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Plasma protein markers for estimating the population genetic structure of Amazon turtle (*Podocnemis expansa* Schweigger, 1812)

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ABSTRACT:

This study aims to test the potential of allelic frequency distributions of plasma proteins analysed by starch gel electrophoresis for identifying Amazon turtle (*Podocnemis expansa* Schweigger, 1812) stocks. Three proteins: transferrin (Tf), albumin (Alb) and general protein (GP), and four enzymes: esterase (EST), phosphoglucomutase (PGM), malate dehydrogenase (MDH) and superoxide dismutase (SOD) from three geographical areas in the Amazon region, totalizing three population samples: 1) Maracarana, Rio Uatumã-AM, 2) Trombetas, Rio Trombetas-PA and 3) Monte Cristo, Rio Tapajós-PA, were tested. Out of 11 presumptive loci detected, seven were monomorphic (*GP-1*, *GP-2*, *EST-1*, *EST-2*, *PGM-1*, *SOD-1* and *SOD-2*) and four polymorphic (*Tf*, *Alb*, *MDH* and *PGM-2*). The chi-square (X^2) analysis for testing the hypothesis of independent segregation between pairs of polymorphic loci in the population samples, revealed no statistically significant differences. Based on Wright's *F*-statistics (F_{IS}, F_{IT} and F_{ST}) the detected average value of F_{IS} = 0.1347, indicates a moderate inbreeding within the population samples, whereas the average value of F_{IT} = 0.1912, indicates a moderately great inbreeding in the total population sample.The detected average value of F_{ST} = 0.0652, indicates a moderate genetic differentiation among the population samples. The pairwise comparisons of F_{ST} point to a moderate differentiation between all comparisons made with the Monte Cristo population sample (F_{ST} ranging from 0.061 to 0.066); but very little differentiation was detected between Maracarana and Trombetas samples (F_{ST} = 0.0035). A limited gene flow among the population samples was detected (*Nm* = 3.58). The UPGMA dendrogram showed the lowest genetic distance between population samples of Maracarana and Trombetas, whereas, the highest genetic distances were detected when these two samples were compared to that of Monte Cristo. Exact test for population differentiation revealed high levels of statistically significant differences in all pairwise comparisons made with the Monte Cristo sample. The data point out a possible existence of distinct subpopulations "stocks" of *P. expansa* in the sampled area, where Monte Cristo sample was always genetically different compared to the other two population samples examined.

Keywords:
Podocnemis expansa, Amazon Basin, plasma proteins, eletrophoresis, population genetic structure.

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INTRODUCTION

Podocnemis expansa (Schweigger, 1812), popularly known as giant Amazon river turtle or also as Amazon turtle in the Brazilian Amazon Basin, belongs to the order Testudines, suborder Pleurodira, family Podocnemididae (Bickham *et al*., 2007). Among the 14 species of turtles existing in the Amazon region, *P. expansa* is recognized to be the largest freshwater turtle of the genus *Podocnemis* in South America, being quite exploited commercially by man (Alho, 1985, Vogt, 2008). Females of this species can measure 50 to 89 cm long, 60 cm wide (Alfinito, 1975; Pritchard and Trebbau, 1984; IBAMA, 1989) and weigh 15 to 80 kg (Mittermeier, 1978; Alho and Pádua, 1982; Pritchard and Trebbau, 1984; IBAMA, 1989). The Amazon turtle is widely distributed and it can be found in the Rios Araguaia and Tocantins (Mittermeier *et al.*, 1980; Ferreira Júnior and Castro, 2003), mouth of the Rio Amazonas, and at least up to Rios Marañón and Morona, in Peru. The geographical distribution of this species also covers the Rios Orinoco and Essequibo, both in Venezuela (Mittermeier *et al.*, 1980; Pritchard and Trebbau, 1984; Iverson, 1992), also including Rios in Bolivia, Colombia and Guyana. In Ecuador, there has been a decrease in the number of *P. expansa* at the Rio Tiputini (Cisneros-Heredia, 2006).

According to Gilmore (1986), the capture of Amazon turtle can be considered the most important ethnozoological activity throughout the Amazon region, back from the pre-Columbian times up to today.

Many adventurers and travelers who roamed South America in the centuries that followed its discovery highlighted several natural spectacles of the Amazon, among which the most impressive were the major agglomerations of the Amazon turtle in large beaches of the Amazon and Orinoco River basins. The spawn of Amazon turtle in large groups, with tens of thousands of females crawling up to the beaches was at the same time a faithful record of the abundance and exuberance of the turtle populations of the New World (Ferreira Junior, 2003).

After European occupation of the Amazon, the consumption of Amazon turtle as subsistence activity turned into an extractive production system with characteristics of mercantilism (1700-1900 approximately), where, the main products were butter and oil produced from the eggs, both used for frying food, household and public lighting (Bates, 1863; Smith, 1974). At that time, the eggs were crushed in a canoe to break the shells. Afterwards, water was added to it making a mixture that was left exposed to the sun for several hours in order to extract the oil floating on the surface. The oil was skimmed off together with shells and boiled before being stored in clay pots (Smith, 1974; 1979; Redford and Robinson, 1991), while, the fat extracted by this process, was mixed with the resin, and used to caulk boats (Smith,1974; 1979). In the eighteenth century, *P. expansa* used to spawn in some beaches near the town of Itacoatiara, State of Amazonas, Brazil, where, due to commercial interests of the Portuguese these places started being named as Royal beach (Pesqueiro Real das Tartarugas). The meat of the Amazon turtle was destined for local consumption, supplying only the regional market and troops of the Province of Rio Negro in Barcelos, State of Amazonas, Brazil (for details see Smith, 1979).

By 1980s, there was a change in the human consumption of the Amazon turtle meat, where this product, which was considered a main item and source of protein for local people, began also to be consumed as a delicacy in the Amazonian people's diet (Alho, 1985). In Manaus, capital city of the State of Amazonas, Brazil, the so-called "tartarugada", a banquet of Amazon turtle meat prepared in different ways, is very common on important occasions. Thus, exploitation, illegal trade and consumption of turtles are socially important for the higher social class, and economically important for the lower social class who catches these animals to supply

the demand from the higher social class (Alho, 1985). In Belém, capital city of the State of Pará, Brazil, in addition to the trade of fat and derivates, the consumption of meat and eggs from the Amazon turtle, is also practiced (Alves and Santana, 2008).

Other by-products, such as shells, fat and viscera of the Amazon turtle are much appreciated, and used in making medicinal ointments, soaps, cosmetics, household items and adornments (Smith, 1974, 1979; Alho, 1985; IBAMA, 1989; Pantoja-Lima, 2007; Alves and Santana, 2008). The Amazon turtle meat besides being consumed as a local delicacy has also been looked for in restaurants (Wetterberg *et al*., 1976; Ferrarini, 1980; Redford and Robinson, 1991).

After several decades of intense exploitation, *Podocnemis expansa* had already been considered as endangered species by the criteria of the International Union for Conservation of Nature and Natural Resources (IUCN). Today, due to protection programmes implemented by the Brazilian federal government, and led by Chico Mendes Institute for Biodiversity (ICMBIO) and the Center for Conservation and Management of Reptiles and Amphibians (RAN) in the Amazon region, this species is no longer threatened. However, this species still shows a low risk of extinction, therefore, it is considered a conservation dependent species according to the IUCN criteria. The *Podocnemis* species are listed in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), published as Annex II, Normative Instruction Number 11/2005, available at the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA).

Protein and isozyme electrophoresis has long been used as a powerful molecular tool for species identification, population genetic structure, conservation and management genetics of plants and animals (Ferguson, 1980; Ferguson *et al.*, 1995, Scaltsoyiannes, 1999, Zeidler, 2000, Ridgway, 2005; Verspoor *et al*., 2005; Sujatha *et al*., 2011). Protein and isozyme molecules known as genetic markers are encoded by structural genes, which play the role of determining the precise sequence of amino acids in their polypeptide chains. Thus, whenever these molecules are electrophoretically analysed, the bands visualized in the gels are assumed as direct products of gene action. These genetic markers when extracted from various biological tissues of animal and vegetable origin (muscle, heart, liver, whole blood, blood plasma, hemolymph, seed, root, leaf, among others) and then analysed by electrophoresis, reveal protein banding patterns which can provide valuable information, primarily regarding the correct identification of species, natural and artificial stocks (Ferguson, 1980; Ferguson *et al.*, 1995). Among the most diverse biological molecules, proteins are essential for the expression of hereditary information. There are tens of thousands of genes coding for unique polypeptide chains, which, either solely or in combined forms, generate an indefinitely great number of protein variants, a variety further embellished by allelic diversity. So far, protein electrophoresis has provided the main source of insight into the genetic character of species in the wild (Verspoor *et al*., 2005).

Protein electrophoresis detects genetic variation that arises from amino acid substitutions generated by base sequence variation. These substitutions alter the charge state or conformational character of the protein and change its mobility when placed in a gel electrophoresis matrix subjected to an electrical field. However, protein electrophoresis presents some limitations, such as not every DNA sequence variation that leads to amino acid changes, not all amino acid changes are detectable by this technique. Moreover, electrophoresis screening can detect only a part of the amino acid sequence variation, which might be present, amino acid variation represents only part of the variation in the DNA, protein-coding regions represent only a small part of the overall genome, only one third of amino

acid substitutions and one tenth of DNA base substitutions will be detected by this method. Despite these limitations, protein electrophoresis analyses are still able to provide valuable insight into the relative amount and distribution of genetic variation which, in the context of population genetic theory, can iteself give significant insight into the character of a given species (for review see Verspoor *et al*., 2005). Even with the advent of DNA molecular markers, protein and isozyme markers when separated by electrophoresis, due to the relative simplicity, speed and relatively low cost of analysis as compared to many others are still being applied in studies of population genetic structure in different taxonomic groups. For instance, electrophoretic studies of proteins and isozymes have successfully been used in: plants (Scaltsoyiannes, 1999; Zeidler, 2000; Veasey *et al.,* 2008); insects (Simon and Hebert, 1995; Scarpassa and Tadei, 2000; Santos *et al*., 2003); coral reafs (Ridgway, 2005); mollusks (Nikiforov and Zvyagintsev, 2008); reptiles (Bock *et al*., 2001); fish (Billington, 1996, Batista and Solé-Cava, 2005; Kuz'min and Kuz'mina, 2005, Verspoor *et al*., 2005; Zawadzki *et al*., 2008); mammals (Erdoğan and Özbeyaz, 2004; Yiğit *et al*., 2007; Tao *et al*., 2007); among many other organisms.

Some studies on the genetic population structure of the Amazon turtle (*Podocnemis expansa*) have already been performed by means of proteins (Teixeira *et al.,* 1996; Moura *et al*., 2011), allozymes (Bock *et al.,* 2001), mitochondrial DNA (mtDNA) and microssatélites (Sites *et al.,* 1999; Pearse *et al*., 2006).

The first study in Amazon turtle using the allele frequency distribution of transferrin (a blood plasma iron binding protein), suggested a free flow of genes in seven population samples from five geographical areas of the Amazon (Teixeira *et al.,* 1996). This finding has recently been confirmed by Moura *et al*., (2011) who were investigating the temporal distribution of the transferrin alleles (Tf^a and Tf^b) in three of the population samples originally examined by Teixeira *et al*., (1996).

The allozyme markers used by Bock *et al*. (2001) showed population genetic structure between Amazon turtle samples collected in Brazil and Peru. While the DNA molecular markers (nuclear and mitochondrial) of this species, analysed by Sites *et al*., (1999) in samples collected from two river systems in the Amazon Basin (Tapajos and Araguaia) suggested extensive within-system gene flow (up to 275 Km) but very little gene flow between river sytems (2400 km). Another study with Amazon turtle based on analysis of mitochondrial DNA and microsatellite markers, from a broad sampling in South America (i.e. 18 different sites in Brazil, Colombia, Peru and Venezuela) strongly indicated the existence of genetic structure between populations (Pearse *et al*., 2006).

With the exception of the works by Teixeira *et al.,* (1996), Bock *et al*., (2001) and Moura *et al*., (2011) there is practically no information on the distribution of allele frequencies at polymorphic loci of proteins applied to the identification, conservation and management of natural stocks of the Amazon turtle (*P. expansa*). Among the various types of biological tissues as sources of protein variants, the circulating blood plasma has a high number of proteins (approximately 300) (Anderson and Anderson, 2002), which can be extracted with relative facility and used in the analysis of genetic variability. Then, the present study aims to test the potential of allelic frequency distributions of plasma proteins separated by means of starch gel electrophoresis for identifying Amazon turtle stocks. This research may also provide a valuable contribution to conservation and management of this species in the region.

MATERIALS AND METHODS

Sampling sites and collection of blood samples

A capture permit for catching wild Amazon turtle (*P. expansa*) was provided by the Brazilian

Institute of Environment and Renewable Natural Resources (IBAMA) through the Biodiversity Authorization and Information System (SISBIO License No. 13081-1 of 19/12/2007). In possetion of this legal support ninety five hatchling specimens of *P. expansa* were collected from three geographical areas in the Amazon region, totalizing three population samples: 1) Maracarana (02º13'S; 58º15'W), Rio Uatumã-AM (30); 2) Trombetas (01°20'S; 56°45'W), Rio Trombetas-PA (34), and 3) Monte Cristo (04°04′S; 55° 38′W), Rio Tapajós-PA (31)(**Fig. 1**).

With the aid of 3 ml disposable syringes, blood samples were taken from the femoral artery and placed in 5 ml BD vacutainer tubes, containing 0.5 ml of 3.8% sodium citrate as anticoagulant. Blood plasma specimens were separated by centrifugation at 3,000 rpm for 20 minutes and stored at -25ºC, until electrophoresis run (Teixeira *et al*., 1996). After blood collection all animals were returned to their respective collection sites.

Electrophoresis procedures

The electrode buffer (0.06 M lithium hydroxide and 0.30 M boric acid) and gel buffer (0.03 M tris (hydroxymethyl) aminomethane and 0.005 M citric acid) containing 1% of the electrode buffer, were prepared according to Ridgway *et al*., (1970). The buffers were adjusted to pH 8.20 with 1 M lithium hydroxide. Sigma starch gels at a concentration of 8.35% were made up in 340 ml of the gel buffer. By means of a continuous

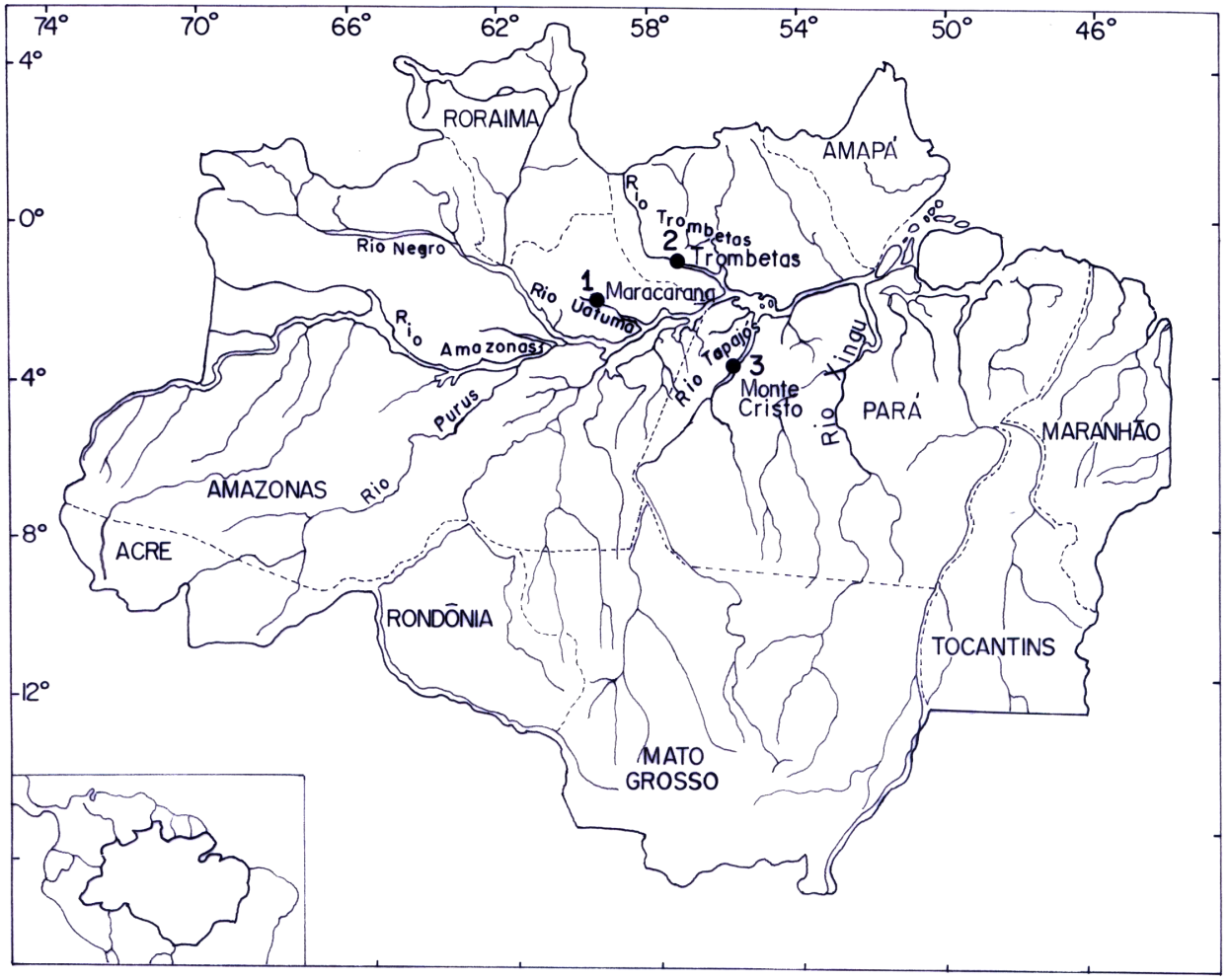

Fig. 1. The geographic sources where the three population samples of *P. expansa*, were captured in the Amazon region: 1) Maracarana, Rio Uatumã; 2) Trombetas, Rio Trombetas; and 3) Monte Cristo, Rio Tapajós. Map modified from Teixeira *et al.*, (1996).

stirring process performed with a simple mechanical stirrer, the starch mixed in buffer was cooked up to boiling point (approximately 90°C) in a 2-liter round-bottomed flask supported by a heating mantle. Then, the cooked gel was degassed with the help of a vacuum pump. The soluble protein supernatants were absorbed into 5 mm x 8 mm rectangular filter papers (Whatman 3 MM or 17 MM), which were then inserted on the gels. The filter paper Whatman 3 MM was used for analysis of esterase (EST), phosphoglucomutase (PGM), malate dehydrogenase (MDH) and superoxide dismutase (SOD); and Whatman 17 MM for analysis of transferrin (Tf), albumin (Alb) and general protein (GP). In the analyses of Tf, Alb and GP, the gels were subjected to a potential of 120 V during the first 45 minutes. The filter paper inserts were removed and the electrophoretic run continued at 250 V for about four hours, until the borate line had moved about 10 cm past the insert line. For the analyses of EST, PGM, MDH and SOD, the gels were subjected to a potential of 150 V for one hour. The filter paper inserts were removed and the electrophoretic run continued for about five hours, until the borate line had moved about 10 cm past the insert line. The alleles were classified alphabetically according to their decreasing electrophoretic mobilities towards the anode. In the analyses of Tf, Alb, PG, MDH and PGM, control plasma of known genotypes were selected and interspersed along the gels in order to genotype the specimens more accurately.

Protein system staining

Transferrin (Tf)

The differential precipitation method with rivanol (2-ethoxy-6,9-diaminoacridine lactate) was used for isolation of plasma transferrin prior to electrophoresis run as described by Jamieson and Turner (1978) with modifications of Teixeira *et al*.,.(1996). Then, plasma samples were treated with 2% rivanol solution at a ratio of 1:1. For the revelation of transferrin molecules the gels were stained with 1% Amido Black, diluted in a solution prepared in 5:5:1 parts of water, methanol and acetic acid, respectively for five minutes, according to Jamieson and Turner (1978). Finally, the gels were washed by successive immersions in plastic vessels containing the aforementioned solution (water, methanol and acetic acid) for approximately 15 hours (Teixeira *et al*., 1996). The *Tf* locus alleles were identified and classified according to Teixeira *et al.,* (1996).

Albumin (Alb)

Given that, usually the protein supernatant obtained by the differential precipitation method with rivanol, contains significant amounts of albumin (Boettcher *et al*., 1958) this method was also tested to isolate albumin molecules. Thus, plasma samples were treated with 1% rivanol solution at a ratio of 1:1 (Jamieson and Turner, 1978) with modifications. For visualization of the albumin molecules, the gels were stained with 1% Amido Black following the procedure of Jamieson and Turner (1978) used for staining transferrin, except for the staining time that lasted from five to seven minutes. The distaining procedure was the same as that previously described for transferrin.

General protein (GP)

For the revelation of bands in the general protein patterns, the gels were stained with the same methodology described previously for transferrin and albumin (i.e. 1% Amido Black).

Nomenclature and enzyme staining

The enzymes were classified according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB. 2009), where each enzyme name is written in full and enzyme abbreviation and enzyme commission number (EC number) in parentheses, as follow: esterase (EST-EC 3.1.1.1), phosphoglucomutase (PGM-EC 2.7.5.1), malate dehydrogenase (MDH-EC 1.1.1.37) and superoxide dismutase (SOD-EC 3.15.1.1).

In order to visualize isozyme bands in the zymograms, the starch gels were immersed and incubated in staining solutions containing chemical components (substrates, coenzymes, electron carriers, buffer solutions, and tetrazolium salts) necessary to detect enzymatic activities as described below for each enzyme system tested.

Esterase (EST – EC 3.1.1.1)

For the revelation of esterase isozyme pattern in the gels the following staining solution was used: 3 ml of 1% α-naphthyl acetate, dissolved in acetone at 50%, 87 ml of distilled water, 100 mg of the dye fast blue RR salt and 10 ml of 0.5 M Tris HCl pH 7.1 (Shaw and Prasad, 1970). The gel slices were placed in plastic vessels, immersed in the above solution and later incubated at 37°C in a laboratory oven for about 30 minutes or until the development of the bands. The staining solution was then discarded and the gels, after being washed with distilled water, were fixed in a 10% glycerol solution prepared in 5:5:1 parts of water, methanol and acetic acid, respectively.

Phosphoglucomutase (PGM – EC 5.4.2.2)

The staining recipe used for revealing the phosphoglucomutase isozyme patterns in the gels was that recommended by Alfenas *et al*., (1991) with modifications: 14 mg of $NADP^+$, 2.8 mg PMS, 14 mg MTT, 28 mg $MgCl_2$, 70 mg of glucose-1-phosphate, 70 ml of 0.2 M Tris-HCl pH 8.0 and 28 ml of glucose-6-phosphate dehydrogenase (G6PDH), which was added last. The gels were placed in plastic vessels containing the staining solution and then incubated in an oven at 37°C for about two hours, or until the development of the bands. The staining solution was discarded and the gels after being washed with distilled water were fixed in a 10% aqueous glycerol solution.

Malate dehydrogenase (MDH – EC 1.1.1.37)

The malate dehydrogenase isozyme patterns were revealed using the following staining solution: 5 mg of NAD^+, 5 mg PMS, 10 mg MTT, 25 mL of 0.5 M malic acid pH 8.0 and 50 mL of 0.1 M Tris HCl pH 8.5 (Allendorf *et al*., 1977) with modifications. The gels were immersed into this solution in plastic vessels and then incubated in an oven at 37°C for about one hour, or until the development of the bands. The staining solution was discarded and the gels after being washed with distilled water were fixed in a 10% glycerol solution prepared in 5:5:1 parts of water, methanol and acetic acid, respectively.

Superoxide dismutase (SOD – EC 1.15.1.1)

The following staining solution was used for visualizing the superoxide dismutase pattern in the gels: 24 mg MTT plus 7 mg PMS dissolved in 70 ml of 0.05 M Tris-HCl pH 7.0 (Allendorf *et al*., 1977) with modifications. The gels were immersed in the aforementioned solution, in plastic vessels and then incubated at 37°C in the dark for approximately 2 hours, or until the development of achromatic bands. The staining solution was discarded and the gels were then washed with distilled water and fixed in a 10% glycerol solution prepared in 5:5:1 parts of distilled water, methanol and acetic acid, respectively.

Statistical analysis

Population genetic analyses of the Amazon turtle were performed using the following statistical programmes: Biosys-2 (Swofford *et al*., 1997), TFPGA 1.3 (Miller, 1997) and POPGENE Version 1.32 (Yeh *et al.,* 1999). The list of statistical analyses is presented below, and the programmes used in each one of them are shown in parentheses: 1) log-likelihood ratio (G) and chi-square (X^2) tests assuming the Hardy-Weinberg equilibrium (POPGENE 1.32 and TFPGA 1.3); 2) estimation of allele frequencies, average heterozygosity, proportion of polymorphic loci and mean number of alleles per locus (POPGENE 1.32 and TFPGA 1.3); 3) estimation of genetic distances according to Rogers (1972) as modified by Wright (1978) (TFPGA 1.3); 4) cluster analysis of genetic distances using the Unweighted Pair-Group Method with Arithmetic

Table 1. Allele frequencies at 11 protein and isozyme loci examined in the blood plasma from three population samples of *Podocnemis expansa*.

Locus and allele	Population samples			
	Maracarana* N=30	Trombetas* N=34	Monte Cristo* N=31	Total N=95
***Tf*^{a}**	0.7333	0.8088	0.7903	0.7789
***Tf*^{b}**	0.2667	0.1912	0.2097	0.2211
***Alb*^{a}**	0.9167	0.9265	0.9516	0.9316
***Alb*^{b}**	0.0833	0.0735	0.0484	0.0684
GP-1	1.000	1.000	1.000	1.000
GP-2	1.000	1.000	1.000	1.000
EST-1	1.000	1.000	1.000	1.000
EST-2	1.000	1.000	1.000	1.000
SOD-1	1.000	1.000	1.000	1.000
SOD-2	1.000	1.000	1.000	1.000
***MDH*^{a}**	0.000	0.000	0.2903	0.0947
***MDH*^{b}**	1.000	1.000	0.7097	0.9053
PGM-1	1.000	1.000	1.000	1.000
***PGM-2*^{a}**	0.7037	0.7273	0.4423	0.6395
***PGM-2*^{b}**	0.2963	0.2727	0.5577	0.3605

***Three, one and five specimens were not genotyped at the locus PGM-2 in Maracarana, Rio Uatumã; Trombetas, Rio Trombetas and Monte Cristo, Rio Tapajós, respectively (see Table 2).**

Average (UPGMA) dendrogram (TFPGA 1.3); 5) exact tests for population differentiation (Raymond and Rousset,1995) for making pairwise population comparisons (TFPGA 1.3); 6) *F*-statistics (F_{IS}, F_{IT} and F_{ST}) of Wright (1978) and estimated gene flow (*Nm*) based on Nei (1978) among the population samples and between pairs of the population samples (POPGENE 1.32); 7) linkage disequilibrium analysis between pairs of polymorphic loci (BIOSYS 2); and 8) homogeneity test for investigating allele frequency distributions among population samples and between pairs of the population samples (BIOSYS 2 and POPGENE 1.32).

Futhermore, the significance of F_{ST} values between population samples was tested using the chi-square contingency test of Workman and Niswander (1970).

RESULTS

Electrophoretic analyses of three protein and four enzyme systems from blood plasma specimens of three Amazon turtle population samples: 1) Maracarana, Rio Uatumã-AM; 2) Trombetas, Rio Trombetas-PA; and 3) Monte Cristo, Rio Tapajós-PA revealed zones of electrophoretic activities in the gels presumably encoded by 11 loci **(Table 1)**. Of these loci, seven proved to be monomorphic (*GP-1*, GP-*2*, *EST-1*, *EST-2*, *PGM-1*, *SOD-1* and *SOD-2*) and four polymorphic (*Tf*, *Alb*, *MDH-1* and *PGM-2*). Detailed descriptions of the electrophoregrams and zymograms follow below.

Protein systems

Transferrina (Tf)

As previously reported by Teixeira *et al*., (1996), the locus *Tf* was found to be polymorphic revealing electrophoretic patterns with three theoretically expected genotypes (*Tf*^{aa}, *Tf*^{ab} and *Tf*^{bb}), presumably encoded by codominant alleles (*Tf*^{a} and *Tf*^{b}). The samples showed a good genetic balance at the intra and inter-population levels, as revealed by chi-square (X^2) and log-likelihood ratio (G) tests **(Table 2)**. Homogeneity test for *Tf* allele frequency distributions among the population samples and between pairs of population samples revealed no statistically significant differences **(Table 3)**.

Albumina (Alb)

The electrophoregrams of albumin revealed a polymorphism with the presence of two genotypes (*Alb*^{aa} and *Alb*^{ab}) presumably encoded by two codominant

Table 2. Genotype and allele frequency distributions at four polymorphic loci in the P. expansa population samples. Chi-square *(X^2)* and log-likelihood ratio (G) tests were applied assuming Hardy-Weinberg equilibrium. Expected numbers of genotypes are shown in parentheses.

	Population samples			Total N=95
	Maracarana N=30	Trombetas N=34	Monte Cristo N=31	
***Tf* genotypes**				
Tf^{aa}	16 (16.0339)	22 (22.1642)	20 (19.2787)	58 (57.5556)
Tf^{ab}	12 (11.9322)	11 (10.6716)	9 (10.4426)	32 (32.8889)
Tf^{bb}	2 (2,0339)	1 (1.1642)	2 (1.2787)	5 (4.5556)
***Tf* allele frequencies**				
Tf^{a}	0.7333	0.8088	0.7903	0.7789
Tf^{b}	0.2667	0.1912	0.2097	0.2211
Hardy-Weinberg test				
d.f.	1	1	1	1
X^2	0.001022	0.0345	0.6332	0.0708
P	0.9745	0.8527	0.4262	0.7902
G	0.001024	0.0355	0.5824	0.0697
P	0.9745	0.8504	0.4454	0.7918
***Alb* genotypes**				
Alb^{aa}	25 (25.1695)	29 (29.1493)	28 (28.0492)	82 (82.4127)
Alb^{ab}	5 (4.6610)	5 (4.7015)	3 (2.9016)	13 (12.1746)
Alb^{bb}	0 (0.1695)	0 (0.1493)	0 (0.092)	0 (0.4127)
***Alb* allele frequencies**				
Alb^{a}	0.9167	0.9265	0.9516	0.9316
Alb^{b}	0.0833	0.0735	0.0484	0.0684
Hardy-Weinberg test				
d.f.	1	1	1	1
X^2	0.1953	0.1690	0.0526	0.4707
P	0.6586	0.6810	0.8186	0.4926
G	0.3642	0.3178	0.1017	0.8822
P	0.5462	0.5729	0.7497	0.3476
***MDH* genotypes**				
MDH^{aa}	0	0	9 (2.5082)	9 (0.8095)
MDH^{ab}	0	0	0 (12.9836)	0 (16.3810)
MDH^{bb}	30	34	22 (15.5082)	86 (77.8095)
***MDH* allele frequencies**				
MDH^{a}	0	0	0.2903	0.0947
MDH^{b}	1.000	1.000	0.7097	0.9053
Hardy-Weinberg test				
d.f.	-	-	1	1
X^2	-	-	32.5034	100.1114
P	-	-	0.0000**	0.0000**
G	-	-	38.3835	60.5680
P	-	-	0.0000**	0.0000**
Population samples				
	Maracarana* N=27	**Trombetas* N=33**	**Monte Cristo* N=26**	**Total* N=86**
***PGM-2* genotypes**				
$PGM\text{-}2^{aa}$	15 (13.2642)	16 (17.4545)	5 (4.9608)	36 (34.4211)
$PGM\text{-}2^{ab}$	8 (11.4717)	16 (13.0909)	13 (13.0784)	37 (40.1579)
$PGM\text{-}2^{bb}$	4 (2.2642)	1 (2.4545)	8 (7.9608)	13 (11.4211)

PGM-2 allele frequencies				
***PGM-2**[a]*	0.7037	0.7273	0.4423	0.6395
***PGM-2**[b]*	0.2963	0.2727	0.5577	0.3605
Hardy-Weinberg test				
d.f.	1	1	1	1
X^2	2.6086	1.4359	0.00097	0.5390
P	0.1063	0.2308	0.9751	0.4628
G	2.4752	1.6216	0.00097	0.5353
P	0.1156	0.2029	0.9751	0.4644

d.f. = degrees of freedom; *Three, one and five specimens were not genotyped at the *PGM-2* locus in Maracarana, Rio Uatumã; Trombetas, Rio Trombetas and Monte Cristo, Rio Tapajós, respectively; ** $P < 0.001$.

alleles (*Alb*[a] and *Alb*[b]) at the locus *Alb*. The theoretically expected genotype (*Alb*[bb]) was not detected in any of the population samples analysed. All samples showed a good genetic balance at the intra and inter-population levels, as revealed by the chi-square (X^2) and log-likelihood ratio (G) tests **(Table 2).** Homogeneity test for *Alb* allele frequency distributions among population samples and between pairs of population samples revealed no statistically significant differences **(Table 3).**

General protein (GP)

Two bands common to all specimens were seen in the gels, in a zone of electrophoretic activity classified as post-albumin (Manwell and Baker, 1970) with electrophoretic migrations immediately prior to albumin, and presumably encoded by the fixed loci (*GP-1* and *GP-2*) **(Table 1).** Some additional bands were seen in the gels, but these were too weakly or diffusely stained for being reliably scored.

Table 3. The allele frequency distributions at four polymorphic loci among the population samples and between pairs of the population samples of *Podocnemis expansa*, were analysed by means of homogeneity tests. Chi-square (X^2), log-likelihood ratio (G) and probability statistical values were calculated by using contingency tables.

		Population samples		
Locus	**Among samples**	**Maracarana vs Trombetas**	**Maracarana vs Monte Cristo**	**Trombetas vs Monte Cristo**
Tf	$X^2_{(2)}$=1.124 *P* = 0.570 $G_{(2)}$ =1.105 *P* = 0.576	$X^2_{(1)}$=1.037 *P* = 0.309 $G_{(1)}$= 1.035 *P* = 0.309	$X^2_{(1)}$= 0.546 *P* = 0.460 $G_{(1)}$ = 0.547 *P* = 0.460	$X^2_{(1)}$=0.069 *P* =0.792 $G_{(1)}$ = 0.069 *P* = 0.792
Alb	$X^2_{(2)}$ = 0.628 *P* = 0.731 $G_{(2)}$ = 0.656 *P* = 0.720	$X^2_{(1)}$=0.042 *P* =0.836 $G_{(1)}$=0.042 *P* = 0.837	$X^2_{(1)}$= 0.608 *P* = 0.436 $G_{(1)}$= 0.613 *P* = 0.434	$X^2_{(1)}$= 0.355 *P* = 0.551 $G_{(1)}$ = 0.359 *P* = 0.549
MDH	$X^2_{(2)}$=41.050 *P* = 0.000*** $G_{(2)}$ =44.375 *P* = 0.000***	§	$X^2_{(1)}$= 20.434 *P* = 0.000*** $G_{(1)}$= 27.392 *P* = 0.000***	$X^2_{(1)}$= 22.915 *P* = 0.000*** $G_{(1)}$ =29.859 *P* = 0.000***
PGM-2	$X^2_{(2)}$=10.315 *P* = 0.006** $G_{(2)}$ =10.112 *P* = 0.006**	$X^2_{(1)}$=0.081 *P* =0.776 $G_{(1)}$ =0.081 *P* =0.776	$X^2_{(1)}$ =6.398 *P* =0.011* $G_{(1)}$ =6.466 *P* =0.011*	$X^2_{(1)}$=8.634 *P* =0.003** $G_{(1)}$ =8.682 *P* = 0.003**
Grand total	$X^2_{(8)}$=53.117 *P* = 0.000*** $G_{(8)}$ =56.248 *P* = 0.000***			

***p < 0.05; **p < 0.01 or ***p < 0.001; § fixed locus in the samples.**

Enzyme systems

Esterase (EST – EC 3.1.1.1)

The esterase enzyme revealed two zones of electrophoretic activity, with a monomorphic isozyme pattern presumably encoded by the same fixed loci (*EST-1* and *EST-2*) in all Amazon turtle samples tested **(Table 1).** The loci (*EST-1* and *EST-2*) were visualized in the gel, in a more cathodic electrophoretic region. The locus *EST-1* was weakly stained with an electrophoretic migration immediately ahead of *EST-2* locus. The locus *EST-2* was strongly stained.

Phosphoglucomutase (PGM – EC 5.4.2.2)

The phosphoglucomutase zymogram showed two loci (*PGM-1* and *PGM-2* (**Fig. 2**). *PGM-1* locus proved to be monomorphic and *PGM-2* polymorphic. *PGM-2* showed three theoretically expected genotypes ($PGM\text{-}2^{aa}$, $PGM\text{-}2^{ab}$ and $PGM\text{-}2^{bb}$) presumably encoded by codominant alleles ($PGM\text{-}2^{a}$ and $PGM\text{-}2^{b}$). The loci (*PGM-1* and *PGM-2*) were detected in a more cathodic region of the gel. *PGM-1* showed a faster electrophoretic migration with a slightly stained band. The locus *PGM-2* despite showing zymogram bands that could be genotyped for most specimens examined, nine specimens could not be genotyped due to the low resolution of the bands. All samples showed a good genetic balance at the intra and inter-population levels, as revealed by the chi-square (X^2) and log-likelihood ratio (G) tests **(Table 2).** However, homogeneity tests revealed statistically significant differences among the population samples and between all pairwise comparisons made with Monte Cristo sample (**Table 3**).

Table 4. Linkage disequilibrium analysis between pairs of polymorphic loci in the population samples of *P. expansa*.

Loci compared	X^2	d.f	*P*
Tf* and *Alb	0.38	1	0.5353
Tf* and *MDH	0.76	1	0.3836
Tf* and *PGM-2	0.21	1	0.6505
Alb* and *MDH	2.98	1	0.0841
Alb* and *PGM-2	0.96	1	0.3261
MDH* and *PGM-2	2.03	1	0.1538

Malato desidrogenase (MDH – EC 1.1.1.37)

The MDH zimogram revealed a zone of electrophoretic activity located in the more cathodic region of the gels, supposedly controlled by *MDH* locus. This locus proved to be polymorphic in the Monte Cristo population sample, with genotypes (MDH^{aa} and MDH^{bb}) produced by action of codominant alleles (MDH^{a} and MDH^{b}) but with the absence of theoretically expected genotype MDH^{ab} **(Fig. 3).** In population samples of Maracarana and Trombetas this locus proved to be fixed for the allele MDH^{b} **(Table 2).** Allele MDH^{a} and genotype MDH^{aa} were detected only in Monte Cristo sample. Due to the enormous deficiency of heterozygotes and the excess of homozygotes (MDH^{aa}) the Monte Cristo population sample and the total population sample are not in Hardy-Weinberg equilibrium, as shown in the chi-square (X^2) and log-likelihood ratio (G) tests **(Table 2)**. The homogeneity tests revealed high levels of statistically significant differences among population samples and all pairwise comparisons made with Monte Cristo sample **(Table 3).**

Superoxide dismutase (SOD – EC 1.15.1.1)

The superoxide dismutase zymogram revealed two zones of electrophoretic activity, with a monomorphic isozyme pattern presumably encoded by two fixed loci (*SOD-1* and *SOD-2*) in all samples tested **(Table 1).** The loci (*SOD-1* and *SOD-2*) were detected in a more cathodic region of the gels. The locus *SOD-1* migrated slightly ahead of the *SOD-2* locus. Eventually, it was noted the presence of satellite bands positioned in front of the locus *SOD-1*.

Linkage disequilibrium

The hypothesis of independent segregation between pairs of the polymorphic loci detected in *P. expansa*, was tested by means of the chi-square (X^2) test. Of the six possible combinations between pairs of loci, there were no statistically significant

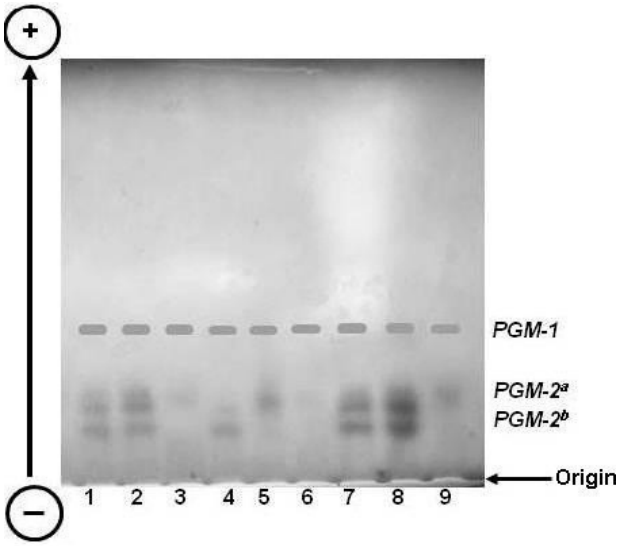

Fig. 2. Zymogram of phosphoglucomutase showing the monomorphic locus *PGM-1*, and the polymorphic locus *PGM-2 in the blood plasma of P. expansa*. The *PGM-2* genotypes from left to right are: *PGM-2*^{aa} (lanes 3, 5, 6 and 9); *PGM-2*^{ab} (lanes 1, 2, 7 and 8); *PGM-2*^{bb} (lane 4).

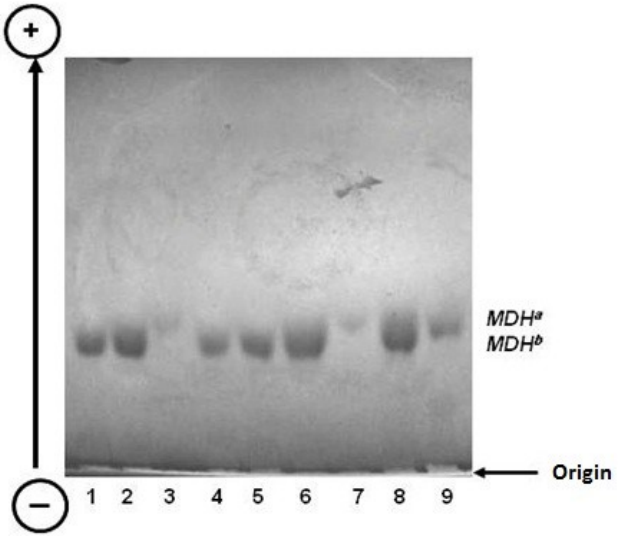

Fig. 3. Zymogram of malate dehydrogenase showing the polymorphism at the locus *MDH* in the blood plasma of *P. expansa*. The genotypes from left to right are: *MDH*^{bb} (lanes 1, 2, 4, 5, 6 and 8); *MDH*^{aa} (lanes 3, 7 and 9).

differences **(Table 4).**

Measures of genetic diversity

The proportion of polymorphic loci (*P*) when calculated by using the 0.95 criterion (i.e. the common allele with a frequency equal to or less than 0.95), showed a *P* of 27.27% for the population samples of Trombetas and Maracarana, and of 36.36% for Monte Cristo and total sample **(Table 5)**. The highest *P* values presented by Monte Cristo and total population samples were due to the polymorpism at the *MDH* locus detected only in the Monte Cristo sample. The average number of alleles per locus in the population samples of Maracarana and Trombetas was 1.27 and in Monte Cristo 1.36. The average observed heterozygosity (*Ho*) in the Maracarana population sample was 0.0785, Trombetas 0.0869 and Monte Cristo 0.0771. The average expected heterozygosity (*He*) in the Maracarana population sample was 0.0874, Trombetas 0.0766 and Monte Cristo 0.1211. As one can see, (*He*) value for the Monte Cristo sample was much higher as compared to the other two samples.

Genetic structure

The estimates of population genetic structure in the population samples performed by using Wright's *F* statistics showed average values of 0.1347 and 0.1912 for F_{IS} and F_{IT}, respectively. The *Alb* locus was the only one to show negative values for F_{IS} and F_{IT}, due to the slight excess of heterozygous individuals in the samples. The average value of F_{ST} was 0.0652 among the population samples (**Table 6**). Maracarana vs Trombetas samples showed an average value of F_{ST} = 0.0035, whereas Monte Cristo vs Maracarana and Monte Cristo vs Trombetas, revealed average F_{ST} values = **0.061** and **0.066,** respectively **(Table 7)**. All pairwise comparisons of F_{ST} values made with Monte Cristo sample were significantly different from zero ($P < 0.001$).

Gene flow estimation expressed as number of migrants per generation (*Nm*) among the population samples was 3.58 (**Table 6**).

Corroborated by the results obtained from homogeneity tests **(Table 3)**, the pairwise comparisons performed by means of exact tests for population

Table 5. Summary of genetic variation at 11 loci in the population samples of *P. expansa*. Standard deviations are shown in parentheses.

	Population samples			Total
	Maracarana	Trombetas	Monte Cristo	
Mean number of turtle per locus	29.6	33.9	30.5	94
Number of locus	11	11	11	11
***Proportion of polymorphic loci (*P*)**	27.27	27.27	36.36	36.36
Average number of alleles per locus	1.27	1.27	1.36	1.36
Number of polymorphic loci	3	3	4	4
Average observed heterozygosity (*Ho*)	0.0785 (0.1442)	0.0869 (0.1668)	0.0771 (0.1553)	0.0811 (0.1533)
****Average expected heterozygosity (*He*)**	0.0874 (0.1632)	0.0766 (0.1439)	0.1211 (0.1933)	0.1004 (0.1631)

****Nei (1973) expected heterozygosity; **P* corresponds to 0.95 criterion.**

differentiation revealed high statistically significant differences between all comparisons made with Monte Cristo sample **(Table 7).**

Genetic distance

The Rogers (1972) genetic distance modified by Wright (1978) revealed the lowest distance between Maracarana and Trombetas population samples, unlike the higher values detected between these samples and Monte Cristo **(Table 8),** as illustrated by the UPGMA dendrogram method **(Fig. 4).** The internal support values of the nodes obtained by bootstrap methods showed a low node bootstrap value of 27.27% between Maracarana and Trombetas samples. A maximum bootstrap value of 100% was found in the node that includes the samples Maracarana, Trombetas and Monte Cristo.

Table 6. Wright's *F*-statistics and gene flow (*Nm*) for each locus in the *P. expansa* population samples.

Locus	F_{IS}	F_{IT}	F_{ST}	**Nm*
Tf	0.0174	0.0233	0.0060	41.65
Alb	-0.0771	-0.0734	0.0034	73.31
GP-1	-	-	0.0000	-
GP-2	-	-	0.0000	-
EST-1	-	-	0.0000	-
EST-2	-	-	0.0000	-
MDH	1.000	1.000	0.2143	0.92
PGM-1	-	-	0.0000	-
PGM-2	0.0198	0.0895	0.0711	3.27
SOD-1	-	-	0.0000	-
SOD-2	-	-	0.0000	-
Mean	0.1347	0.1912	0.0652	3.58

***Nm* = Gene flow estimated from F_{ST}= 0.25 (1 -F_{ST}) / F_{ST}.**

DISCUSSION

Linkage disequilibrium

In population genetic studies typically using a system of multiple polymorphic gene loci, it is necessary to subject these loci to the test of linkage disequilibrium, since genetic coadaptation may exist between some alleles, but not between others within the same population. In this circumstance, certain alleles of a given locus can also be coadapted with others of distinct loci (Ayala and Kiger, 1980; Allendorf and Luikart, 2007).

Given that, the alleles of the polymorphic loci examined here in the Amazon turtle (*P. expansa*) population samples, are not associated (Table 4) and

Table 7. Exact tests for population differentiation (Raymond and Rousset, 1995) based on the allele frequency distributions at polymorphic loci were applied for making pairwise comparisons in the *P. expansa* population samples (lower half of matrix). In addition, F_{ST} values (upper half of matrix) and corresponding *Nm* values (between parentheses) are shown. The F_{ST} values significantly different from zero are shown in bold.

	Population samples		
	Maracarana	**Trombetas**	**Monte Cristo**
Maracarana	-	0.0035 (71.15)	**0.061** (3.62)
Trombetas	$X^2_{(6)}$= 2.230 *P* = 0.897	-	**0.066** (3.54)
Monte Cristo	$X^2_{(8)}$ = 31.230 *P* = 0.0001*	$X^2_{(8)}$= 33.023 *P* = 0.0001*	-

***p < 0.001.**

presumably are segregating independently (i.e. no epistasis), they can be used in future population genetic studies of this species, requiring gametic equilibrium between loci, as basic premise. Therefore, the use of these loci as genetic markers for investigating Amazon turtle probably should not bring about any influence in the estimation of parameters on the population genetic structure of this species. Nevertheless, we cannot completely rule out the possibility that some of these loci are in linkage disequilibrium with other loci that have not been examined thus far, due to evolutionary processes, such as: inbreeding, genetic drift, natural selection, population bottleneck and Wahlund effect (Ayala and Kiger, 1980; Durand *et al*., 2003; Hartl, 2008).

Hardy-Weinberg equilibrium

The Hardy-Weinberg test is a simplified statistical method commonly used to describe population genetic characteristics by means of the binomial expansion: $(p + q)^2 = p^2 + 2pq + q^2 = 1$, where p and q, correspond the allelic frequencies of the two different alleles of a given population presumably in genetic equilibrium. This method allows one to describe a population by its allele and genotype frequencies displayed at each gene locus, and the effects of natural selection. The main assumption of Hardy-Weinberg Law is that in an infinitely large population in the absence of evolutionary processes: mutation, migration, drift and natural selection; gene frequencies will remain constant across generations. This law also says that if the crossings occur at random, the genotypic frequencies are related to the gene frequencies through the aforementioned binomial expansion (Ayala and Kiger, 1980; Ferguson, 1980; Allendorf and Luikart, 2007).

Of the four polymorphic loci analysed (*Tf*, *Alb*, *MDH*, *PGM-2*) only *MDH* locus showed Hardy-Weinberg disequilibrium in the Monte Cristo population sample, with repercussion in the total population sample (Table 2). Among the factors that normally bring about genetic imbalance in gene loci, are: 1) adaptive differences between genotypes (Nevo *et al*., 1984); 2) possible existence of other loci in linkage disequilibrium with a particular locus under study (Futuyma, 1997); 3) selection against heterozygous individuals (Gillespie, 1998; Beiguelman, 2008); 4) inbreeding (Wright, 1921); 5) Wahlund effect (i.e. physical mixtures of genetically distinct populations) (Smith *et al*., 1981); and 6) production of a non-functional protein (i.e. a null allele) (Ferguson, 1980). With the exception of the null allele and Wahlund effect (factors not observed in the present *MDH* analysis), one or more of the aforementioned factors, acting in combination, could be affecting the genotypic and allelic distributions in the locus *MDH*. Therefore, a broader sampling should be conducted in the occurrence area of

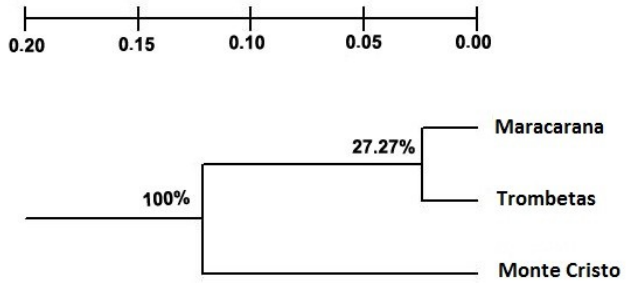

Fig. 4. The UPGMA dendrogram based on Rogers (1972) genetic distance modified by Wright (1978) between *P. expansa* population samples.

P. expansa, with analyses including preferably the loci examined in this study, and extra loci, in order to better understand this peculiar genetic imbalance detected in the Monte Cristo population sample. Additionally, it is necessary to conduct further studies with historical series of the molecular genetic markers already examined in *P. expansa* (Moura *et al*., 2011, and references therein); to assess what alleles at certain loci could be increasing or decreasing in frequency over subsequent generations of this species.

Measures of genetic diversity

The knowledge of allele frequency distributions is essential in researches of genetic variability and population genetic structure. This kind of research contributes significantly to the knowledge of the evolutionary history of a given population, therefore, may indicate the action of certain evolutionary mechanisms on the population under study, such as inbreeding, genetic drift, founder effect and population bottlenecks. Additionally, by comparisons of allele frequencies it is possible to demonstrate whether certain populations have similar evolutionary histories (Hartl, 2008).

The genetic variation of a population is usually measured by the proportion of polymorphic loci (*P*), average heterozygosity per locus (*H*), number of polymorphic loci and average number of alleles per locus (Nei, 1975).

Among these measures of genetic variation, heterozygosity is the most informative, and is therefore preferred by most population geneticists, and less affected by sample size. It is a good measure of variation because it estimates the probability that two alleles drawn at random from a population are different. The polymorphism of a population is, moreover, an imprecise measure of genetic variation due to the arbitrariness in the choice of the polymorphism criterion to be used (0.95 or 0.99), i.e. the most common allele with frequency no greater than 0.95 or 0.99. For example, a certain locus presenting two alleles with frequencies 0.95 and 0.05, while another presenting 20 alleles, each with a frequency of 0.05 are equally considered polymorphic under the 0.95 criterion of polymorphism (Nei, 1975; Ayala and Kiger, 1980).

Table 8. Rogers (1972) genetic distance modified by Wright (1978) between the *Podocmenis expansa* population samples.

	Population samples		
	Maracarana	**Trombetas**	**Monte Cristo**
Maracarana	-	-	-
Trombetas	0.0240	-	-
Monte Cristo	0.1195	0.1230	-

In the present study, the average expected heterozygosity (*He*) of *P. expansa* in the Maracarana population sample was 0.0874, Trombetas 0.0766 and Monte Cristo 0.1211. The population sample of Monte Cristo showed the higher variation between the average observed heterozygosity (*Ho*) and the average expected heterozygosity (*He*) (0.0771 and 0.1211, respectively) **(Table 5)**. This was noted due to the level of polymorphism detected in the *MDH* locus, where the allele frequencies of MDH^a MDH^b were 0.29 and 0.71, respectively, only in this sample. As a result, a great variation between these measures *Ho* and *He* was also observed in the total sample. The value for *He* in the poplation sample of Monte Cristo (0.1211) was much higher than the values revealed in the two other samples analysed in the present study, Maracarana (0.0874) and

Trombetas (0.0766); and in the population samples examined by Bock *et al*., (2001), from Peru (0.043) and Brazil (0.072- 0.078). However, these values are within the range estimated for the group of reptiles, where, in studies of protein-isozyme markers, this measure ranged from 0.050 to 0.124 (Chakraborty *et al*., 1980; Ferguson, 1980; Allendorf and Luikart, 2007).

The polymorphism (*P*) in *P. expansa* was estimated to be 0.17 (Bock *et al*., 2001). This value is well below the values estimated in the *P. expansa* population samples examined in the present study, which was 0.27 in Maracarana and Trombetas, and 0.36 in Monte Cristo. It should be emphasized that the Monte Cristo sample is showing a polymorphism 33% higher, in relation to the samples of Maracarana and Trombetas, and 122% higher in relation to the samples examined by Bock *et al*., (2001). In addition, the samples of the present study showed polymorphism values above that shown in reptiles (*P* = 0.23) (Ferguson, 1980; Ayala and Kiger, 1980; Hartl, 2008).

The Monte Cristo population sample of *P. expansa* with an average number of alleles per locus equal to 1.36, exceeded the value of 1.27 detected in the Trombetas and Maracarana samples.

This prominence in the measures of genetic variation of *P. expansa* displayed by Monte Cristo (i.e. population sample collected from the Rio Tapajós) in relation to the other population samples investigated in the present study, is consistent with the results of microsatellite and mitochondrial DNA of this species presented by Sites *et al*., (1999) and Pearse *et al*., (2006). Sites *et al*., 1999 found a higher allelic richness and a larger number of haplotypes in the *P. expansa* population sample from Rio Tapajós in relation to the population sample from Rio Araguaia. Pearse *et al*., (2006) confirmed this much higher genetic diversity of Tapajós compared to most other Brazilian population samples collected from Amazon areas.

Genetic structure

In order to generate information on the genetic structure of natural populations for breeding and genetic conservation of species, many researchers have used Wright's *F* statistics (Dias, 1998). The parameter used to estimate the gene fixation indexes resulting from biparental inbreeding is the *F* coefficient (F_{IS}, F_{IT} and F_{ST}) of Wright (1951) defined as the correlation between the alleles in the gametes that form a zygote (Robinson, 1998). F_{IS} is known as the inbreeding index of individuals relative to its subpopulation (it estimates the deviation from Hardy-Weinberg genotypic frequencies in the subpopulation); F_{IT} is the inbreeding in the population (estimated deviation from Hardy-Weinberg equilibrium in the entire population); and F_{ST} is the inbreeding due to the differentiation among subpopulations in relation to the total population (divergence of allele frequencies among populations) (Frankham *et al*., 2008).

According to Wright (1978) *F* equal to 0.25 is considered an arbitrary value above which there is very great genetic differentiation among populations; values in the range 0.15 to 0.25 indicate moderately great genetic differentiation; 0.05 to 0.15 indicate moderate genetic differentiation; and genetic differentiation is, however, by no means negligible if *F* is as small as 0.05 or even less. By using Wright (1978)'s criterion the average value of F_{IS} = 0.1347 obtained in *P. expansa* in the present study indicates a moderate inbreeding within the population samples of this species **(Table 6)**. The average value of F_{IT} = 0.1912 indicates that inbreeding in the total sample population was moderately great. The average value of F_{ST} = 0.0652 points to a genetic differentiation moderate among population samples. The loci that contributed most to genetic differentiation among the samples were *MDH* and *PGM-2*. The *MDH* locus in particular stood out as having a great deficiency of heterozygous individuals in the population sample of Monte Cristo, which reflected in the total population

sample **(Table 2)**. In addition, statistically significant differences were detected in the allele distributions of these loci, as demonstrated in the homogeneity tests **(Table 3)**.

The values of F_{IS}, F_{IT} and F_{ST} in population studies of *Pseudemys scripta* using allozymes were 0.183, 0.142 and 0.048, respectively (Scribner *et al*., 1986), and approach those values observed in the present work for *Podocnemis expansa*.

According to Wright (1969 cited in Frankham *et al*., 2002, p. 328) one migrant per generation would be enough to avoid a complete genetic differentiation among idealized populations. Theoretically, the number of migrants $Nm \geq 4$ means the occurrence of panmixia among populations. While, $Nm \leq 2$ indicates a moderate genetic divergence; however, considerable variation exists for these values of *Nm* among organisms, providing a considerable scope for genetic divergence that results from random genetic drift (Hartl, 2008).

Sites *et al.,* (1999) found a value of $Nm = 1.67$ in *P. expansa*, reflecting a moderate genetic divergence among the population samples studied. Valenzuela (2001) estimated a value of $Nm \approx 4.60$, revealing a moderate genetic divergence among population samples of Rios Caquetá (Colombia), Tapajos and Araguaia (Brazil). In the present study an average value of $Nm = 3:58$, was estimated in the total population sample of this species **(Table 6)**, which resulted in a moderate genetic divergence as shown above ($F_{ST} = 0.0652$). In the pairwise population sample comparisons, Maracarana vs Trombetas, the detected value of $Nm = 71.15$ provides strong evidence for panmixia between these two samples examined. In contrast with the *Tf* locus data presented by Teixeira *et al*., (1996), a limited gene flow was detected in the pairwise population sample comparisons, Monte Cristo vs Maracarana and Monte Cristo vs Trombetas ($Nm = 3.62$ and 3:54, respectively). This gene flow limitation also led to a moderate genetic differentiation detected in the aforementioned pairwise sample comparisons, Monte Cristo vs Maracarana and Monte Cristo vs Trombetas, with F_{ST} values = 0.061 and 0.066, respectively **(Table 7)**. In this context, the following question comes up: what is the *Nm* in the natural populations *of P. Expansa*. The data presented here, based only on a limited number of loci and population samples examined, plus the paucity of data available in literature for this species regarding genetic structure, do not allow answering this question. Currently what can be clearly seen is that, the greatest genetic differentiation revealed in the present study when Monte Cristo (i.e. population sample collected from the Rio Tapajós) was compared to the other samples, shows to be congruent with the DNA data published by Pearse *et al*., (2006), who compared Tapajós with other population samples from the Amazon basin.

Genetic distance

Genetic distance measuments have long been applied as one of the major tools for investigating the genetic differentiation between populations. These measurements that use gene and genotypic frequencies obtained through protein electrophoresis technique from individuals taken at random in natural populations, are able to provide an estimate of intra and interspecific differentiation. These frequencies can be transformed into a series of indexes that allow estimating the degree of similarity or genetic distance between species and populations (for review see Nei, 1975; Wright, 1978; Ayala and Kiger, 1980; Ferguson, 1980; Solferini and Selivon, 2001).

When genetic distance, genetic identity, or other measurements of genetic similarity have been compiled for all possible pairs of populations or species, the data are best presented in the form of a matrix. Then, for visual display of the results, dendrograms can be constructed. One of the most useful dendrograms for electrophoretic data is the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) (Ferguson, 1980). This method is mainly applied because it assumes

an evolutionary constant rate in each branch of the tree and works poorly when this assumption is violated (Hartl, 2008).

The matrix of the estimated genetic distances between population samples of *P. expansa*, showed the lowest value in the pairwise comparison Maracarana vs Trombetas **(Table 8)**. These findings suggest that these samples are presumably part of a large panmictic population, i.e. belonging to the same stock, with this fact being supported by the large estimated number of migrants per generation between them (*Nm* = 71.15). It is noteworthy that tagged specimens of Amazon turtle, from the Rio Trombetas were already found near the town of Itacoatiara, in the state of Amazonas (Alfinito *et al*., 1976). This fact could explain a possible flow of animals from the Rio Trombetas towards Rio Uatumã (river from where the Maracarana population sample was caught). Considering that these rivers are tributaries of the same bank (i.e. left bank) of the Amazon River, such a flow would be facilitated, resulting in a greater homogeneity between these sampled areas **(Table 3, Table 7)**.

It also should be highlighted that the greatest distance values were detected between the pairwise population sample comparisons made with Monte Cristo, meaning restricted flow of genes between Monte Cristo vs Maracarana (*Nm* = 3.62) and Monte Cristo vs Trombetas (*Nm* = 3.54) **(Table 7, Table 8)**. In fact, statistically significant differences were detected in these comparisons **(Table 3)**.

The dendrogram **(Fig. 4)** generated from the data of Table 8, in a way, seems to represent graphically the ancestry relationships and genetic structure of the *P. expansa* population samples examined.

CONCLUSION

1) The present electrophoretic data based on plasma protein markers point out a possible existence of distinct subpopulations "stocks" of *P. expansa* in the sampled area, where Monte Cristo (population sample from the Rio Tapajós), was always genetically different compared to the other two population samples examined.

2) Due to the reasonably high degree of polymorphism revealed in plasma proteins of *P. expansa*, an extension of the present research is highly recommended in order to detect a greater number of polymorphic loci and apply them as genetic markers in population studies of this species.

3) Even with the advent of modern DNA based markers, the protein and isozyme markers separated by electrophoresis technique, remain a powerful and effective tool in studies of population genetic structure, and, therefore, it should never be underestimated. The relative simplicity, speed and low cost of protein electrophoresis in relation to many other molecular techniques, still makes it very popular and useful in many laboratories worldwide.

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