Morphometric and genetic differentiation among populations of *Eupemphix nattereri* (Amphibia, Anura, Leiuperidae) from central Brazil

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ABSTRACT. To assess genetic structure and phenotypic diversity of *Eupemphix nattereri* Steindachner, 1863, morphometric and molecular analyses were carried out for nine populations from the State of Goiás. A total of 11 morphometric traits were evaluated and genetic information was estimated using RAPD markers. Genetic and phenotypic distances were determined as a function of geographical origin. Correlation among genetic, morphometric, micro, and macroenviromental were analyzed by the Mantel test. Genetic data indicated high levels of genetic diversity (Φ_{st}= 0.3) among the nine populations. Mantel tests did not reveal a significant positive correlation between genetic and geographical distances, indicating that locally geographical populations were not genetically similar, even in distances smaller than 50 km. Discriminant analysis on 11 morphometric measurements showed a high divergence among the nine populations. However, a marginally significant correlation (P=0.08) between genetic and morphometric distances was found. The observed correlation was not causal in terms of the relationship between phenotype and genotype, but indicated common spatial structures. Thus, our results suggest that isolation-by-distance processes may explain population divergence in *Eupemphix nattereri*.

KEYWORDS. *Eupemphix nattereri*, morphology, RAPD markers, gene flow, population structure.

RESUMO. Diferenciação genética e morfométrica em populações de *Eupemphix nattereri* (Amphibia, Anura, Leiuperidae) do Brasil Central. Visando conhecer a estrutura genética e a diversidade fenotípica da espécie *Eupemphix nattereri* Steindachner, 1863, análises morfométricas e moleculares foram realizadas em nove populações do Estado de Goiás. Onze caracteres morfométricos foram avaliados e a diversidade genética foi estimada com o uso de marcadores RAPD. As matrizes de distâncias genéticas e fenotípicas foram correlacionadas com as distâncias geográficas e dados macro e microambientais, utilizando o teste de Mantel. Em relação aos caracteres genéticos, foram encontrados altos níveis de diversidade (Φ_{st}= 0,3) entre as nove populações. Não houve, entretanto, uma correlação positiva significativa entre as distâncias genéticas e geográficas, indicando que populações geograficamente próximas não seriam geneticamente similares, mesmo estando situadas entre distâncias menores do que 50 km. Uma análise discriminante dos onze caracteres morfométricos evidenciou também uma elevada divergência entre as nove populações. Entretanto, foi encontrada uma correlação quase significativa (P=0,008) entre as distâncias genéticas e morfométricas. A correlação observada não foi casual entre fenótipo e genótipo, mas indicou estruturas espaciais comuns. Desta forma, nossos resultados sugerem que processos de isolamento por distância poderiam explicar a divergência populacional na espécie *Eupemphix nattereri*.

PALAVRAS-CHAVE. *Eupemphix nattereri*, morfologia, marcadores RAPD, fluxo gênico, estrutura populacional.

At present there are approximately 6,300 species of anuran amphibians over the world (IUCN, 2004), and the largest diversity is found in the Neotropics (Duellman, 1988). Brazil represents one of the most diverse countries, with 747 species (SBH, 2005). In State of Goiás, more than 70 anuran species are known to occur, corresponding to 9.4% of Brazil's anuran diversity. However, the anuran fauna remains largely unknown in the central areas of Brazil (Brandão & Araújo, 1998).

Despite the high levels of species diversity, little is known about the population structure of amphibians in South America, due to the scarcity of data referring to the dynamics of the group (Myers *et al*., 2000; Young *et al*., 2001). Many studies on population genetic structure of amphibians sustain the idea that among vertebrates, amphibians exhibit relatively low vagility and are highly philopatric (Blaustein *et al*., 1994; Seppã & Laurila, 1999; Shaffer *et al*., 2000; Lampert *et al*., 2003), allowing the accumulation of genetic and morphological differences among populations (Blouin & Brown, 2000; Camp *et al*., 2000; Méndez *et al*., 2004). Consequently, amphibians have recently become a focus in studies trying to understand ecological and evolutionary processes (Zeisset & Beebee, 2003; Eterovick *et al*., 2005; Funk *et al*., 2005).

The family Leiuperidae comprises 7 genera, and 48 species, occurring all over the Neotropical region and including species that have terrestrial, fossorials, arboreal, and aquatic habits (Frost *et al*., 2006).

The genus *Eupemphix* (Leiuperidae) was described by Steindachner (1863) to include the species *Eupemphix nattereri*, whose type locality is Cuiabá, Mato Grosso, Brazil (Nascimento *et al*., 2005). *Eupemphix nattereri* is a frog largely distributed in South America, ranging from the east of Paraguay to central and southeast Brazil (Cei, 1980; Frost, 2004). At the beginning of the rainy season, males and females are found reproducing in permanent and temporary ponds in open areas

(BRASILEIRO *et al*., 2005). The breeding season can extend for many months, characterizing the reproductive pattern as explosive (WELLS, 1977).

Analyses of intraspecific geographical variability in morphology have often revealed extensive variation in body size among amphibians (BABIK & RAFINSKI, 2000; CASTELLANO *et al*., 2000; SCHÄUBLE, 2004). Body size is a fundamental morphological trait, important in a physiological, ecological, and behavioral context of a species (SCHÄUBLE, 2004). However, causes and maintenance of geographical variation in morphology are likely to be complex and not always well understood (MALHOTRA & THORPE, 1997).

Studies based on molecular markers, such as RAPD (Random Amplified Polymorphic DNA) have contributed in a complementary manner to the understanding of amphibian population genetics all over the world (RAFINSKI & BABIK, 2000; TRAKIMAS *et al*., 2003; ZEISSET & BEEBEE, 2003). RAPD are based on randomic amplifications of DNA fragments by small primer sequences of approximately 8 to 10 base pairs (RABOUAM *et al*., 1999). Dominant markers such as RAPD can be easily developed, even for species without prior genetic information at low costs (MUELLER & WOLFENBARGER, 1999). Consequently, RAPD markers represent molecular tools that help answer questions about variations among individuals and populations (VON EGGELING & SPIELVOGEL, 1995; CUSHWA & MEDRANO, 1996; HASSANIEN *et al*., 2004).

The current paper reports on the association among morphometric, genetic, macroenvironmental, and microenvironmental data of nine populations of *Eupemphix nattereri*, from the State of Goiás. Our hypothesis is that it is possible to infer the population structure of *Eupemphix nattereri* by investigating the levels of similarity and genetic diversity between and among populations of this anuran. Thus, it was determined the extent of genetic differentiation of *Eupemhix nattereri* in Central Brazil using RAPD markers and, it was also evaluated the potential correlations among morphological variation, abiotic factors, and genetic differentiation.

MATERIALS AND METHODS

Study Area. All nine study areas were located in State of Goiás, central Brazil, between parallels 14º N and 19º S and the meridians 52.5º W and 40º E, occupying an area of 281,250 Km^2. The vegetation of these areas is composed by different physiognomies of the Cerrado biome, forming savanna-like vegetation in the well-drained interfluves, and gallery forests along streams and rivers (RATTER *et al*., 1998).

A total of 132 specimens of *E. nattereri* were collected at the nine municipalities (Fig.1), during the rainy season of 2002-2004, between October and March. Sampling localities, sample sizes, coordinates, micro environmental characteristics of each municipality are listed in Table I. Voucher individuals were deposited in the Zoological Collection of the Universidade Federal de Goiás (ZUFG).

Morphometric analysis. Eleven external body measurements were taken from each of the 132 adult specimens of *E. nattereri*. All the measurements are expressed in millimeters. Measured specimens were fixed in 10% formalin and maintained in 70% ethyl alcohol. For measurements we used an ocular micrometer in a stereomicroscope and calipers to the nearest 0.05 mm. Raw morphometric data were log-transformed to perform a parametric statistical analysis. The descriptions of each measurement are listed in Table II.

Molecular data. Genomic DNA was purified from 20mg of frozen liver with DNA purification Kit, according to manufacture's procedures. Amplification reactions were performed in a final solution of 20µl with 2.5 ng of genomic DNA, 10X reaction buffer, 2mM of $MgCl_2$, 0.26 mM of each dNTP, 10 ng of a single primer (Operon Technologies), 0.5 U of Taq DNA-polymerase and double distiled H_2O. The cycling amplification parameters were: initial denaturation at 96ºC for 3 min, followed by 40 cycles of denaturation for 1 min at 92ºC, annealing for 1 min at 35º C and elongation for 1 min at 72ºC. Ten microliters of each reaction product were loaded in a horizontal 1.5% agarose gel and electrophoresed at 5V/cm. The images obtained by each gel were captured using a video-documentation system and analyzed with Image Master 1D software (Total Lab, Amersham Pharmacia Biotech, USA).

A total of 40 primers were tested to select those with the best amplification pattern. The presence of a determined locus was confirmed by the software Master 1 D (Total Lab, Amersham Pharmacia Biotech, USA) by verifying the peaks of the bands during gel analyses. Loci with peaks smaller than 20 pixels, after two repetitions, were excluded from the analyses (Fig. 2).

After this initial evaluation, eigth primers (Tab. III) were used in the analysis of DNA polymorphism between and among populations. The eigth RAPD primers produced a total of 82 distinct, reproducible bands that

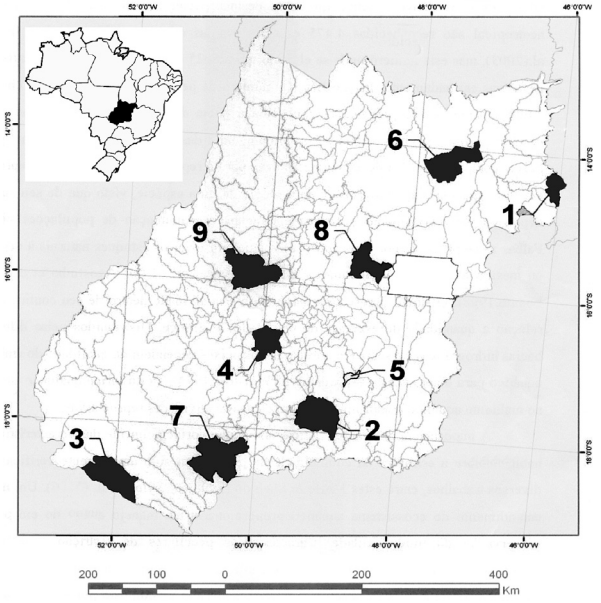

Fig. 1. Map of the State of Goiás, showing the nine sample sites of *Eupemphix nattereri* Steindachner, 1863 (1, Mambaí; 2, Morrinhos; 3, Aporé; 4, Palmeiras de Goiás; 5, Cristianópolis; 6, Alto Paraíso; 7, Quirinópolis; 8, Cocalzinho de Goiás; 9, Goiás).

Table I. Geographical and environmental data for the sampling sites of *Eupemphix nattereri* Steindachner, 1863 populations in Central Brazil (1, Mambaí; 2, Morrinhos; 3, Aporé; 4, Palmeiras de Goiás; 5, Cristianópolis; 6, Alto Paraíso; 7, Quirinópolis; 8, Cocalzinho de Goiás; 9, Goiás).

Municipalities	N	Latitude	Longitude	Air temperature (°C)	Humidity (%)	Elevation(m)
1	20	14º29'16''	46º06'47''	32	51	709
2	24	17º43'54''	49º06'03''	24	80	771
3	13	18º57'55''	51º55'35''	25	60	538
4	10	16º48'18''	49º55'33''	28	84	596
5	13	17º11'96''	48º42'14''	20	70	802
6	21	14º07'57''	47º30'36''	25	67	1186
7	13	18º26'54''	50º27'06''	27	63	541
8	12	15º47'40''	48º46'33''	25	85	1152
9	08	15º56'04''	50º08''25''	24	73	802

Table II. Standardized morphometric measurements carried out on specimens of *Eupemphix nattereri* from central Brazil.

Measurement	Abbreviation	Description
Snout-vent length	SVL	From tip of snout to posterior margin of vent
Femur length	FL	Cloacae to Knee
Head Wide	HW	Measured between mean edges of head
Anterior interorbital distance	AID	Distance between anterior edges of eyes
Eye-nostril distance	END	Distance between anterior edge of eye and posterior edge of nostrils
Snout-nostril distance	SND	Distance between anterior egde of snout and posterior edge of nostrils
Foot length	F	Tip of longest toe to back of heel
Tibia length	TL	Knee to heel
Head length	HL	Distance between anterior and posterior edge of head
Interorbital distance	IO	Shortest distance between eye sockets
Tympanum diameter	TD	Maximum distance between rims of tympanum

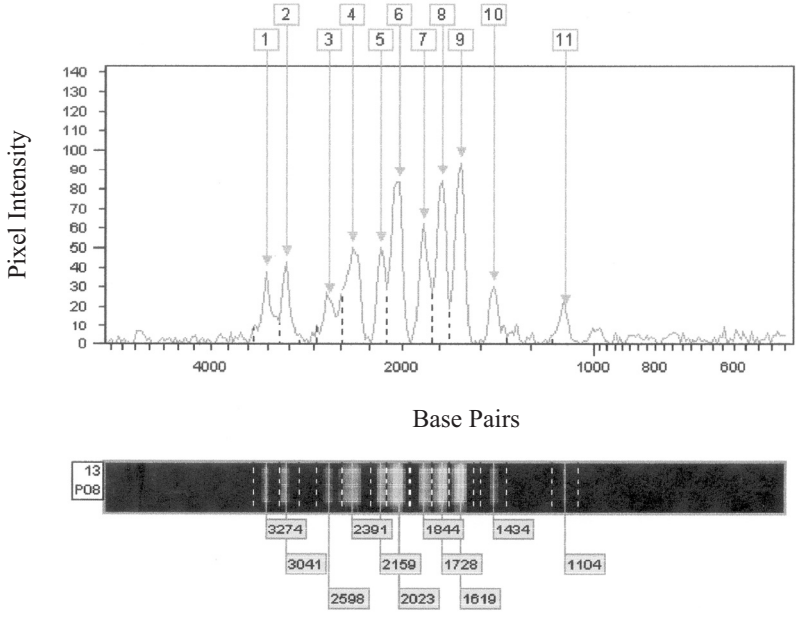

Fig. 2. Results of the RAPD marker analysis for the primer OPA13, obtained from one individual of *Eupemphix nattereri* Steindachner, 1863 from Palmeiras municipality, central Brazil, using the software Image Master 1D (Amersham Pharmacia Biotech, USA). The amplicons varied from 1.104 to 3.274 bp and band intensity ranged from 25 to 90 pixels for eleven amplified *loci*. Peak cut off < 20 pixels (1, *locus* of 3274 bp; 2, *locus* of 3041 bp; 3, *locus* of 2598 bp; 4, *locus* of 2391 bp; 5, *locus* of 2159 bp; 6, *locus* of 2023 bp; 7, *locus* of 1844 bp; 8, *locus* of 1728 bp; 9, *locus* of 1619 bp; 10, *locus* of 1434 bp; 11, *locus* of 1104 bp).

comprised the complete data set. A binary matrix was constructed from the gel readings, where the individuals were genotypically characterized for presence (1) and absence (0) of bands.

Statistical analysis. To determine the degree of morphological differentiation between and among populations, and to identify which biometric parameter most contributed for the variation in morphology, analyses of variance (ANOVA) were performed. Components of variance of ANOVA model II permitted the estabilishment of the variation between and among local populations, for each trait. Patterns of morphometric

discrimination were examined by canonical analysis or multiple discriminant analysis (NEFF & MARCUS, 1980).

The eleven morphometric characteristics were log-transformed, the eigenvalues and eigenvectors were obtained by the product of the variance-covariance matrix between and among populations (NEFF & MARCUS, 1980). The Mahalanobis distance squared (MDS) obtained by the canonical scores, was used to visualize the pattern of morphometric differentiation among the nine populations. All analyses were performed using the software SYSTAT/SYSGRAPH (BROWNE & MELS, 2000).

Analysis of molecular variance (AMOVA) of the genetic matrix was obtained by Euclidean distances between all pairs of haplotypes (EXCOFFIER *et al*., 1992). AMOVA was performed by partitioning the total variation among and within population, using that Euclidean distances. Total variation was expressed by the Φ_{st} coefficient and the significance was tested using 1,000 permutations. All statistical analyses were performed with WINAMOVA software, from L. Excoffier (University of Geneva). Genetic similarity dendograms among the nine populations were constructed by using the Φ_{st} coefficients and the UPGMA grouping method (SNEATH & SOKAL, 1973). The significance of the dendogram was tested by a cophenetic correlation with 5,000 permutations, using the software NTSYS 1.5 (ROHLF, 1989).

Mantel test (MANLY, 1997) using NTSYS 1.5 (ROHLF, 1989) was used to determine the significance of correlations using matrices of pairwise distances between populations, with 1,000 randomizations. Microenvironmental variables used in the analyses included: relative air temperature, air humidity, and altitude. Macroenvironmental data were obtained from the Atlas of Biosphere (SET, 2002), using a grid formate of *ArcView* archive. All cells from the grid contained the coordinates for each municipality.

The following distance matrices were used in these analyses: (a) the morphological matrix of morphometric distances (Mahalanobis distances) among populations; (b) the genetic matrix obtained by AMOVA; (c) the geographical distance matrix; (d) the matrix of Euclidean distances among populations, using standardized macroenvironmental data (media=0 and variance=1); and (e) the matrix of microenvironmental data.

Table III. Selected primers, primers sequences, and the number of polymorphic *loci* used in the population study of *Eupemphix nattereri* Steindachner, 1863 from central Brazil.

Primers	Primers sequences 5´→3´	Polymorphic loci
OPA13	CAGCACCCCAC	14
OPB04	GGACTGGAGT	7
OPB06	TGCTCTGCCC	7
OPB07	GGTGACGCAG	13
OPB10	CTGCTGGGAC	10
OPB11	GTAGACCCGT	12
OPB18	CCACAGCAGT	6
OPC20	ACTTCGCCAC	12
Total		81

RESULTS

ANOVA detected differences among populations (Tab. IV) related to the 11 morphometric characteristics. Canonical Variates Analysis confirmed the significant difference among populations (λ de Wilks= 0.17; P= 0). The first canonical variable (VC1) detected differences among the nine populations mainly for SVL, HW and HL. The second canonical variables (CV2) which indicate limb measurements, such as FL, F and TL, exhibited positive correlations, demonstrating morphometric variation among anuran populations (Tab. V). The scores of the two canonical variables for each population are shown in Fig. 3.

The size of the amplicons varied from 100 to 2,000 base pairs. The number of RAPD fragments by primer varied from 6 to 14 (Tab. III). AMOVA indicated a variation of 70% between populations and 30% among populations (Tab.VI). The Φ_{st} coefficient indicated a significant difference among populations, suggesting low gene flow among individuals and high fidelity to the breeding site. UPGMA showed genetic distances among the nine populations (Fig. 4) and the cophenetic correlation coefficient was r=0.80.

Macroenviromental data had mean pluviometric indexes of 91.9 (SD=$\pm$6.8), annual temperatures of 23ºC (SD= $\pm$0.7), and relative air humidity of 68.5%

Table IV. Results of Analysis of Variance (ANOVA) for 11 morphometric traits of *Eupemphix nattereri* Steindachner, 1863, including variance components among populations (V%) and the levels of significance at 95% and 99% (*, P<0.05; **, P<0.01; ***, NS=non significant).

Morphometric variables	F	V(%)
SVL	8.7**	35.8
HW	6.8**	30.2
FL	4.1**	20.9
F	5.0**	24.1
TL	3.0**	16.3
IO	2.9**	15.7
TD	3.4**	7.9
SND	4.0**	20.4
END	2.0*	11.4
HL	4.7**	23.2
AID	0.9***	5.7

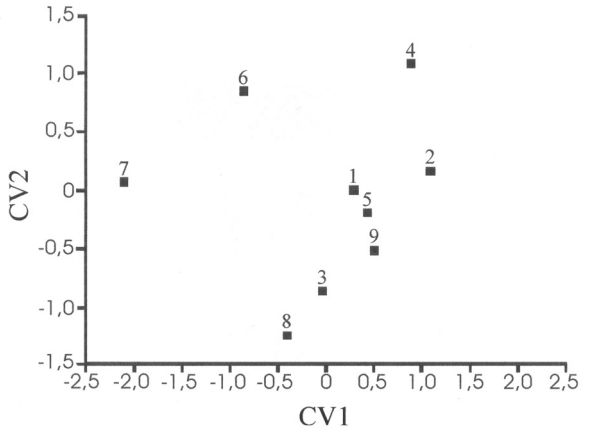

Fig. 3. Results of Canonical Variable Analysis, showing the relative position of each population of *Eupemphix nattereri* Steindachner, 1863. Numbers 1 to 9 refer to the populations listed on table I.

(SD=±2.0). Mean Air temperatures, humidity, and altitude were 25.5ºC (SD=±3.3), 70% (SD=±11), and 789 m (SD=±239), respectively.

Results of the correlation matrix obtained by Mantel test are summarized in Fig. 5. There was a significant statistical correlation (r=0.75; P=0.001) between geographical and macroenvironmental matrixes (pluviometric indexes, mean temperature, and air humidity). A marginally positive correlation (P=0.08) was found between morphometric and genetic variation (Fig. 6), which could be explained by evolutionary forces, selecting the genetic make up responsible for morphological traits in *E. nattereri*. No correlation was observed between morphometric variation and

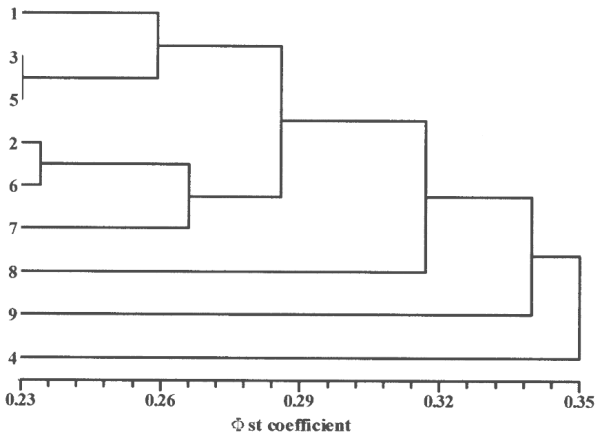

Fig. 4. Dendogram of genetic distance among nine populations of *Eupemphix nattereri* Steindachner, 1863, defined by UPGMA clustering method, based on genetic divergence (Φ_{st}).

Table V. Canonical coefficientes of original morphometric traits of *Eupemphix nattereri* Steindachner, 1863 logarithmized at the first two axes.

Variables	CV1	CV2
SVL	-0.72	0.26
HW	-0.63	-0.05
FL	-0.33	0.45
F	-0.26	0.47
TL	-0.16	0.41
IO	-0.27	0.12
TD	-0.40	-0.09
SND	-0.43	0.28
END	-0.32	-0.05
HL	-0.52	-0.05
AID	-0.15	0.14

Table VI. Analysis of Molecular Variance (AMOVA) based on 82 polymorphic RAPD *loci* for nine populations of *Eupemphix nattereri* Steindachner, 1863 from Central Brazil (DF, degree of freedom).

Source of variation	DF	Sum of squares	Mean Square	Variance	Total variance (%)	P	Φ_{st} coefficient	Bartlet´s statistics
Population	8	777.1	97.1	5.8	29.9%	<0.001	0.29	0.8
Individual	123	1665.9	13.5	13.5	70.1%			

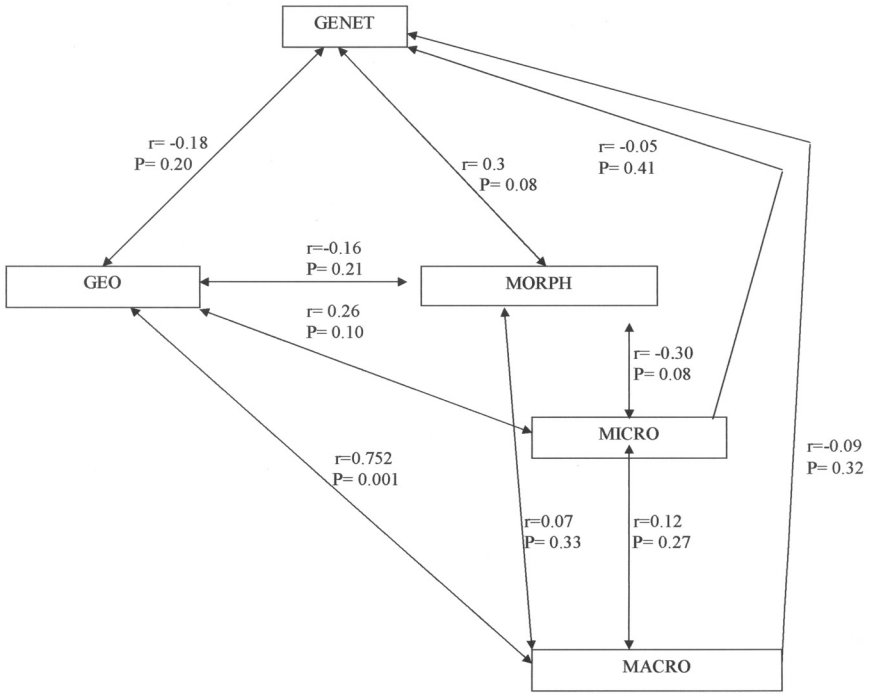

Fig. 5. Resume of Mantel test performed among geographical (GEO), genetic (GENET), morphometrical (MORPH), microenvironmental (MICRO) and macroenvironmental (MACRO) data.

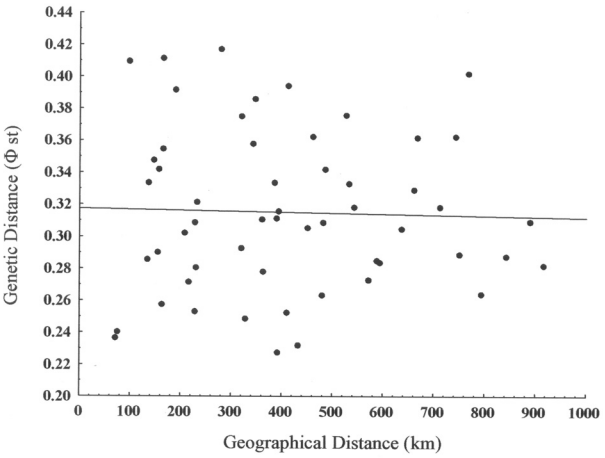

Fig. 6. Graphical representation of Mantel test between geographical and genetic distances for *Eupemphix nattereri* Steindachner, 1863 population in Central Brazil.

microenviromental data. Although significant correlations were not obtained between morphometric distance and macroenviromental data, all nine populations of *E. nattereri* reported here did not present a spatial pattern with respect to genetic and geographical distances.

DISCUSSION

Body size and head length were the two variables that mostly contributed for significant differences related to morphometric traits, as observed for other groups (CASTELLANO & GIACOMA, 1998; CASTELLANO *et al.*, 1999; BABIK & RAFINSKI, 2000; CASTELLANO *et al.*, 2000; MÉNDEZ *et al.*, 2004, ROSSO *et al.*, 2004). According to our results, the variation in morphological traits did not present a spatial pattern, showing a substantial overlap in overall morhometric variables of the studied populations.

In a study performed with 22 populations of *Physalaemus cuvieri* Fitzinger, 1826 also from the Cerrado, showed that individuals located in dryer sites in the north of State of Goiás had larger sizes (Juliano & Bastos, unpublished data). However, the difference in body size along a latitudinal gradient of humidity could happen because animals from wet climates reproduce earlier than populations in dryer climates; thus, size is only a consequence of climate-driven reproductive activity (CASTELLANO *et al.*, 2000). Other component that may influence morphological variation in amphibians is the specific vegetation at each area. Most morphological traits correlates with one another, either body shape or body size varies according to their habitat (ROSSO *et al.*, 2004). In our study, all populations of *E. nattereri* were sampled in areas of Cerrado. It is known that this vegetation is a mosaic, and even in the closest areas there is a wide variation in the kind of vegetation throughout Cerrado ecossystems (BRASILEIRO *et al.*, 2005).

In anuran amphibians, adult body sizes depend on many factors including time of metamorphosis, growth rate before and after maturity, age of maturity, and longevity (ROSSO *et al.*, 2004). Moreover, genetic and environmental differences can cause diversity in ontogenetic development, resulting in body size variation. Thus, besides ecological factors, genetic, physiological and ontogenetic factors may also explain the wide range of trait variations found in *E. nattereri*. Our data suggest that ecological and genetic factors act in synergism to increase specimen's variation. Because of the complex patterns of morphometric traits, the environmental variables used in this study could not account for the intricate steps beyond the evolutionary mechanisms of Cerrado's anurans. Consequently, the correlation observed between phenotypic and environmental matrixes of distances was only marginal.

It must be consider that other factor in local scales, not analyzed in this study, such as pond temperatures and pH, during the development of tadpoles could influence morphometric traits, as described to *Rana arvalis* (RÄSÄNEN *et al.*, 2003). The water has a potential effect on morphology and larval development and, therefore, is a relevant factor affecting body size in ectotherms (Atkinson, 1996).

With respect to the genetic differentiation, high level of diversity was found (Φ_{st}=0.3) among the studied populations. There was not a statistically significant correlation between genetic and geographical distances, indicating that sites geographically close were not genetically similar. Many studies of intraspecific genetic variation in anurans have been conducted along extended scales, of many kilometers, and their results show a substantial variation among populations (LAMPERT *et al.*, 2003; TRAKIMAS *et al.*, 2003; ZEISSET & BEEBEE, 2003; PALO *et al.*, 2004; TELLES, 2005). Thus, our data pointed out a strong evidence to support the previous statement even for places situated as close as 50 km.

According to NEWMAN & SQUIRE (2001), there are explanations, other than just limited gene flow, both genetic and evolutive for local differentiation in neutral markers. At fine spatial and temporal scales, where mutation is less likely to be an important factor, population genetic structure depends on the relative rates of gene flow and dynamics of population size, which determine the rate of genetic drift and turnover of local populations. Even when there is some exchange of individuals among populations, if effective population size is small, fine-scale differentiation may result from high rates of drift and that may be the case for the populations used in this study.

Therefore, a statistical significant correlation, between morphometric and genetic data in the populations of *Eupemphix nattereri* indicates that the divergence among populations of this species must follow the model of isolation by distance, and even morphometric and genetic variations could be influenced by a spatial structure, although in a fine scale. The results of the current study showed no sufficient evidence to provide no more than a tentative explanation for the spatial structure of *E. nattereri* population in central Brazil. However, as genetic variation is probably neutral and therefore could not be associated to morphometric variation, the trends for a positive correlation could only be explained by isolation by distance.

In conclusion, the results of the current study indicated limited gene flow among the populations of

Eupemphix nattereri. Several recent studies showed that amphibians avoid open habitats, as fields and roads, moving mostly in forest areas (MADISON & FARRAND, 1998; ROTHERMEL & SMELISTCH, 2002). This peculiar behaviour occurs because of increased risks of desiccation and predation in open areas (SPEAR *et al*., 2005). As the sample sites were representative of Cerrado biome, characterized by open areas, amphibian behaviour could be responsible for the reduced gene flow, causing simultaneously genetic and morphological differentiation among populations.

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