gene flow, and (iii) To assess the utility of the microsatellite system for forensic purposes by defining the population of origin of 26 captive individuals located at the Estación de Biología Tropical Roberto Franco (EBTRF).

2.2 Methods

2.2.1 Microsatellites

2.2.1.1 Sampling

Blood samples from 124 individuals of *Podocnemis vogli* were collected from the following four natural populations: Paz de Ariporo, (Casanare) (22), Puerto López, (Meta) (10), Unillanos, (Meta) (20), San Martin (30) and Puerto Carreño, (Vichada) (16) and 26 individuals in captivity from Estación de Biología Tropical Roberto Franco (EBTRF), located in Villavicencio, Meta.

The 98 samples from natural population were used to analyze the genetic diversity, demographic parameters, and the analysis of the isolation by distance. However, the individuals from EBTRF were used to define their possible region of origin.

2.2.1.2 Population differentiation

To assess population differentiation, a genetic approach as well as statistics methods were performed. Firstly, the genetic approach used the Bayesian clustering approach implemented in the software STRUCTURE 2.3 (Pritchard et al., 2000), which evaluates population differentiation by assigning individuals to different clusters based on their genotypes' tendency to the HW equilibrium and Linkage Disequilibrium (Pritchard et al., 2000). The settings to run the program were: (i) an admixture model, which assumes that individuals could bring part of their genome from ancestral populations (Hubisz et al., 2009) and (ii) Allele Frequencies Correlated, which considers that the frequencies are similar to ancestral population (Pritchard et al., 2000). Additionally, the software ran with a

burning length of 20000 and 100000 Markov Chain reps after-burn. Besides, the iteration k populations assigned were between 1 and 7 with an iteration of 20. The number of k was estimated through the ΔK statistic using Evanno method (Evanno et al., 2005), and MedMeaK' (median of means), 'MaxMeaK' (maximum of means), 'MedMedK' (median of medians) and 'MaxMedK' (maximum of medians) which deal with uneven sampled size (Puechmaille, 2016), implemented in the online program Structure Harvester.

Secondly, the following statistics methods do not implement genetic assumptions; a Principal Component Analysis (PCA) and a Discriminant Analysis of Principal Components (DAPC), performed using the ADEGENET package in R software. The DPCA was evaluated up to K= 35 groups and 3 discriminate analyzes (Jombart & Collins, 2017).

Additionally, the analyzes of molecular variance (AMOVA) were implemented in ARLEQUIN (Excoffier et al., 1992) and were used to detect the source of genetic variability in different hierarchical levels of population structure. Also, this software calculates the F_{ST} and R_{ST} to assess the genetic differentiation among genetic population.

2.2.1.3 Isolation by distance

To assess whether there is a correlation of the genetic differentiation and geographical distance, the estimation of isolation by distance was performed using the mantel test, implemented in the software IBM (Bohonak, 2004). The geographical distance was measured linearly between the geographically referenced sampling points and the genetic distance considered the F_{ST} for each population pair.

2.2.1.4 Genetic variation

To assess genetic diversity, the software of ARLEQUIN v3.11 (Excoffier et al., 2005) was used to calculated the Expected heterozygosity (H_E) and Observed heterozygosity (H_O) under Hardy-Weinberg equilibrium. The genetic parameters such as allelic richness (A_R), and the number of alleles per population (N_A) was determined in the software FSTAT (Goudet, 2001). Additionally, GENALEX 6.1 (Peakall & Smouse, 2012) was used to calculate the number of private allelic (PA) and the frequency of the private allelic per population (Paf).

2.2.1.5 Bottleneck

The presence of a recent bottleneck in a population was determined by the analysis of significant heterozygosity excess using the software BOTTLENECK 1.2. (Cornuet & Luikart, 1996; Piry et al., 1999). The first method implemented in this software was Wilcoxon's signed rank is the most used test because it is powerful and robust in detecting bottleneck with less than 20 polymorphic loci (Piry et al., 1999). A second method, the allele frequency test, determinates whether there is a shift mode in the distribution of allele frequency, which indicates a bottleneck (Piry et al., 1999). Establishing the mutational model before detecting a bottleneck is essential, because it modifies the relationship between the extent of heterozygosity and the number of alleles (Cornuet & Luikart, 1996). The mutational models are: the stepwise mutation models (SMM), and two-phase model mutation (TMP), which define that the size of the alleles is the result of multiple mutational steps with one or more repeat units added or subtracted (Kosman & Jokela, 2019). The TPM model is more recommended for microsatellites using the next parameters: 12 in variance and 5% for multiple mutational step and 95% single-step mutation (Piry et al., 1999).

2.2.1.6 Migration

The Bayesian method for evaluating migration is incorporated in the software BAYESASS, which uses individual genotypes to estimate migration across population (Wilson & Rannala, 2003). The software carried out ten independent runs among all the genetic cluster. Firstly, it ran with the default parameters and then, with 10.000.000 iterations for MCMC, 100000 burin-in and sampling the chain every 1000 iterations. Finally, the seed was modified at random to confirm the same results in all runs.

2.2.1.7 Inbreeding

To know the presence of inbreeding per genetic population, the value of the F_{IS} was calculated using the software ARLEQUIN (Excoffier et al., 1992).

2.2.2 Mitochondrial DNA

2.2.2.1 PCR amplification for the Cytochrome *b* gene (Cyt *b*)

To preliminary evaluate previous genetic variation between sampling localities, 16 samples of the Cyt *b* gen were amplified as follows: three from Paz de Ariporo,

(Casanare), three from the EBTRF, three from Puerto López, (Meta), two from Unillanos (Villavicencio, Meta), four from Puerto Carreño (Vichada) and one from the Rio Humadea, vereda Santa Helena San Martin, (Meta), available in the GenBank of NCBI. The PCR used the primer pairs: Cytb G + mt-E-Rev (Fritz et al., 2006; Spinks et al., 2004) and mt-c2 + mt-f-na (Fritz et al., 2006), which amplified around 1094 pb. The PCR was run in the Eppendorf master cycler, with a final volume of 25 µL, which contains: 12.5 µL of My Tag HS MIX (2X), 0,2 µM of reverse primer of each locus, 0,2 µM of forward primer of each locus, 200 ng of DNA and completed with ppH_2O up to 25 µL. The PCR thermocycle was as follows: Initial denaturing for 5 min at 94°C, followed by 40 cycles with denaturing for 45s at 94 °C, annealing temperature at 53 °C for 45 s and final elongation of at 72 °C for 90s.

2.2.2.2 Analysis data for Cytochrome *b* gene

The Cyt *b* gene was sequenced through the capillary electrophoresis technique, using BigDye Terminator v3.1 of Applied Biosystems done in SSiGMol (Servicio de Secuenciación y Análisis Molecular) in the Instituto de Genética, Universidad Nacional de Colombia, Bogotá. The sequence editing was performed using the software Bioedit and Chromas v2.23 (Technelysium, Australia), and the alignment was done in MEGA 10.2 (Tamura et al., 2011). The mitochondrial Cyt *b* gene coding sequences in *Podocemis vogli* were verified through alignment in Basic Local Alignment Search Tool (BLAST).

The haplotype diversity and nucleotide diversity were estimated using DNASP 5.0 software (Librado & Rozas, 2009). A haplotype network was constructed to reveal the different haplotypes, as well as the degree of differentiation between them and their relation. The parsimony network was done using the algorithm TCS, which infers genealogical information from the nucleotide sequence (Clement et al., 2000) through the software PopART v1.7 (Leigh & Bryant, 2015).

2.3 Results

2.3.1 Microsatellites

2.3.1.1 Population differentiation

Structure Selector revealed two different inferences of population structure comprising five genetic clusters (K=5) through the assessment of MedMeaK, MaxMeaK, MedMedK, MaxMedK and four genetic clusters (K=4) through the analysis of ΔK statistic (Figures 1 & 2). The correspondence between individuals and the two inferences differs mainly that the individuals from Puerto López (Meta) and Unillanos (Villavicencio, Meta) belong to two different cluster genetic where K=5 or one cluster genetic where K=4. In general, for both cases, all individuals from San Martin (Meta), 13 individuals from Puerto Carreño (Vichada) and all individuals from Paz de Ariporo (Casanare) + two individuals from Puerto Carreño (Vichada) conformed three different genetic clusters.

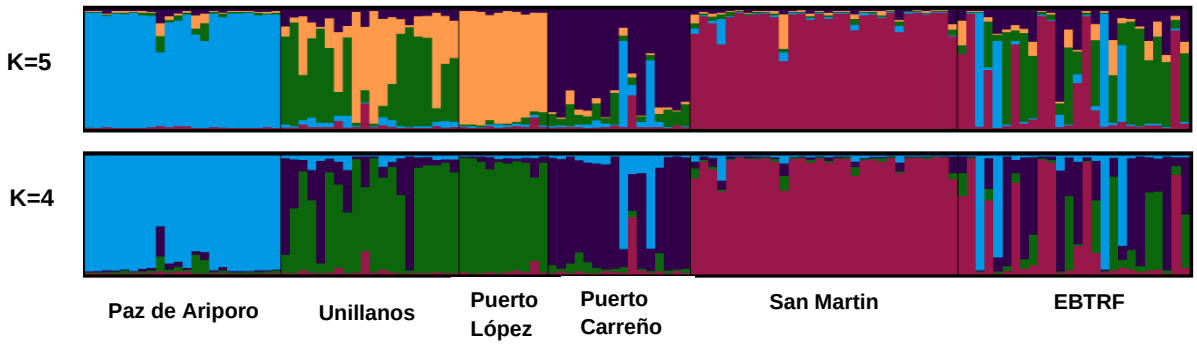


Figure 1. Barplot generated by Structure Selector, showing four clusters (K=4) and five (K=5) inferred from the of ΔK statistic and from MedMeaK, MaxMeaK, MedMedK, MaxMedK. This analysis was based on ten loci of unlinked microsatellite. Each individual from six collected localities is represented by a bar with a subdivision into k color corresponding to the estimated membership. EBTRF: Estación de Biología Tropical Roberto Franco.

Particularly, the individuals in captivity from Estación de Biología Trópical Roberto Franco presented high estimation of membership for the four different genetic cluster (Figure 1). For K=5, four individuals presented a greater ancestry for cluster I (Paz de Ariporo), 12 individuals for cluster II (Unillanos), one individual for cluster IV (Puerto Carreño) and nine individuals for cluster V (San Martin). In contrast, for K=4, four individuals have the highest inferred ancestry value for cluster I (Paz de Ariporo), five individuals for cluster II

(Unillanos and Puerto López), nine individuals for cluster III (Puerto Carreño) and eight for cluster IV (San Martin) (Table 1).

Table 1. The inferred ancestry values of 26 individuals from Estación de Biología Trópical Roberto Franco. Values of Q in bold are the highest inferred ancestry values per cluster and individual.

Individual	Inferred Ancestry Value at K=5						Inferred Ancestry Value at K=4			
	Q1	Q2	Q3	Q4	Q5		Q1	Q2	Q3	Q4
1	0.005	0.070	0.218	0.088	**0.618**		0.006	0.193	0.133	**0.667**
2	0.007	0.011	0.008	0.009	**0.964**		0.008	0.009	0.012	**0.971**
3	**0.960**	0.008	0.010	0.009	0.014		**0.965**	0.010	0.008	0.016
4	0.018	0.400	0.040	0.050	**0.491**		0.025	0.093	0.264	**0.617**
5	**0.722**	0.039	0.012	0.221	0.006		**0.843**	0.015	0.136	0.006
6	0.009	**0.817**	0.025	0.127	0.023		0.010	0.034	**0.928**	0.028
7	0.034	0.086	0.041	0.124	**0.715**		0.040	0.074	0.109	**0.777**
8	0.015	**0.787**	0.031	0.123	0.043		0.015	0.074	**0.863**	0.047
9	0.009	**0.566**	0.108	0.241	0.075		0.012	0.238	**0.675**	0.075
10	0.010	0.020	0.010	0.010	**0.950**		0.010	0.013	0.015	**0.961**
11	0.009	0.048	0.013	0.022	**0.908**		0.009	0.023	0.033	**0.935**
12	0.098	0.048	0.018	**0.824**	0.012		0.127	0.011	**0.846**	0.015
13	0.013	**0.707**	0.126	0.130	0.025		0.015	**0.613**	0.339	0.033
14	0.079	0.163	0.012	0.345	**0.402**		0.048	0.013	**0.703**	0.235
15	0.014	0.019	0.010	0.014	**0.943**		0.015	0.009	0.029	**0.947**
16	0.038	**0.595**	0.128	0.091	0.148		0.046	**0.418**	0.354	0.181
17	**0.949**	0.014	0.008	0.013	0.016		**0.957**	0.011	0.013	0.020
18	0.021	**0.427**	0.279	0.240	0.032		0.019	**0.799**	0.154	0.027
19	**0.669**	0.174	0.043	0.063	0.051		**0.760**	0.063	0.118	0.059
20	0.013	**0.912**	0.014	0.037	0.024		0.023	0.144	**0.795**	0.039
21	0.007	**0.874**	0.016	0.088	0.014		0.009	0.053	**0.920**	0.018
22	0.013	**0.598**	0.138	0.223	0.028		0.015	**0.646**	0.306	0.034
23	0.015	**0.811**	0.099	0.048	0.026		0.026	**0.651**	0.281	0.043
24	0.007	**0.585**	0.051	0.351	0.006		0.007	0.027	**0.960**	0.006
25	0.013	0.074	0.038	0.045	**0.830**		0.015	0.074	0.073	**0.838**
26	0.014	**0.615**	0.083	0.245	0.043		0.017	0.416	**0.511**	0.056

A) K=5 **B) K=4**

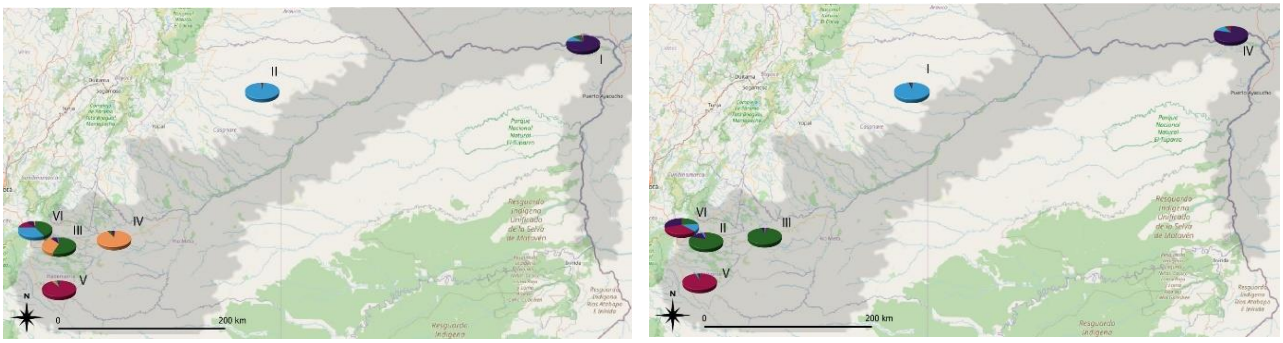


Figure 2. Estimated membership of the four and five genetic cluster represented by a pie chart. Paz de Ariporo, Casanare (I), Unilllanos, Meta (II), Puerto López, Meta (III) and Puerto Carreño, Vichada (IV), San Martin, Meta (V) EBTRF, Meta (VI). Distribution of the species in gray (sensu Rhodin et al 2021).

Likewise, the scatter plot of PCA revealed that the general variation is described between individuals from the region of Casanare (Paz de Ariporo), Meta (Unillanos, EBTRF, Puerto López) and Vichada (Puerto Carreño) (Figure 3A). Therefore, the mayority of individuals from Meta presented a lower differentiation. However, the fourth cluster were statistically supported by the DPCA (Figure 3B and 3C) with similar distribution of the individuals of the clusters defined by STRUCTURE.

The AMOVA test including the five clusters revealed a significant variation of 11.71% attribute to differences in the allelic frequency among population (F_{ST} = 0.11708; P-value < 0.001). This result was similar from K=4 (F_{ST} = 0.11140; P-value < 0.001) (Supplementary Table S1). Additionally, the amount of differentiation between all pairs of clusters with both Ks, the values of F_{ST} and R_{ST} revealed a relatively low but statistically significant differentiation (P-value< 0.05) (Table 2). Notably, the genetic population of San Martin present the most genetic differentiation with both Ks (F_{ST}>0.1248; P-value<0.001). In contrast, with K=5, Unillanos (III) and Puerto Lopez (IV) had the lowest F_{ST}.

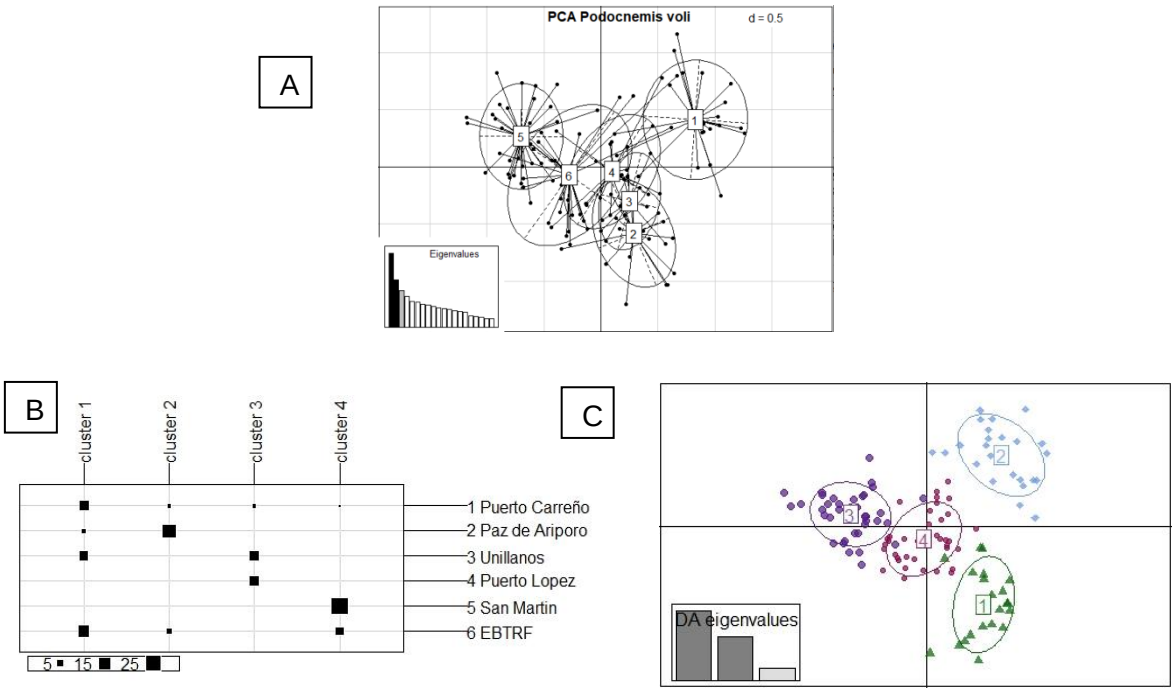


Figure 3. A) Scatter plot of the Principal Component Analysis (PCA) for the genetic differentiation of *Podocnemis vogli* across ten microsatellite loci for the five collecting sampling: Casanare (1), Unilllanos (2), EBTRF (3), Puerto López (4) and Puerto Carreño (5). B) Correspondence between the collected samples with the cluster inferred using Discriminate Principal Component Analysis (DPCA). The squares correspond to the number of individuals which below the inferred cluster. C) Scatter plot of the DPCA showing the four inferred clusters.

Table 2. Pairwise F_{ST} values (below diagonal) and Pairwise R_{ST} values (above diagonal) between four cluster of *Podocnemis vogli*. Values in bold were statistically significant (P-value <0.05).

	Puerto Carreño	***Paz de Ariporo***	***Unillanos***	***Puerto López***	***San Martin***
K=5					
Puerto Carreño	-	**0.1797**	**0.0713**	**0.0906**	**0.1559**
Paz de Ariporo	**0.0973**	-	**0.1730**	**0.2054**	**0.3584**
Unillanos	**0.0901**	**0.0875**	-	**0.1559**	**0.3558**
Puerto López	**0.0805**	**0.0790**	**0.0541**	-	**0.1559**
San Martin	**0.1318**	**0.1641**	**0.1642**	**0.1248**	-
K=4					
	Puerto Carreño	**Paz de Ariporo**	**Unillanos + Puerto López**	**San Martin**	
Puerto Carreño	-	**0.2063**	**0.0510**	**0.1997**	
Paz de Ariporo	**0.0993**	-	**0.1154**	**0.3462**	
Unillanos + Puerto López	**0.0583**	**0.0635**	-	**0.1899**	
Puerto Carreño	**0.1300**	**0.1607**	**0.1272**	-	

2.3.1.2 Isolation by Distance

There is no evidence of correlation between the geographical distance and the genetic distance for five genetic population (Z=-368159.75, r=-0.1094, P-value=0.490) and four genetic population (Z=-3498170.73, r=-0.0184, P-value=0.387). Thus, the geographical distance does not explain the genetic differentiation between the genetic populations.

2.3.1.3 Bottleneck

In general, the genetic clusters presented between 6 and 8 loci with an excess of heterozygosity. The Wilcoxon test were significative for the genetic clusters of Puerto Carreño (Cluster I) and Paz de Ariporo (Cluster II) (P-value <0.05) for K=5 y K=4, indicating the presence of a recent bottleneck (Table 3). Additionally, the allele frequency test showed a presence of bottleneck in all genetic populations; however, this result must be taken with caution due to the low number of samples for each population.

Table 3. Bottleneck results following the model of microsatellite evolution two phase model (TPM) for all alleles in the four cluster and five cluster. The significant value (P-value < 0.05) for the Wilcoxon test in bold. P: Probability of no significant heterozygosity excess; LH_{exc}: number of loci with heterozygosity excess.

	Sign test		***Wilcoxon test***	***Test of Allele frequency***
Cluster	***Obs LH_{exc}***	**Exp *LH_{exc}***	**P**	
K=5				
Puerto Carreño	7	5	**0.010**	Shifted
Paz de Ariporo	7	5.63	**0.042**	Shifted
Unillanos	8	5.76	0.080	Shifted
Puerto López	7	5.67	0.116	Shifted
San Martin	6	4.99	0.180	Shifted
K=4				
Puerto Carreño	7	4.92	**0.018**	Shifted
Paz de Ariporo	7	5.63	**0.042**	Shifted
Unillanos + Puerto López	7	5.76	0.138	Normal
San Martin	6	5.02	0.180	Shifted

2.3.1.4 Migration

Table 4. Estimation of migration rate among four and five genetic cluster in *Podocnemis vogli.*

	Puerto Carreño	***Paz de Ariporo***	***Unillanos***	***Puerto López***	***San Martin***
K=5					
Puerto Carreño	0.824(0.985)	0.106(0.099)	0.021(0.020)	0.025(0.025)	0.024(0.021)
Paz de Ariporo	0.013(0.012)	0.949(0.023)	0.012(0.012)	0.012(0.012)	0.013(0.013)
Unillanos	0.021(0.019)	0.179(0.090)	0.718(0.040)	0.063(0.088)	0.020(0.019)
Puerto López	0.019(0.019)	0.154(0.101)	0.016(0.016)	0.733(0.096)	0.077(0.073)
San Martin	0.010(0.010)	0.012(0.011)	0.010(0.009)	0.011(0.010)	0.957(0.019)
K=4					
	Puerto Carreño	**Paz de Ariporo**	**Unillanos + Puerto López**	**San Martin**	
Puerto Carreño	0.737(0.068)	0.190(0.085)	0.053(0.053)	0.020(0.019)	
Paz de Ariporo	0.013(0.012)	0.959(0.022)	0.014(0.013)	0.014(0.013)	
Unillanos + Puerto López	0.013(0.013)	0.148(0.113)	0.780(0.128)	0.059(0.051)	
Puerto Carreño	0.010(0.009)	0.011(0.011)	0.031(0.017)	0.949(0.021)	

A) K=5 **B) K=4**

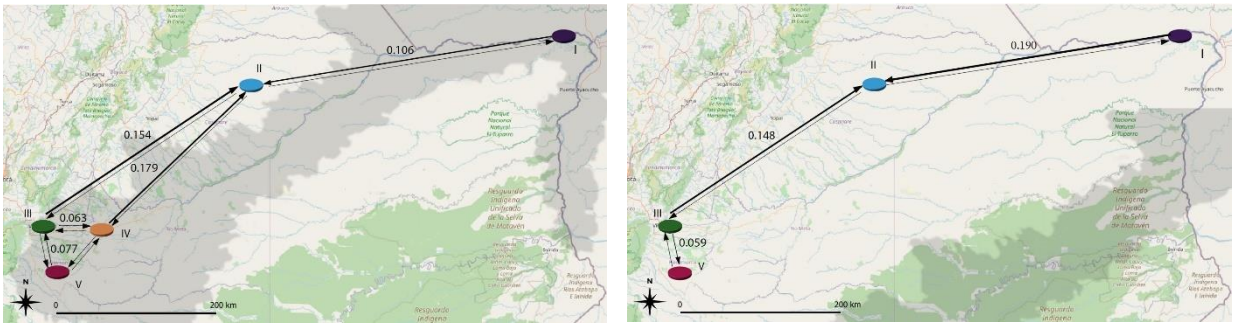


Figure 4. Representation of the migration rate among five (A) and five genetic cluster (B) in *Podocnemis vogli.* Puerto Carreño, Vichada (I), Paz de Ariporo, Casanare (II), Unilllanos, Meta (III), Puerto López, Meta (IV) and San Martin (V).

The migration rate among four and five genetic clusters was low and asymmetric in most cases (Table 4). The highest migration rate, above 0.1, was directed to the cluster II (Paz de Ariporo), where K=4 and K=5 (Figure 4), indicating a unidirectional flow of individuals moving through this cluster and the presence of source-sink relationship.

2.3.1.5 Inbreeding

There is no evidence of inbreeding with F_{IS} value negative and not significative (P-value > 0.05) in most of the genetic population for the four cluster and five cluster, indicating an excess heterozygosity presented in each genetic population. Except for the cluster in Unillanos in the analysis for five cluster with F_{IS} of 0.012 (P-value < 0.05).

2.3.1.6 Genetic diversity

The ten unlinked loci of microsatellites with no significant deviations of Hardy-Weinberg proportions after Bonferroni correction revealed important genetic information (Table 5). The allelic diversity described by the allelic richness and the number of alleles per cluster was low with a mean of 4.48 number of alleles and 3.87 of allelic richness for five cluster and 5.07 and 4.79 for four clusters, respectively. Furthermore, the observed heterozygosity was on average 0.638 and the expected heterozygosity was 0.554 for both Ks, indicating a high heterozygosity. Remarkably, the highest number of private alleles was found in the cluster of Puerto Carreño.

Table 5. Genetic parameters inferred from ten microsatellites. Sample size (N), mean number of alleles (N_A), allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), number of private allele (PA), private alleles frequency range (PAf), P-value > 0.05 for all values of F_{IS}.

CLUSTER	Microsatelites (nADN)							
	N	N_A	A_R	H_O	H_E	PA	Paf	F_{IS}
K=5								
Puerto Carreño (I)	14	4.5	4.3	0.661	0.536	7	0.058	-0.343
Paz de Ariporo (II)	24	4.3	3.5	0.581	0.528	5	0.067	-0.090
Unillanos (III)	12	4.2	4.0	0.638	0.600	3	0.056	0.012
Puerto López (IV)	18	4.7	4.06	0.741	0.624	5	0.067	-0.202
San Martin (V)	30	4.7	3.5	0.567	0.480	5	0.065	-0.222
Total	98					25		
Mean		4.48	3.87	0.638	0.554	5	0.063	
K=4								
Puerto Carreño (I)	16	5.7	5.34	0.646	0.588	11	0.051	-0.161
Paz de Ariporo (II)	24	4.3	5.66	0.581	0.528	5	0.667	-0.123
Unillanos (III)	27	5.4	4.40	0.720	0.621	5	0.044	-0.155
San Martin (V)	31	4.9	3.77	0.565	0.479	5	0.061	-0.202
Total	98					26		
Mean		5.07	4.79	0.628	0.554	6.5	0.206	

2.3.2 Cytochrome *b*

The Cyt *b* sequences were of 1094 pb without any stop codon along the translated sequence. The alignments were obtained from 16 sequences, which revealed five distinct haplotypes slightly differentiated between two to three mutational steps (Table 6; Figure 3). The parsimony network revealed one shared haplotype in individuals from Puerto López (Meta), Unillanos (Villavicencio, Meta), Puerto Carreño (Vichada) and Paz de Ariporo (Casanare). Furthermore, differentiated, and exclusive haplotypes were revealed in individuals from Paz de Ariporo (Casanare), Puerto Carreño (Vichada), San Martin (Meta) and EBTRF; one individual for each locality.

Table 6. Genetic parameters inferred from 16 sequences of Cyt *b.* Sample size (N), number of haplotypes (h) haplotype diversity (hd), nucleotide diversity (p), haplotypes (H).

		Cytochrome b (mtDNA)			
Locality	**N**	**H**	**hd**	**Pi**	**H**
Unillanos, Meta	2	1	0	0	2
EBTRF, Meta	3	2	0.667	0.0005	2, 3
Puerto López, Meta	3	1	0	0	2
Paz de Ariporo, Casanare	3	2	0.667	0.0001	2, 5
Puerto Carreño, Vichada	4	2	0.5	0.0004	2, 4
San Martín, Meta	1	1	-	-	1
Total	**16**				

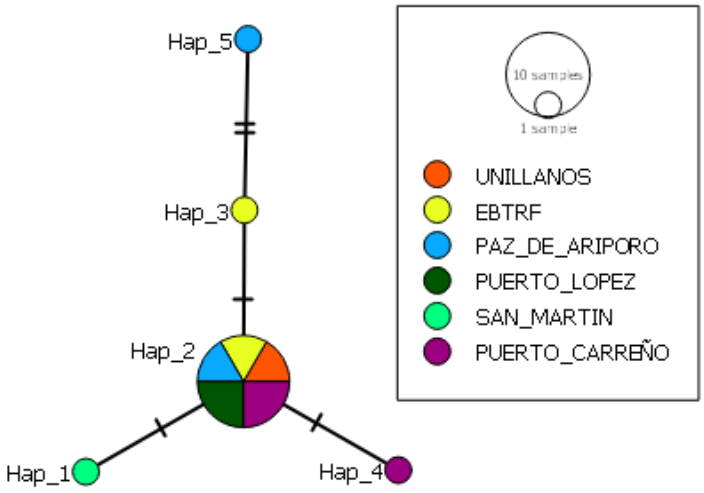


Figure 3. Parsimony haplotype network for sequences of Cyt *b* of *Podocnemis vogli*. The circles represent each haplotype and the lines connecting each haplotype correspond to mutational steps. Circle size shows the haplotype frequency, and each color indicates the collected sampling. Estación de Biología Tropical Roberto Franco (ETBRF).

2.4 Discussion

Evidence of past and present population structure along the different river drainages

The STRUCTURE analyses reveled a recent population structure formed by the five sampling regions which represent five different genetic population (K=5) or four genetic populations where most individuals from Puerto López and Unillanos (Meta) are part of a unique genetic population (K=4). Additionally, there is low but statistically significant differentiation between the populations (K=5: F_{ST}=0.1170, P-value<0.001; K=4: F_{ST}=0.11140, P-value<0.001). For K=5, the lowest differentiation was presented between the cluster from Puerto López and Unilllanos with an F_{ST}=0.0541 and a high admixture of genetic pool of Puerto Lopez in Unillanos, which for K=4 conform one group. This result could be explained by the proximity of the geographic location. However, the database had an uneven sampling which could deal with the assessment of MedMeaK, MaxMeaK, MedMedK, MaxMedK (Puechmaille, 2016), which give a result of K=5. This could contribute for a better approximation for the clusters.

On the other hand, the Cyt *b* provides useful information about genetic diversity, genetic structure and genetic differentiation (Castilla et al., 1998; Patwardhan et al., 2014). Vargas-Ramírez et al. (2017) inferred from sequences of Cyt *b* that *Podocnemis lewyana* presented low genetic variation with only two haplotypes along their distribution range in Colombia. In this study, the analysis of Cyt b gene in *Podocnemis vogli* preliminary revealed a low genetic diversity and weak but present past genetic differentiation. The haplotype network showed the presence of exclusive haplotypes for the geographic populations from Puerto Carreño, San Martin, Paz de Ariporo and ETBRF. However, a common haplotype shared by all localities except from San Martin was also detected. We do not reject the possibility that this locality also presents the same haplotype since only one sequence from this locality was analyzed. The presence of private haplotypes from different populations revealed by the preliminary analysis of the mtDNA marker, suggest that the species had population structure for a long time.

Detection of populations decline due to anthropic effects

The generation of the recent genetic clusters can be influenced by three important aspects. 1) The movement capacity of the specie: this species present low displacement around 5 km per month in a short area close to floodplain savannahs and creeks, as reported by the first and only radio detection study for this species (Pinzón Arias et al., 2017), which would imply the low displacement of them along their distribution.

2) The characteristic geographic of the Orinoquia Region: this region in Colombia presents a great extension of 34.720.820 ha, composed of a savanna and a water network of rivers, streams, pipes and lakes, which flux to East of Colombia and is the habitat of *Podocnemis vogli*. The natural barriers as big rivers as the Meta River can contribute to their genetic differentiation and low migration between population (Manel et al., 2003) due to individuals are seldom seen in the big rives. However, the streams, pipes and lakes hydrological system may allow migration. On the other hand, geographic distance did not explain the genetic differentiation (P-value > 0.05).

3) Finally, the human intervention: the presence of physical barriers such as roads, cities, buildings, the deterioration of their habitat and the traffic trade can reduce population, the migration rate and displacement the turtle to small areas. The detection of the major and asymmetrical migration rate with a source-sink relationship ($m < 0.106$; K=4 and K=5) to the genetic populations of Paz de Ariporo from Puerto Carreño, Puerto Lopez and Unillanos could be explained by the traffic trade.

Furthermore, there is evidence of bottlenecks in the genetic population of Puerto Carreño (K=5: A_R=4.3, H_O= 0.661; K=4: A_R=5.34, H_O= 0.646) and Paz de Ariporo (K=5: A_R=3.5, H_O= 0.581; K=4: A_R=5.66, H_O= 0.581) with low allelic richness in contrast with the observed and expected heterozygosity. The reduction of allelic diversity faster than the heterozygosity is a sign of presence of bottlenecks (Cornuet & Luikart, 1996). The presence of the bottleneck is most probably the consequence of deep anthropogenic effects in P*odocnemis vogli* such as alteration and destruction of their natural habitat and the removal of individual as food and economic exploitation.

Previous genetic evaluations in threatened continental chelonian species, revealed different levels of genetic diversity and genetic structure (Table 7). In species related to

Podocnemis vogli, the most extreme case was identified in *Podocnemis lewyana,* with the lowest genetic diversity and heterozygosity (A_R=2.24; H_O=0.33) (Vargas-Ramírez et al., 2011). In contrast, high genetic diversity has been identified in *Podocnemis unifilis (*A_R= 7.25; H_O= 0.69) and *Podocnemis expansa* (A_R=6.4; H_E=0.75, H_O=no data) (Escalona et al., 2009; Pearse et al., 2006). Despite that *Podocnemis vogli* is believed to be more abundant and less threatened than those species, presented less allelic richness and observed heterozygosity (K=5: A_R=3.87, H_O=0.638; K=4: A_R=4.79, H_O=0.628).

Table 7. Genetic parameters inferred from microsatellite in *Podocnemis vogli* and other continental chelonian species (from Vargas-Ramírez et al., 2011). N sample size: H_O observed heterozygosity; H_E unbiased expected heterozygosity; A_R allelic richness; h haplotype diversity; p nucleotide diversity.

TAXON	Region		*N*	Loci	H_O	H_E	A_R	Source
Astrochelys radiata	Madagascar		323	13	0.76	0.80	8.90	Rioux Paquette et al. (2009)
Emydoidea blandingii	Nova Scotia, Canada		129	5	0.55	0.55	8.00	(Mockford et al., 2005)
Emys orbicularis	Western, central and southern Europe		382	8	0.63	0.72	5.15	(Pedall et al., 2011)
Emys trinacris	Sicily, Italy		31	8	0.55	0.68	4.86	(Pedall et al., 2011)
Gnpherus berlandieri	Texas, USA		138	8	0.47	0.52	5.21	Fujii and Forstner (2010)
Graptemys geographica	Ontario, Canada		109	5	0.65	0.63	4.15	(Bennett et al., 2010)
Malaclemys terrapin	Gulf and Atlantic coasts, USA		320	6	0.76	0.79	7.44	(Hauswaldt & Glenn, 2005)
Podocnemis unifilis	Amazon and Orinoco Basins		312	5	0.69	0.70	7.25	(Escalona et al., 2009)
Terrapene ornate	Illinois, Nebraska, USA		74	11	0.57	0.72	9.27	(Kuo & Janzen, 2004)
Chelonoidis nigra vandenburghi	Galápagos Islands, Ecuador		162	10	0.60	0.68	8.7	(Beheregaray et al., 2003)
Emys orbicularis	Spain		261	7	0.72	0.71	5.27	(Velo-Antón et al., 2008)
Macrnchelys temminckii	Mississippi River, USA		195	7	0.36	0.37	2.38	(Echelle et al., 2010)
Podocnemis expansa	Amazon and Orinoco Basins		453	9	—	0.75	6.4	(Pearse et al., 2006)
Podocnemis lewyana	Colombia		147	10	0.33	0.32	2.24	(Vargas-Ramírez et al., 2011)
Podocnemis vogli	**Orinoco Basins in Colombia**	**K=5**	**98**	**10**	**0.64**	**0.55**	**3.87**	**This study**
		K=4	**98**	**10**	**0.63**	**0.55**	**4.79**	

The values of heterozygosity from the microsatellite data revealed a high level of genetic diversity in *Podocnemis vogli* but the allelic richness was low compared with most of the

species described in the Table 7. Additionally, this situation is also presented in other family of turtles with serious declination of their population such as *Astrochelys radiata* (A_R=8.90; H_O=0.75), *Chelonoidis nigra* (A_R=8.7; H_O=0.6) and *Macrochelys temminckii* (A_R=2.38; H_O=0.36). *Podocnemis vogli* presents similar results in its genetic diversity with the specie *Graptemys geographica* from Ontario, Canada (A_R= 4.15; H_O= 0.65). In this case, the specie showed no genetic differentiation despite the evidence of barriers to the gen flow (Bennett et al., 2010).

Conservation implications

The five identified genetic population of *Podocnemis vogli* correspond to Management units (MU) for conservation. This information is important because it can identify priority ecosystems for the long-term conservation of this species.

Using the evaluated microsatellite loci allowed us to infer the genetic identity of the 26 individuals from the *exsitu* population present at the EBTRF. Consequently, those captive individuals can be released into the natural population in almost all population except in Puerto Lopez (Table1). There are reports from local news that hundreds of individuals of *Podocnemis vogli* are transferred and released in other geographic locations to restore population (SuVersión, 2017; Violeta, 2020). Recently, 100 individuals have released in the Reserve La Esperanza, Caño Chiquito in Casanare in 2017 (SuVersión, 2017) and 500 individuals from Paz de Ariporo in Casanare were released in the Macuate Nature Reserve, Caño Negro in Arauca at the end of 2020 (Violeta, 2020). However, this conservation program did not consider the genetic composition of the individuals. The microsatellite system developed in this study constitute a powerful tool not only to evaluate many more populations of *P. vogli* throughout the species distribution range, but to assign the population of origin of the captive individuals present in reception centers.

At present, *Podocnemis vogli* are categorized as Low concern (LC) at the regional level (Morales-Betancourt, Lasso, et al., 2015), but at global conservation has not been evaluated yet (IUCN, 2021). However, the International Union for Conservation of Nature Tortoise and Freshwater Turtle Specialist Group (TFTSG) has been listed as Vulnerable (VU) (Rhodin et al., 2018). In addition, this species is regulated under Appendix II of the Convention on International Trade for Endangered Species (CITES, 2019). Therefore, we

suggest a new evaluation and designation of this species into a new threat categorization species at the regional level, considering the genetic results such as the presence of genetic structure and recent bottleneck which indicate population reduction due to anthropic factors.

3. Conclusions

This research project constitutes the first population genetic assessment for *Podocnemis vogli*.

- In Chapter 1, a ten microsatellite loci system was methodically standardized to be used for a subsequent population genetics evaluation.
- In chapter 2, the population genetics evaluation was performed using the microsatellite system as well as Cyt *b* sequences mtDNA and 98 individuals from five sampling localities covering a great area of the species distribution range and 26 individuals in captivity.
- Present and past Genetic structure was revealed using the ten microsatellite loci and the Cyt b sequences, respectively. The genetic structure comprised five populations matching four sampling localities. Furthermore, intermediate to high genetic diversity was also identified for those populations, but low allelic richness. This results plus the identification of bottlenecks in two populations, indicate that the assessed populations show deep signatures of population decline most probably caused by human intervention. Asymmetrical and low migration between populations was also identified. Additionally, by using the ten microsatellite loci a successful population assignation of the 26 individuals from the ex-situ population at the EBTRF was performed.

Based on those results, the following conservation and management guidelines for the species are proposed:

1. A reassessment of the regional threat category should be urgently performed.
2. Four Independent Management Units (sensu Morritz, 1994) for conservation should be stablished to preserve the already identified genetic diversity.
3. The environmental corporations at the studied localities should be informed about the species‘ nowadays situation. In coordination with them, an objective and focused environmental education campaign should be urgently set up, to communicate that populations of the species are declining and to stablish agreements for community-based conservation initiatives.

4. In coordination with environmental regional corporations, further assessment of the power of the microsatellite system in evaluating the probable population of origin of confiscated individuals should be carried out.

Finally, to complete the population genetic evaluation for *P. vogli* aiming at improving the understanding of the genetic dynamics of the species throughout its entire distribution range, it is crucial to analyze many more samples from more localities in Colombia and Venezuela.

A. Supplementary table 1: Hierarchical Analyses of Molecular Variance for five genetic clusters.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among population	4	52.324	0.28371 Va	11.71
Within individuals	193	412.918	2.13947 Vb	88.29
Total	197	465.242	2.42319	
Fixation Indices	FST=0.11708			

B. Supplementary table 2: Hierarchical Analyses of Molecular Variance for four genetic clusters.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among population	3	45.640	0.27092 Va	11.14
Within individuals	192	414.942	2.16115 Vb	88.86
Total	195	460.582	2.43208	
Fixation Indices	FST= 0.11140			

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