

## Genetic variability in species of bats revealed by RAPD analysis

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**ABSTRACT.** Random amplified polymorphic DNA molecular marker was utilized as a means of analyzing genetic variability in seven bat species: *Molossus molossus*, *M. rufus*, *Eumops glaucinus*, *E. perotis*, *Myotis nigricans*, *Eptesicus furinalis*, and *Artibeus planirostris*. The determination of genetic diversity was based on 741 bands produced by a 20-random primer set. Only eight bands were considered monomorphic to one species. The greatest number of bands and the most polymorphic condition were exhibited by *M. molossus*, followed by *M. nigricans*, *A. planirostris*, *E. furinalis*, *E. glaucinus*, *M. rufus*, and *E. perotis*. Nei's genetic diversity index in the seven species considering the 20 primers was not greater than 0.22, but some primers were capable of detecting values between 0.39 and 0.49. Nei's unbiased genetic distance values and the UPGMA clustering pattern show that *M. molossus* and *M. rufus* have a close genetic relationship, unlike that observed between *E. perotis* and *E. glaucinus*. The latter was clustered with *A. planirostris* and *E. furinalis*. The low values for genetic diversity and distance observed indicate a genetic conservatism in the seven species.

The fluorescent *in situ* hybridization experiments did not confirm a monomorphic condition for the eight bands identified, demonstrating that the monomorphic bands obtained by random amplified polymorphic DNA are insufficient for the identification of bat species.

**Key words:** Chiroptera, Fluorescent *in situ* hybridization, Random amplified polymorphic DNA, Genetic variation, Bats

## INTRODUCTION

*Artibeus* (Phyllostomidae), *Molossus*, *Eumops* (Molossidae), *Myotis*, and *Eptesicus* (Vespertilionidae) are among the most variable genera of Chiroptera. Recognition of the species of these genera is based, in general, on morphological and cranial characteristics. However, such characteristics are of limited value because they reveal considerable intraspecific variation and differences among species are small (Freeman, 1981; Wetterer et al., 2000; López-González and Presley, 2001; Ruedi and Mayer, 2001; Lim et al., 2004; Bickham et al., 2004; Timm and Genoways, 2004). Despite the evident effort to better understand systematic relationships among the diverse species, some issues about systematic relationships as well as problems with identification remain unsolved.

In modern studies on the phylogeny and taxonomy on the intraorder level, the molecular analysis role of PCR-based markers has been of great significance. For example, molecular markers have been obtained using the following methods: random amplified polymorphic DNA (RAPD), SSR-PCR, Sine-PCR, and AFLP, as well as others involving amplification and sequencing of nuclear and mitochondrial genes (Wright et al., 1999; Corneli and Ward, 2000; Bannikova et al., 2002; Kawai et al., 2002; Newton et al., 2003; Lim et al., 2004; Callejas et al., 2004; Panaram and Borowsky, 2005).

In Chiroptera, new characteristics available as a result of molecular approaches have helped to give new strength to systematic studies as well as to knowledge concerning the evolutionary potential of populations and species (Wright et al., 1999; Ditchfield, 2000; Kawai et al., 2003; Hooper et al., 2003; Newton et al., 2003; Van Den Bussche and Hooper, 2004; Dávalos and Jansa, 2004; Porter and Baker, 2004; Ferreira et al., 2005). Despite the fact that most molecular investigations have attempted to characterize mitochondrial DNA sequences, the RAPD method is among the molecular tools that provide most information about some vertebrate species, especially with regard to the assessment of genetic variation within species.

The RAPD technique detects randomly amplified polymorphic DNA fragments in PCR with a single arbitrary primer of 8-10 bp (Williams et al., 1990). The number of fragments amplified and the degree of polymorphism in eukaryotic species depend on the nucleotide sequence, the secondary structure and the number of primers used for each RAPD assay. These features of the RAPD assay make it possible to detect DNA polymorphism in the absence of specific nucleotide sequence information. Thus, the RAPD-PCR method has been used successfully to detect genetic variation within and between related species and populations of different organisms, making it possible to conduct molecular phylogenetic studies (Callejas and Ochando, 1998; Mamuris et al., 1999; Prioli et al., 2002; Almeida et al., 2001, 2003; Hassanien et al., 2004).

Bats are of particular interest to evolutionary biology since they have a capacity for long-distance dispersal through flight and, indeed, there are differences at the intraspecific level in molecular patterns and genetic variation that could be markedly unlike those of other small mammals that do not fly.

In general, high dispersal abilities are associated with a low population structure (Bohonak, 1999), which has been reported for some mobile species, including birds (Avice, 1994), cetaceans (Larsen et al., 1996) and bats (McCracken and Gassel, 1997; Petit and Mayer, 1999; Ditchfield, 2000; Burland and Wilmer, 2001).

Studies describing molecular patterns of intraspecific geographical differentiation in bats have indicated a low level of genetic divergence and a limited geographical structure in species with continental distribution (Patterson et al., 1992; Sinclair et al., 1996). However, high-intraspecific divergence levels with clearly defined geographical structuring have also been observed. These different results can be attributed to the different molecular markers used in the various studies.

There are very few estimates of genetic variability determined by variable markers such as RAPD. In the present study, therefore, the RAPD procedure was used in order to characterize genetic variability for seven species of bats belonging to five genera (*Artibeus planirostris*, *Molossus molossus*, *Molossus rufus*, *Eumops glaucinus*, *Eumops perotis*, *Myotis nigricans*, and *Eptesicus furinalis*), and also to identify a species-specific marker which would then be employed as a probe in fluorescent *in situ* hybridization (FISH) experiments.

## MATERIAL AND METHODS

### DNA extraction and random amplified polymorphic DNA analysis

The present study analyzed ten specimens belonging to each of six species: *Artibeus planirostris*, *Molossus molossus*, *Molossus rufus*, *Eumops glaucinus*, *Eumops perotis*, and *Myotis nigricans*, and six specimens of *Eptesicus furinalis* collected in a single locality (São José do Rio Preto 20°48'S, 49°24'W, São Paulo State). Total genomic DNA was isolated from lung and liver tissues according to Sambrook et al. (1989). The specimens analyzed are deposited in the Chiroptera Collection of São Paulo State University (UNESP) at São José do Rio Preto, Brazil.

A set of 20 random decanucleotide primers (Invitrogen do Brasil) that produced accurate bands (Table 1) was used in the detection of polymorphism among populations. Amplification conditions were according to Williams et al. (1990) with some modifications. The reactions were performed in a total volume of 15  $\mu$ L containing 25 ng genomic DNA, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP, 50 pmol of a single 10-base primer, 0.5  $\mu$ g/mL BSA, and 1 U Taq DNA-polymerase. PCR was performed in a thermocycler (Perkin Elmer 2400) programmed at 94°C for 3 min, followed by 43 cycles of 30 s at 94°C, 1 min at 35°C and 2 min at 72°C. After the last cycle, there was a 5-min extension at 72°C.

RAPD-PCR technical limitations were resolved following a strict protocol with standardized conditions, repeating the amplification reactions two or more times.

The RAPD products were resolved by vertical electrophoresis on 8% polyacrylamide gels, run with 1X TBE buffer (Tris, boric acid, 0.5 M EDTA, pH 8.0) and subsequently stained with silver nitrate.

Bands occurring only in the ten specimens of a particular species were recognized as

**Table 1.** Codes and sequences of the random primers used for detection of genetic variation.

Primer code	Sequence (5' to 3')	Primer code	Sequence (5' to 3')
P1	GAA ACG GGT G	P11	CAG GCC CTT C
P2	CAG CAC CCA C	P12	TGG GGC TGT C
P3	GTT GCG ATC C	P13	CCC GTT GCC T
P4	ACA CCG GAA C	P14	GTC CGG AGT G
P5	GAC TGC CTC T	P15	TTC AGG GCA C
P6	GTG ACC GAG T	P16	TGT GGC AGC A
P7	AGC AGC GCA C	P17	ACA GCC TGC T
P8	AGA CGA TGG G	P18	GGT CGA TCT G
P9	GAC GTG GTG A	P19	GGG TAA CGC C
P10	GTG AAT GCG G	P20	TGC GAG AGT C

monomorphic (species-specific) and excised from polyacrylamide gels, reamplified in the presence of biotinylated nucleotide (14-dATP; Gibco), and the DNA used as a probe in FISH.

### Data analysis

RAPD patterns of specimens were determined by direct comparison of the amplified DNA electrophoresis profiles. Fragments were scored as 1 if present or 0 if absent, based on a molecular size standard marker, and the data obtained were analyzed as binary variables. Each band was considered to be an allele of a locus. The number and frequencies of polymorphic loci, gene diversity indices and unbiased genetic distances were estimated according to Nei (1978) using POPGENE 1.31 software (Yeh and Boyle, 1997). Clustering was performed by the unweighted pair-group method of analysis (UPGMA) with statistical support obtained by bootstrapping with 1000 interactions (PAUP, Version 4.0; Swofford, 2002).

### Fluorescent *in situ* hybridization

FISH was performed to verify the specificity of species-specific bands. The probes consisting of the biotinylated monomorphic fragments were applied onto the interphasic nucleus of specimens of the band-carrying species and other species analyzed. Cellular suspensions were obtained from primary fibroblast cultures according to Morielle-Versute and Varella-Garcia (1995). Dividing cells were prepared following mitotic arrest with colchicine ( $4 \times 10^{-4}$  mol/L) for 30 min, hypotonic treatment (10% sodium citrate) for 30 min, and fixation in 3:1 methanol:acetic acid (v/v). Prior FISH slides were pretreated with RNase (0.1 mg/mL) and pepsin (0.008%) and denatured in 70% formamide/2X SSC, pH 7.0, at 72°C for 2-3 min. The probe consisting of 1 µg of the biotinylated fragments combined with 50 µg salmon sperm DNA and 25 µg mouse Cot-1 DNA, was denatured in a water bath at 72°C for 5 min, quickly chilled on ice, centrifuged for 2 s and applied to selected areas of the slide (27 mL/slide on 22 x 50-mm coverslips). Hybridization was performed for 16 h at 37°C in a humidified chamber. Slides were washed three times, for 5 min each time, in 50% formamide/2X SSC and in 2X SSC at 37°C. Finally, they were washed twice in 4X SSC, 0.05% v/v Tween 20, pH 7.0, at room temperature. Detec-

tion was performed with fluorescein-isothiocyanate conjugated to avidin. Cells were counter-stained with propidium iodide in fluorescence antifade solution (0.3 mg/mL). Analysis was performed in a Zeiss Axiophot fluorescence microscope with interference filter sets for fluorescein-isothiocyanate and Texas-red.

## RESULTS AND DISCUSSION

### Banding polymorphism patterns

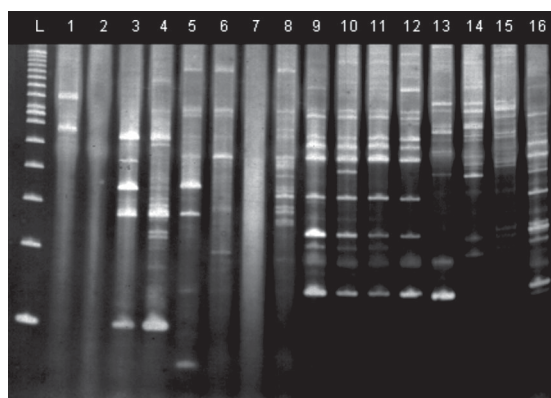
Using RAPD-PCR in order to analyze intra- and interspecific genomic polymorphism in Chiroptera, it was possible to obtain a significantly greater number of amplified fragments. The 20 primers examined produced a total of 741 scorable bands (Table 2). On average, 25 to 48 RAPD fragments with a length of 200 to 1000 bp were detected. The number of bands was variable in each species. *M. molossus* was the species that produced the greatest number of bands (369), and *E. glaucinus* the lowest (239). The number of polymorphic bands and the relative and average frequencies also varied.

**Table 2.** Primers, number of polymorphic bands and polymorphism frequencies observed in the seven species analyzed.

Primer	Species (number of polymorphic/number of bands)							Total
	<i>M.m.</i>	<i>M.r.</i>	<i>E.g.</i>	<i>E.p.</i>	<i>M.n.</i>	<i>E.f.</i>	<i>A.p.</i>	
1	12/17	17/18	13/13	4/10	13/13	13/14	10/10	33
2	17/18	13/17	17/17	15/17	10/22	11/13	5/5	38
3	22/24	8/8	11/13	3/5	16/20	13/15	13/13	48
4	12/18	8/11	6/12	7/11	19/23	13/14	16/16	42
5	10/15	6/8	15/15	13/15	23/24	8/10	9/9	39
6	23/25	12/16	12/12	8/16	7/12	13/15	8/8	39
7	24/28	15/19	16/17	15/20	21/26	14/16	12/12	45
8	18/26	15/26	23/23	17/22	23/29	10/12	19/19	42
9	14/17	6/6	14/14	4/10	16/18	11/13	10/10	38
10	20/22	12/13	8/12	2/7	18/18	9/17	13/14	41
11	13/14	4/10	15/15	13/16	8/18	12/14	17/17	35
12	17/18	13/15	7/7	7/13	10/12	18/21	10/10	33
13	17/17	11/12	7/7	6/12	9/13	15/17	14/14	36
14	13/13	9/10	11/11	6/10	16/17	6/7	16/16	38
15	7/9	9/11	11/11	15/15	18/22	11/13	7/9	35
16	16/19	9/9	1/3	2/8	14/15	12/15	16/17	29
17	17/19	9/16	12/12	8/9	17/20	7/9	12/13	35
18	18/19	15/15	9/13	5/11	16/16	14/14	11/11	35
19	19/20	9/9	1/3	11/13	17/18	15/19	16/17	35
20	10/11	11/11	7/9	2/7	6/6	8/9	8/8	25
Polymorphism (%)	43.0%	28.5%	29.4%	23.1%	40.1%	31.4%	32.9%	

*M.m.* = *Molossus molossus*; *M.r.* = *M. rufus*; *E.g.* = *Eumops glaucinus*; *E.p.* = *E. perotis*; *M.n.* = *Myotis nigricans*; *E.f.* = *Eptesicus furinalis*; *A.p.* = *Artibeus planirostris*.

The most polymorphic species was *M. molossus* (43%), followed by *M. nigricans* (40.1%), *A. planirostris* (32.9%), *E. furinalis* (31.4%), *E. glaucinus* (29.4%), *M. rufus* (28.5%), and *E. perotis* (23.1%). The observed DNA polymorphism did not only consist of the presence or absence of fragments with a particular length in the RAPD patterns, but also of a change in the intensity of amplification of fragments with the same length (Figure 1). Only eight (1.1%) bands were monomorphic for one of the seven species: four in *M. nigricans* (P7 - 130 bp, P6 - 150 bp, P11 - 226 bp, and P2 - 275 bp), two in *M. molossus* (P4 - 485 bp and P6 - 133 bp), one in *E. glaucinus* (P4 - 231 bp), and one in *E. furinalis* (P7 - 110 bp). These fragments were isolated, reamplified and utilized as a probe in FISH analysis.



**Figure 1.** RAPD patterns exhibited by four individuals of *Myotis nigricans* (lanes 1-4), *Eumops glaucinus* (lanes 5-8), *E. perotis* (lanes 9-12) and *Eptesicus furinalis* (lanes 13-16) using primer P1. L = molecular weight marker (ladder 100 bp, GIB BRL).

The results observed show that the RAPD-PCR assay is capable of revealing polymorphism in species of bats. All 20 random decanucleotide primers produced at least one polymorphic fragment. Some authors have pointed out that, although RAPD-PCR is a powerful technique for detecting random amplified polymorphic DNA and several reasons exist for the amplification of DNA regions, the main shortcoming of this technique is its sensitivity to changes in reaction conditions, where the use of markers often results in imperfect estimation of genetic distances between taxa of supraspecies rank (Rothuizen and Van Wolferen, 1994). In our experiments the results obtained by optimized and repeatable conditions made differences in banding patterns an improbable RAPD artifact.

### Gene diversity and genetic distance

The 741 polymorphic bands were taken as RAPD markers and combined in a simple matrix of binary data, with scores for the presence or absence of bands. The Nei gene diversity index within each species was not greater than 0.22, which was observed in *M. molossus* specimens. For the total specimens (66), the index was 0.36. Despite these low values for gene diversity, some primers were capable of detecting values between 0.39 and 0.49 (primers 2, 7, 16, and 8).



Estimates of Nei's unbiased genetic distances between the seven species, and the UPGMA clustering pattern are shown in Table 3 and Figure 2. The distances were low. The highest genetic distance was observed between populations of *E. perotis* and *M. rufus* (0.199) and the lowest between *E. glaucinus* and *A. planirostris* (0.066). The UPGMA dendrogram shows that only the taxa of *Molossus* were clustered together, indicating the close genetic relationship among *Molossus* species. Despite the taxonomic proximity between *E. glaucinus* and *E. perotis*, they were not clustered together. *E. glaucinus* was clustered with *A. planirostris* and *E. furinalis*.

**Table 3.** Nei's (1978) genetic distance between the seven species analyzed.

Species	<i>M.m.</i>	<i>M.r.</i>	<i>E.g.</i>	<i>E.p.</i>	<i>M.n.</i>	<i>E.f.</i>	<i>A.p.</i>
<i>M.m.</i>	****						
<i>M.r.</i>	0.079	****					
<i>E.g.</i>	0.113	0.119	****				
<i>E.p.</i>	0.186	0.199	0.104	****			
<i>M.n.</i>	0.185	0.172	0.141	0.192	****		
<i>E.f.</i>	0.147	0.151	0.109	0.159	0.155	****	
<i>A.p.</i>	0.104	0.089	0.066	0.150	0.132	0.088	****

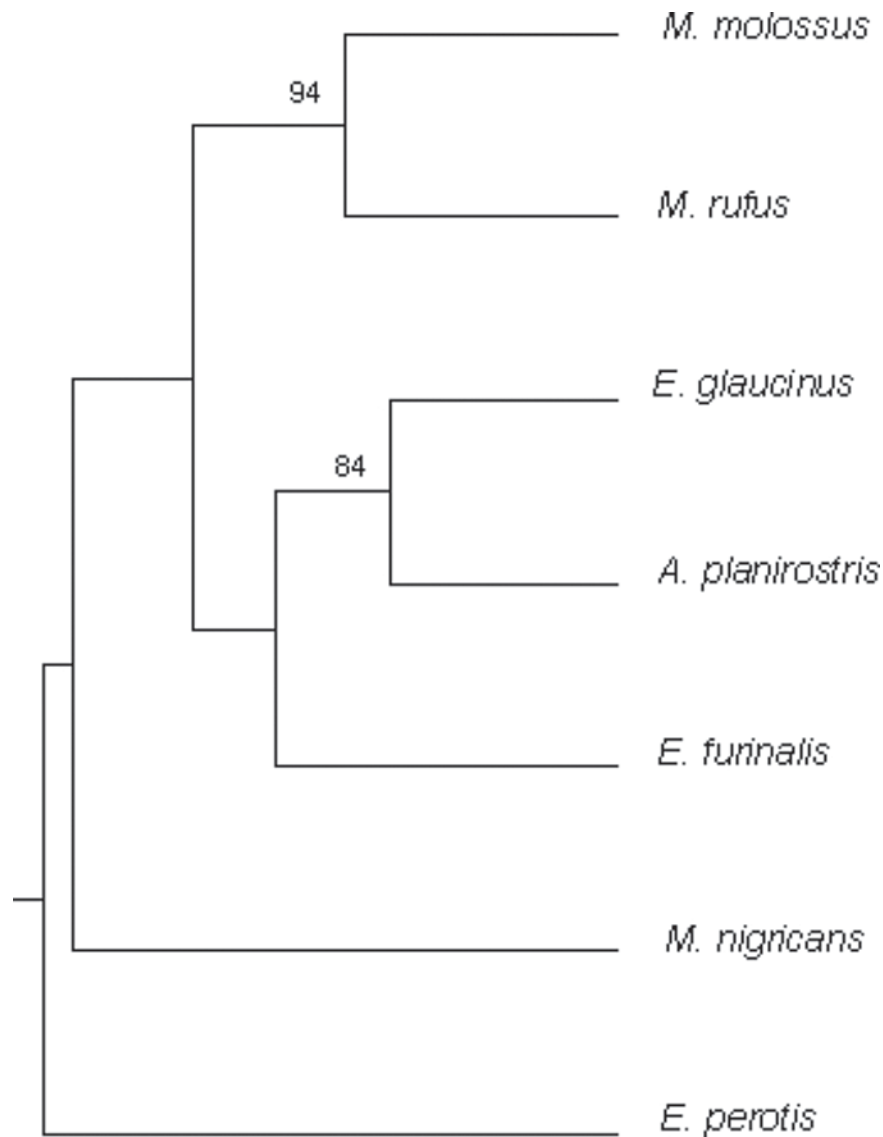
For species abbreviations, see legend to Table 2.

The low RAPD divergence between individuals and species, as derived from the Nei estimate and the UPGMA tree, indicated that the seven species of bats studied show conservatism in RAPD fragment sequences, reinforcing the high genetic similarity estimated by inter- and intraspecific mitochondrial DNA variation, with average sequence divergence usually between 1 and 2.5% (Ditchfield, 2000). However, the genetic variability detected by RAPD demonstrated that there are differences in genetic structure between congeneric species, species of the same family and species of different families. Although much still remains to be understood, RAPD-PCR alleles have been considered to derive from repetitive regions of the genome (Haymer, 1994), and are thus assumed to be neutral markers.

Intra- and interspecific genetic differentiation can be driven by ecological, evolutionary and historical factors, and the knowledge of the effects of genetic variability in species of bats is important for an understanding of how diversity is distributed among populations and species of volant mammals (Miller-Butterworth et al., 2003).

The differences between related bat species have been interpreted as indicating different dispersal capabilities and different levels of gene flow, a scenario that is congruent with documented differences in wing morphology, colony size and number of subspecies (Newton et al., 2003).

It has been pointed out that, although many bat populations show near-panmixia, there is always some degree of population structure due to features of their mating systems or philopatry (Rivers et al., 2005), seen particularly in populations of vespertilionid bats (*Myotis nattereri* and *M. bechsteinii*), which demonstrate an autumn "swarming," a seasonal event that involves the visitation of large numbers of individuals of temperate bat species to underground sites prior to



**Figure 2.** Dendrogram of genetic similarity for the seven species based on the UPGMA method.

hibernation. In these populations, the peculiar genetic feature is high gene diversity caused by mating at autumn swarming sites, allowing interbreeding between bats from otherwise isolated summer colonies (Parsons et al., 2002; Kerth et al., 2003; Veith et al., 2004; Rivers et al., 2005).

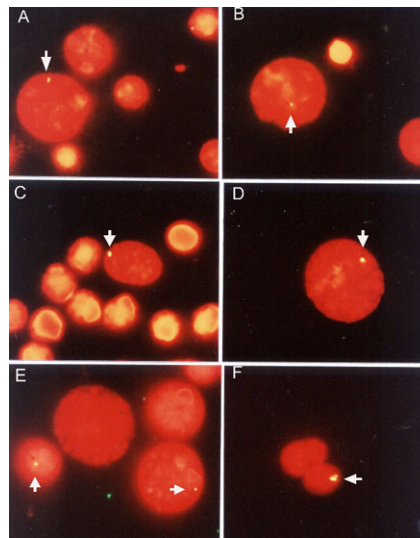
Miller-Butterworth et al. (2003) argue that examining patterns of inter-population genetic diversity in bats can provide valuable information about historical and current evolutionary processes affecting a species. The authors found that genetic structure correlates with local biomes and differentiation in wing morphology, indicating that bats may adapt to local environmental conditions surrounding their roosts.



Despite the morphological variation among species, the differences in polymorphism frequencies and genetic distances observed among the species analyzed in the present study can be interpreted in the same sense. The similarities observed between congeneric and non-congeneric species may be a reflection of adaptation to local biome, occurring in the evolutionary process affecting a species.

### Fluorescent *in situ* hybridization with random amplified polymorphic DNA products

Each of the fragments identified as species-specific DNA sequences (monomorphic) was expected to hybridize with a genome region of that species where it was identified. Despite the monomorphic condition, when the fragments were used as probes in the FISH procedures, they hybridized with nuclear DNA of different species, and did not confirm band specificity. Only one fragment (P4 - 231 bp) was observed hybridized to the nucleus of the species that produced it (*E. glaucinus*) and one of its congeneric species (*E. perotis*). The other seven fragments hybridized with the nucleus of different species (Figure 3).



**Figure 3.** Fluorescent *in situ* hybridization in interphasic nucleus (arrows) with monomorphic fragments probe. A-D: *Eumops glaucinus* nucleus with probes of 110, 231, 130, and 133 bp, respectively. E: *Artibeus planirostris* nucleus with probe of 485 bp. F: *Eptesicus furinalis* nucleus with probe of 275 bp.

One of the critical steps in RAPD analysis is to identify the desired DNA from among contaminating sequences of similar length. Studies involving molecular characterization of the polymorphic fragments have demonstrated that amplified bands often contain more than one sequence arising from technical error, DNA of commensal organisms and fragment rearrangements during PCR amplification (Gu et al., 1999; Rabouam et al., 1999). Despite the use in the present study of polyacrylamide gels, the matrix that produces the highest band resolution level, the results presented here reinforce previous observations that, due to the non-specific nature of RAPD, a single primer can produce similar size amplification products from various regions of

the genome, which may not be resolved on 2% agarose gels, commonly used for the analysis of RAPD-PCR.

FISH is a sensitive method for the identification of homologies of specific DNA sequences with cell genome and chromosomes (Bentivegna et al., 2001). In bats, FISH analysis demonstrated that monomorphic bands obtained by RAPD are not sufficient to identify species. Thus, our results may not be generally applicable to the use of RAPD for the identification of species. However, they show that the use of FISH in RAPD studies may be suitable as a complementary technique in analyses of vertebrate groups where the RAPD-PCR technique alone had been employed for the purpose of species identification.

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