Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Assessing genetic variability in bat species of Emballonuridae, Phyllostomidae, Vespertilionidae and Molossidae families (Chiroptera) by RFLP-PCR

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Genet. Mol. Res. 7 (4): 1164-1178 (2008)
Received July 31, 2008
Accepted August 18, 2008
Published October 28, 2008

ABSTRACT. A PCR-RFLP analysis of the restriction pattern in nuclear (RAG2) and mitochondrial (12S/16S) gene sequences of bat species from the Molossidae, Phyllostomidae, Vespertilionidae, and Emballonuridae families produced a large number of fragments: 107 for RAG2 and 155 for 12S/16S combined in 139 and 402 haplotypes, respectively. The values detected for gene variation

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were low for both sequences (0.13 for RAG2 and 0.15 for 12S/16S) and reflected their conservative feature, reinforced by high values of inter- and intraspecies genetic identity (70-100%). The species with a high gene divergence were variable in the analyses of RAG2 (*Eumops perotis*, *Artibeus lituratus*, and *Carollia perspicillata*) and of 12S/16S (*Nyctinomops laticaudatus*, *C. perspicillata*, and *Cynomops abrasus*), and furthermore, one of them, *C. perspicillata,* also showed the highest intraspecific variation. The species that exhibited the lowest variation for both genes was *Molossus rufus*. In the families, the highest variation was observed in the Molossidae and this can be attributed to variation exhibited by *Eumops* and *Nyctinomops* species. The variations observed were interpreted as a natural variability within the species and genus that exhibited a conserved pattern in the two gene sequences in different species and family analyzed. Our data reinforce the idea that the analyses of mitochondrial and nuclear genes contribute to our knowledge of the diversity of New World bats. The genetic variability found in different taxa suggests that an additional diversity, unnoticed by other methods, can be revealed with the use of different molecular strategies.

Key words: Gene variation; RAG2 gene; 12S/16S gene; PCR-RFLP; Microchiroptera

INTRODUCTION

The order Chiroptera is the second largest order in Mammalia, accounting for about 1116 species (Simmons, 2005a,b; Simmons et al., 2008). They are recorded from all areas of the world except the Arctic and Antarctic and few isolated oceanic islands (Koopman, 1993; Nowak, 1999; Simmons, 2005a,b).

The classification of the highest level of Chiroptera was based on morphological data. More recently, Simmons and collaborators (Simmons, 1998; Simmons and Geisler, 1998, 2002) conducted phylogenetic analyses of family-level relationships based on a variety of different methods for treating taxonomic polymorphisms. They recognized Microchiroptera comprising two infraorders (Yinochiroptera and Yangochiroptera) and seven superfamilies (Emballonuroidea, Rhinopomatoidea, Rhinolophoidea, Noctilionoidea, Nataloidea, Molossoidea, and Vespertilionoidea). However, some other studies including molecular analysis have strongly contradicted many of these groupings (Hutcheon et al., 1998; Kirsch et al., 1998; Van Den Bussche and Hoofer, 2000; Hoofer and Van Den Bussche, 2001; Murphy et al., 2001; Springer et al., 2001; Teeling et al., 2000, 2002, 2003).

Despite that the evolutionary relationships of the order are not fully characterized, there is a consensus at the infraordinal level concerning the monophyletic origin and relationships among the 17 families of Microchiroptera (Smith, 1976; Van Valen, 1979; Novacek, 1980; Pierson et al., 1986; Teeling et al., 2000, 2002, 2005).

The greatest bat diversity is in the Neotropics, with at least 83 genera and 288 species recorded (Mickleburgh et al., 2002; Simmons, 2005a,b; Reis et al., 2007), and despite the large number of morphological characters currently used for the classification and phylogenetic reconstruction of the species of bats, the results of most studies are still incongruent (Koopman, 1970; Nowak, 1999; Simmons, 1998, 2000; Wetterer et al., 2000; Jones et al., 2002). Because of this, many comparative studies to identify the evolutionary patterns within the order have been conducted, and among them the molecular studies have been prominent.

The development of techniques to study DNA gave rise to an enormous advancement in our knowledge of the evolution and genetics of several groups of animals. Although direct DNA sequencing in the past few years has been largely used to detect polymorphisms, the studies with restriction fragment length polymorphism (RFLP) are useful in the detection of polymorphisms for a larger number of individuals, which is sometimes not possible in DNA sequencing.

RFLP of mitochondrial DNA (mtDNA) and nuclear DNA has proved to be a valuable tool for population genetic and systematic studies in several mammal groups including bats (Morales and Bickham, 1995; Wetterer et al., 2000; Papasotiropoulos et al., 2002; Hartl et al., 2005).

The knowledge of the variation and behavior of variable molecular characters, and the identification of genetic markers for a particular species, provide the estimation of the evolutionary history of the species, such as the size of the population, origin, mating structure, and the establishment of a biological clock (molecular), and play a significant role in the population studies of several organisms and their biogeography.

For this reason, the present study aimed to investigate 23 species of bats belonging to the Molossidae, Phyllostomidae, Vespertilionidae and Emballonuridae families, applying the RFLP-polymerase chain reaction (PCR) technique, with mitochondrial and nuclear gene segments, to evaluate the structure and genetic behavior of the sequences in specimens of these complex families of bats.

MATERIAL AND METHODS

Examined taxa

A total of 88 specimens representing 13 genera and 23 bat species belonging to the Molossidae (*Cynomops abrasus*, *C. planirostris*, *Eumops auripendulus*, *E. bonariensis*, *E. glaucinus*, *E. perotis*, *Molossops temminckii*, *Molossus rufus*, *M. molossus*, *Nyctinomops laticaudatus*, and *N. macrotis*), Phyllostomidae (*Artibeus lituratus*, *A. planirostris*, *Carollia perspicillata*, and *Phyllostomus discolor*), Vespertilionidae (*Eptesicus furinalis*, *Lasiurus cinereus*, *L. ega*, *L. blossevilii*, *Myotis nigricans*, and *M. riparius*) and Emballonuridae (*Peropteryx macrotis* and *Rhynchonycteris naso*) families were analyzed. They were from the States of São Paulo, Mato Grosso and Mato Grosso do Sul. One to five individuals from 18 species, representing 11 genera, were utilized in the analyses of the nuclear RAG2 gene (43 specimens), and one to seven individuals from 23 species, representing 13 genera (88 specimens), for the mitochondrial 12S/16S gene (Table 1).

Table 1. Discrimination of the animals analyzed in the present study by family, species, record number, origin, and procedures performed.

Family	Species	Record	Origin	Procedures performed	
				12S/16S gene	RAG2 gene
Molossidae	*Cynomops abrasus* (5)	CFC270	São José do Rio Preto-SP[10]	+	-
		CFC787	São José do Rio Preto-SP[10]	+	-
		CFC788	São José do Rio Preto-SP[10]	+	-
		CFC789	São José do Rio Preto-SP[10]	+	-
		CFC790	São José do Rio Preto-SP[10]	+	-
	Cynomops planirostris (1)	CFC212	Serra das Araras-MT[14]	+	-
	Eumops auripendulus (3)	CFC392	São José do Rio Preto-SP[10]	+	+
		CFC399	Botucatu-SP[1]	+	-
		CFC414	São José do Rio Preto-SP[10]	+	+
	Eumops bonariensis (1)	CFC459	Caiman-Miranda-MS[13]	+	+
	Eumops glaucinus (5)	CFC441	Nova Aliança-SP[7]	+	+
		CFC444	São José do Rio Preto-SP[10]	+	-
		CFC539	São José do Rio Preto-SP[10]	+	-
		CFC556	Nova Granada-SP[8]	+	+
		CFC676	São José do Rio Preto-SP[10]	+	+
	Eumops perotis (5)	CFC384	Bady Bassitt-SP[2]	+	+
		CFC413	São José do Rio Preto-SP[10]	+	+
		CFC508	Mirassol-SP[5]	+	+
		CFC630	Mirassol-SP[5]	+	-
		CFC633	São José do Rio Preto-SP[10]	+	-
	Molossops temminckii (7)	CFC347	São José do Rio Preto-SP[10]	+	-
		CFC373	Campo Grande-MS[12]	+	-
		CFC528	Mirassol-SP[5]	+	-
		CFC546	Mirassol-SP[5]	+	-
		CFC631	Mirassol-SP[5]	+	-
		CFC653	Olímpia-SP[9]	+	-
		CFC654	Mirassol-SP[5]	+	-
	Molossus rufus (5)	CFC391	São José do Rio Preto-SP[10]	+	+
		CFC434	São José do Rio Preto-SP[10]	+	+
		CFC436	São José do Rio Preto-SP[10]	+	-
		CFC609	Nova Aliança-SP[7]	+	+
		CFC675	Guapiaçu-SP[4]	+	+
	Molossus molossus (5)	CFC558	São José do Rio Preto-SP[10]	+	+
		CFC560	Bady Bassitt-SP[2]	+	+
		CFC677	São José do Rio Preto-SP[10]	+	-
		CFC726	São José do Rio Preto-SP[10]	+	+
		CFC805	São José do Rio Preto-SP[10]	+	+
	Nyctinomops laticaudatus (6)	CFC251	São José do Rio Preto-SP[10]	+	+
		CFC298	São José do Rio Preto-SP[10]	+	-
		CFC393	São José do Rio Preto-SP[10]	+	+
		CFC397	São José do Rio Preto-SP[10]	+	+
		CFC737	São José do Rio Preto-SP[10]	+	+
		CFC768	São José do Rio Preto-SP[10]	+	+
	Nyctinomops macrotis (6)	CFC519	Campo Grande-MS[12]	+	-
		CFC520	Campo Grande-MS[12]	+	-
		CFC521	Campo Grande-MS[12]	+	-
		CFC601	Campo Grande-MS[12]	+	-
		CFC602	Campo Grande-MS[12]	+	-
		CFC624	Campo Grande-MS[12]	+	-
Vespertilionidae	*Eptesicus furinalis* (4)	CFC294	São José do Rio Preto-SP[10]	+	+
		CFC295	São José do Rio Preto-SP[10]	+	-
		CFC297	Cedral-SP[3]	+	-
		CFC299	São José do Rio Preto-SP[10]	+	+

Continued on next page

Table 1. Continued.

Family	Species	Record	Origin	Procedures performed	
				12S/16S gene	RAG2 gene
	Lasiurus cinereus (1)	CFC267	Nova Aliança-SP[7]	+	+
	Lasiurus ega (1)	CFC396	São José do Rio Preto-SP[10]	+	+
	Lasiurus blossevilii (1)	CFC367	Cedral-SP[3]	+	+
	Myotis nigricans (6)	CFC545	São José do Rio Preto-SP[10]	+	+
		CFC569	São José do Rio Preto-SP[10]	+	-
		CFC648	São José do Rio Preto-SP[10]	+	-
		CFC700	São José do Rio Preto-SP[10]	+	-
		CFC720	São José do Rio Preto-SP[10]	+	-
		CFC782	Neves Paulista-SP[6]	+	-
	Myotis riparius (1)	CFC579	São José do Rio Preto-SP[10]	+	-
Phyllostomidae	*Artibeus lituratus* (7)	CFC235	São José do Rio Preto-SP[10]	+	+
		CFC280	São José do Rio Preto-SP[10]	+	+
		CFC281	São José do Rio Preto-SP[10]	+	-
		CFC282	São José do Rio Preto-SP[10]	+	+
		CFC427	Neves Paulista-SP[6]	+	-
		CFC617	São José do Rio Preto-SP[10]	+	-
		CFC796	São José do Rio Preto-SP[10]	+	-
	Artibeus planirostris (6)	CFC271	Neves Paulista-SP[6]	+	+
		CFC272	Neves Paulista-SP[6]	+	+
		CFC374	Campo Grande-MS[12]	+	+
		CFC445	São José do Rio Preto-SP[10]	+	-
		CFC484	São José do Rio Preto-SP[10]	+	+
		CFC541	Nova Granada-SP[8]	+	-
	Phyllostomus discolor (4)	CFC477	São José do Rio Preto-SP[10]	+	+
		CFC480	São José do Rio Preto-SP[10]	+	-
		CFC548	São José do Rio Preto-SP[10]	+	+
		CFC795	São José do Rio Preto-SP[10]	+	-
	Carollia perspicillata (4)	CFC433	São José do Rio Preto-SP[10]	+	-
		CFC759	São José do Rio Preto-SP[10]	+	+
		CFC772	São José do Rio Preto-SP[10]	+	+
		CFC774	São José do Rio Preto-SP[10]	+	+
Emballonuridae	*Peropteryx macrotis* (1)	CFC247	Serra das Araras-MT[14]	+	+
	Rhynchonycteris naso (3)	CFC213	Serra das Araras-MT[14]	+	+
		CFC783	Aquidauana-MS[11]	+	+
		CFC785	Aquidauana-MS[11]	+	-
Total	23 species	-	-	88	43

+ = satisfactory; - = unsatisfactory.

Locality	Longitude	Latitude
[1]Botucatu-SP	-48.4450	-22.8858
[2]Bady Bassitt-SP	-49.4453	-20.9181
[3]Cedral-SP	-49.2683	-20.9028
[4]Guapiaçu-SP	-49.2203	-20.7950
[5]Mirassol-SP	-49.5211	-20.8192
[6]Neves Paulista-SP	-49.6297	-20.8461
[7]Nova Aliança-SP	-49.4961	-20.0158
[8]Nova Granada-SP	-49.3142	-20.5339
[9]Olímpia-SP	-48.9147	-20.7372
[10]São José do Rio Preto-SP	-49.3794	-20.8197
[11]Aquidauana-MS	-55.7872	-20.4711
[12]Campo Grande-MS	-54.6464	-20.4428
[13]Miranda-MS	-54.3783	-20.2406
[14]Serra das Araras-MT	-56.8333	-15.2500

Source: IBGE.

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Genomic DNA extraction and amplifications

Nuclear and mtDNA variation was analyzed by RFLP, performed on PCR amplified products.

Genomic DNA was extracted from 5 to 12 g liver, kidney, lung or muscles, fresh or stored frozen following the protocol described by Sambrook et al. (1989), without phenol.

The nuclear RAG2 segments were amplified using the primers RAG2 R1 (5' GGC TGG CCC AA(AG) AGA TCC TG 3') and RAG2 F1 (5' G(AG)A AGG ATT TCT TGG CAG GAG T 3'), according to Lewis-Oritt et al. (2001), and the mitochondrial sequence 12S/16S with primers 12S/16S F (5' TGG GAT TAG ATA CCC CAC TAT 3') and 12S/16S R (5' TGA TTA TGC TAC CTT TGC ACG GT 3'), according to Morales et al. (1993). PCR amplifications were performed in a Perkin Elmer - Gene Amp PCR System thermocycler.

Restriction digests

Digests with 10 restriction endonucleases were performed to determine the haplotypes in the sequences of 88 specimens. The PCR products were subjected to single (*Rsa*I and *Eco*RI) and double digestions (*Eco*RI plus *Hae*III, *Hin*dIII, *Pst*I, *Xho*I, *Dra*I, *Sca*I, *Dde*I, or *Bg*lII) on 6-μL aliquots of PCR products and 3 U restriction enzyme, and the conditions and buffers used were those recommended by the manufacturers. The digested PCR products were separated on ethidium bromide-stained 1.5% agarose gels. A 100-bp ladder as molecular weight marker was used to determine fragment lengths.

Statistical and genealogical analysis

The resulting fragment patterns were used to identify the RAG2 and 12S/16S haplotypes and construct two binary matrices of presence (1) or absence (0) of fragments produced by the digestions. Genetic relationships among populations and species were estimated using the PopGene 1.31 software (Yeh et al., 1999) and unweighted pair group method with arithmetic means algorithm (Sneath and Sokal, 1973), based on Nei's mathematical model (1978). The five following parameters were analyzed for the detection of gene variation (h) and genetic identity (I): 1) gene variation detected by 10 enzymes for the 88 specimens for the 12S/16S gene segment and 43 specimens for RAG2 gene segment; 2) diversity index (gene variation = h) of RAG2 and 12S/16S genes for species with three or more specimens analyzed; 3) frequency of polymorphic loci; 4) genetic identity in individuals from the species that had at least three specimens analyzed, and 5) genetic identity in 18 species for the RAG2 gene and 23 species for the 12S/16S gene, without considering the number of specimens.

RESULTS

The amplified segments of the nuclear gene RAG2 were 1400 bp and of the 12S/16S mitochondrial gene were 1500 bp. At least one to five specimens with a total of 43 individuals representing 18 species were used in the analysis of the RAG2 gene, and at least one to seven specimens with a total of 88 individuals representing 23 species were used in the 12S/16S gene analysis.

RFLP patterns of RAG2 and 12S/16S genes

The fragments obtained after digestions of PCR products with the 10 endonucleases showed different sizes and variation in the intensity of bands, even in the ones with the same molecular weight. Furthermore, a different cut pattern was observed for the RAG2 gene, which exhibited a smaller number of fragments, when compared to the 12S/16S gene. The digestion pattern of the gene segments was similar in the majority of the individuals from the same species. The comparisons of the patterns among different species revealed a variation in the size of fragments, showing evidence of differences in position and/or number of the restriction sites. However, in specimens of congeneric species, the restriction pattern was similar.

In all digestions, there were 107 different fragments combined in 139 haplotypes for the RAG2 gene and 155 fragments combined in 402 haplotypes for the 12S/16S gene (Table 2).

The assessment of the data meeting in binary matrices evaluated by the Popgene software showed the variation of the haplotypes within and among species. The mean index of gene diversity in the nuclear gene (h: 0.13) was lower than in the mitochondrial gene (h: 0.15) (Table 2).

Table 2. Number of fragments (NF) and haplotypes (NH) and Nei's (1973) genetic variation (h) produced by 10 endonucleases in single- plus double-digestions of the nuclear RAG2 and mitochondrial 12S/16S gene segments.

Gene	Number of specimens	NF	NH	h
RAG2	43	107	139	0.13
12S/16S	88	155	402	0.15

The values of h, frequency of polymorphic loci and I for species with three or more specimens analyzed in the RAG2 gene and in the 12S/16S gene are presented in Tables 3 and 4, respectively. It resulted in analyses of 8 species for the RAG2 gene and 16 species for the 12S/16S gene.

When individual species are considered, the values of gene variation and frequency of polymorphic loci were variable for the same gene among species and in two different genes within the species.

The highest index of gene diversity and frequency of polymorphisms for the RAG2 gene was exhibited by *E. perotis* (h: 0.12; 28%), followed by *A. lituratus* and *C. perspicillata*, which showed the same frequency of polymorphic loci (h: 0.12; 26%). The lowest index of diversity and frequency of polymorphisms was observed in *M. rufus* (h: 0.06; 17%). The species with the highest diversity and polymorphisms for the 12S/16S gene was *N. laticaudatus* (h: 0.16; 44%), followed by *C. perspicillata* (h: 0.15; 35%) and *C. abrasus* (h = 0.13, 35%). The lowest variation was in *M. rufus* (h: 0.06; 17%) followed by *P. discolor* (h: 0.07; 15%).

The value of I observed in the species was high, and varied in the species and genes from 81 to 91% for RAG2 and from 80 to 92% for 12S/16S (Tables 3 and 4). The highest variation frequency in the identity was shown by *C. perspicillata*, in which they ranged from 74 to 97% for the RAG2 gene and by *N. laticaudatus*, ranging from 70 to 97% for the 12S/16S gene, followed by the specimens of *M. nigricans* (Vespertilionidae) ranging from 74 to 95%.

The smallest variation was exhibited by *A. lituratus* (I: 80-84%), followed by *M. molossus* (I: 87-92%) in the RAG2 gene, and *E. auripendulus* (I: 84-88%) followed by *M. rufus* (I: 90-95%) in the 12S/16S mitochondrial gene.

The results of the present study show that the intraspecific values of genetic identity were not influenced by geographical origin of the animals. There were no noticeable differences between

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the highest and lowest values of genetic identity, as observed for *N. macrotis* (82-100%, 12S/16S gene), in which all individuals were collected in Campo Grande, MS, for *N. laticaudatus* (85-100%, RAG2 gene and 70-97%, 12S/16S gene), in which all specimens were captured in São José do Rio Preto, SP, for *M. temminckii* (87-97%, 12S/16S gene), with individuals from São José do Rio Preto, SP, Mirassol, SP, Olímpia, SP and Campo Grande, MS, and for *E. glaucinus* (82-96%, 12S/16S gene), collected in São José do Rio Preto, SP, Nova Aliança, SP and Nova Granada, SP.

Table 3. Gene variation (h) and genetic identity (I) values found in the 8 species with three or more specimens based on the analysis of fragments produced for the RAG2 gene after single- and double-digestions.

Family	Species	Number of specimens analyzed	h	Percent of polymorphic loci	Mean	I	
						Higher	Lower
Molossidae	*Eumops glaucinus*	3	0.09	20%	0.86	0.91	0.82
	Eumops perotis	3	0.12	28%	0.81	0.90	0.74
	Molossus rufus	4	0.06	17%	0.91	0.98	0.84
	Molossus molossus	4	0.08	20%	0.89	0.92	0.87
	Nyctinomops laticaudatus	5	0.09	23%	0.88	1.00	0.85
Phyllostomidae	*Artibeus lituratus*	3	0.12	26%	0.82	0.84	0.80
	Artibeus planirostris	4	0.07	19%	0.90	0.95	0.85
	Carollia perspicillata	3	0.12	26%	0.83	0.97	0.74
Total	8 species	29	0.13	91%	-	-	-

Table 4. Gene variation (h) and genetic identity (I) values found in the 16 species with three or more specimens based on the analysis of fragments produced for the 12S/16S gene after single- and double-digestions.

Family	Species	Number of specimens analyzed	h	Percent of polymorphic loci	Mean	I	
						Higher	Lower
Molossidae	*Cynomops abrasus*	5	0.13	35%	0.83	0.91	0.75
	Eumops auripendulus	3	0.09	21%	0.86	0.88	0.84
	Eumops glaucinus	5	0.09	23%	0.88	0.96	0.82
	Eumops perotis	5	0.13	33%	0.84	0.90	0.80
	Molossops temminckii	7	0.07	19%	0.92	0.97	0.87
	Molossus rufus	5	0.06	17%	0.92	0.95	0.90
	Molossus molossus	5	0.12	33%	0.85	0.93	0.75
	Nyctinomops macrotis	6	0.07	19%	0.91	1.00	0.82
	Nyctinomops laticaudatus	6	0.16	44%	0.81	0.97	0.70
Phyllostomidae	*Artibeus lituratus*	7	0.13	35%	0.84	0.95	0.77
	Artibeus planirostris	6	0.12	33%	0.86	0.92	0.77
	Carollia perspicillata	4	0.15	35%	0.80	0.88	0.74
	Phyllostomus discolor	4	0.07	15%	0.90	0.94	0.87
Vespertilionidae	*Myotis nigricans*	6	0.13	34%	0.84	0.95	0.74
	Eptesicus furinalis	4	0.06	19%	0.91	0.96	0.86
Emballonuridae	*Rhynchonycteris naso*	3	0.11	24%	0.84	0.92	0.77
Total	16 species	81	0.15	99%	-	-	-

Considering all species and the set of genera that form different families, Molossidae had the highest variability when compared with other families. The identity index among species ranged from 84 to 100% for the RAG2 gene and 79 to 99% for the 12S/16S gene. The most conserved family was Phyllostomidae, ranging from 95 to 99% for the RAG2 gene and 97 to 99% for 12S/16S (Tables 5 and 6).

Table 5. Mean values of Nei's (1978) genetic identity for the 18 species studied for the nuclear RAG2 gene.

	Molossidae							Phyllostomidae				Vespertilionidae					Emballonuridae	
ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	****	0.84*	0.99	0.95	0.90	0.91	0.92	0.87	0.85	0.88	0.85	0.85	0.84	0.76*	0.87	0.81	0.87	0.87
2		****	0.88	0.88	0.91	0.91	0.92	0.87	0.86	0.88	0.86	0.80	0.82	0.78	0.86	0.90	0.81	0.85
3			****	0.97	0.90	0.90	0.92	0.86	0.85	0.87	0.83	0.84	0.823	0.78	0.85	0.81	0.85	0.87
4				****	0.94	0.93	0.94	0.90	0.89	0.92	0.88	0.86	0.87	0.80	0.91	0.87	0.90	0.92
5					****	0.99	0.99	0.95	0.93	0.96	0.94	0.88	0.91	0.80	0.95	0.90	0.90	0.93
6						****	1.00*	0.96	0.94	0.97	0.94	0.90	0.94	0.81	0.97*	0.91	0.93	0.95
7							****	0.95	0.94	0.97	0.93	0.90	0.92	0.82	0.95	0.90	0.92	0.95
8								****	0.99*	0.98	0.97	0.91	0.94	0.86	0.95	0.91	0.93	0.95
9									****	0.97	0.95*	0.89	0.93	0.85	0.95	0.91	0.92	0.95
10										****	0.97	0.91	0.94	0.84	0.97	0.92	0.95	0.96*
11											****	0.88	0.92	0.80	0.94	0.91	0.90*	0.92
12												****	0.94*	0.83	0.91	0.83	0.88	0.89
13													****	0.79*	0.94	0.87	0.93	0.92
14														****	0.83	0.81	0.80	0.81
15															****	0.89	0.93	0.95
16																****	0.86	0.88
17																	****	0.93*
18																		****

1 - *E. auripendulus*; 2 - *E. bonariensis*; 3 - *E. glaucinus*; 4 - *E. perotis*; 5 - *M. molossus*; 6 - *M. rufus*; 7 - *N. laticaudatus*; 8 - *A. lituratus*; 9 - *A. planirostris*; 10 - *C. perspicillata*; 11 - *P. discolor*; 12 - *M. nigricans*; 13 - *E. furinalis*; 14 - *L. ega*; 15 - *Lasiurus* sp; 16 - *L. cinereus*; 17 - *Peropteriyx macrotis*; 18 - *R. naso*. *Represent the lower and higher inter- and intrafamilial values.

Table 6. Mean values of Nei's (1978) genetic identity for the 23 species studied for the mitochondrial 12S/16S gene.

	Molossidae											Vespertilionidae						Phyllostomidae				Emballonuridae	
ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	****	0.90	0.91	0.88	0.84	0.89	0.93	0.93	0.95	0.94	0.88	0.94	0.85	0.92	0.90	0.95	0.91	0.94	0.94	0.93	0.93	0.86	0.94
2		****	0.88	0.86	0.79	0.85	0.89	0.91	0.91	0.89	0.84	0.89	0.82	0.88	0.86	0.90	0.82	0.89	0.89	0.88	0.88	0.79	0.91
3			****	0.87	0.86	0.92	0.93	0.94	0.93	0.92	0.87	0.91	0.82	0.88	0.90	0.91	0.86	0.90	0.91	0.89	0.90	0.82	0.92
4				****	0.79*	0.83	0.90	0.90	0.89	0.87	0.79	0.87	0.80	0.84	0.89	0.88	0.82	0.88	0.89	0.88	0.88	0.81	0.89
5					****	0.94	0.88	0.87	0.89	0.90	0.88	0.88	0.79	0.84	0.85	0.88	0.84	0.88	0.88	0.87	0.87*	0.77	0.87
6						****	0.93	0.93	0.95	0.95	0.93	0.93	0.83	0.89	0.89	0.93	0.88	0.92	0.92	0.91	0.91	0.79	0.91
7							****	0.97	0.98	0.97	0.88	0.98	0.87	0.94	0.95	0.97	0.92	0.97	0.96	0.96	0.96	0.82	0.96
8								****	0.98	0.95	0.90	0.96	0.84	0.92	0.93	0.95	0.88	0.94	0.94	0.93	0.94	0.81	0.94
9									****	0.99*	0.93	0.97	0.86	0.94	0.94	0.98	0.91	0.96	0.97	0.94	0.97	0.83	0.96
10										****	0.94	0.97	0.88	0.94	0.93	0.99	0.93	0.97*	0.97	0.95	0.97	0.82	0.97
11											****	0.91	0.79	0.87	0.85	0.92	0.85	0.90	0.91	0.88	0.91	0.76	0.89
12												****	0.89	0.95	0.95	0.98*	0.93	0.97	0.97	0.96	0.97	0.82	0.97
13													****	0.84*	0.86	0.87	0.85	0.87	0.86	0.86	0.86	0.73*	0.87
14														****	0.90	0.96	0.88	0.94	0.95	0.93	0.95	0.81	0.94
15															****	0.94	0.89	0.93	0.93	0.93	0.92	0.79	0.94
16																****	0.94	0.97	0.98	0.96	0.98	0.84	0.98*
17																	****	0.91	0.91	0.90	0.91	0.77	0.91
18																		****	0.98	0.99*	0.98	0.87	0.97
19																			****	0.98	0.98	0.85	0.97
20																				****	0.97*	0.84	0.97
21																					****	0.84	0.98
22																						****	0.86*
23																							****

1 - *C. abrasus*; 2 - *C. planirostris*; 3 - *E. auripendulus*; 4 - *E. bonariensis*; 5 - *E. glaucinus*; 6 - *E. perotis*; 7 - *M. temminckii*; 8 - *M. rufus*; 9 - *M. molossus*; 10 - *N. laticaudatus*; 11 - *N. macrotis*; 12 - *E. furinalis*; 13 - *L. ega*; 14 - *Lasiurus* spp; 15 - *L. cinereus*; 16 - *M. nigricans*; 17 - *M. riparius*; 18 - *A. lituratus*; 19 - *A. planirostris*; 20 - *C. perspicillata*; 21 - *P. discolor*; 22 - *P. macrotis*; 23 - *R. naso*. *Represent the lower and higher inter- and intrafamilial values.

The pairwise comparisons of the families demonstrated that the Molossidae and Vespertilionidae families were the most diverse for the RAG2 gene ranging from 76 to 97% and Vespertilionidae and Emballonuridae for the 12S/16S gene ranging from 73 to 98%. This diversity between species of the three families could be easily observed in the species *L. ega* (Vespertilionidae) when compared to *Eumops* (Molossidae) where the identity indexes ranged from 76 to 80% for the RAG2 gene and when compared to *P. macrotis* (Emballonuridae) where the identity index was 73% for the 12S/16S gene. The families with the lowest variation were Phyllostomidae when compared to Emballonuridae for the RAG2 gene, ranging from 90 to 96%, and Phyllostomidae when compared to Molossidae, ranging from 87 to 97% for the 12S/16S gene (Tables 5 and 6).

DISCUSSION

The difference in the number of haplotypes detected by RFLP analysis for RAG2 (139 haplotypes) and 12S/16S (402 haplotypes) genes was significant. It was very representative considering the sequence size (approximately 1400 bp for RAG2 and 1500 bp for 12S/16S) along with the number of individuals analyzed for each sequence, demonstrating the importance and efficiency of the enzymes and gene sequence utilized.

The highest number of fragments and haplotypes generated by mtDNA when compared to the RAG2 nuclear gene can be attributed to a higher number of individuals analyzed for this gene, but not exclusively.

Different categories of sequences have distinct properties. In many groups, including mammals, the rate of nucleotide substitution among mitochondrial protein-coding genes is generally more rapid than the rate of nucleotide substitution among protein-coding regions of nuclear genes (Vawter and Brown, 1986). The rapid evolutionary rate exhibited by the mitochondrial genome makes it quite sensitive in detecting recent genetic bottlenecks due to lack of recombination effects (Brown et al., 1982; Effemberger and Suchentrunk, 1999). Hence, it is fairly sensitive for historical population developments. However, it should be emphasized that this molecular marker represents only a small portion of the total genome diversity.

The nuclear genome is highly desirable as an alternative dataset to independently test mtDNA hypotheses and to essentially counteract criticisms of mtDNA. Nuclear DNA sequences have been used to address phylogenetic questions pertaining to relationships above the genus level (Perasso et al., 1989). The utility of the RAG2 gene for resolving higher-level relationships and diversification patterns of bats has already been pointed out by some authors (Baker et al., 2000; Van Den Bussche et al., 2002; Hoofer et al., 2003; Jones et al., 2005), as well as by Teeling et al. (2000) and Murphy et al. (2001) for other mammalian taxa. Some of these studies verified that the value of mitochondrial sequences in phylogenetic analyses is further enhanced when they are collected in conjunction with nuclear sequences, because they provide an independent estimate of phylogenetic relationships, which can be compared with estimates based on nuclear sequences (Lewis-Oritt et al., 2001; Springer et al., 2001).

Despite differences between the two genomes, the values of genetic variability detected by both genes (h: 0.13 for RAG2 and h: 0.15 for 12S/16S) were similar indicating that they are good markers for the evaluation of genetic variability in bat species.

Despite the variation observed in band intensity that was probably due to the differences in the number of fragments and the incomplete digestion of the original fragment, the

conservatism in the cut pattern of congeneric species pointed to a molecular similarity in the sequences and fragments.

The values of I for the RAG2 and 12S/16S genes were high among individuals of the same species and in the majority of them. This feature indicates that haplotypes produced by different enzymes are conserved and display low variation. The same is observed in the analysis of gene variation among species, since the variation indexes were not more than 0.12 for the RAG2 gene (*E. perotis*, *A. lituratus* and *C. perspicillata*) and 0.16 for the 12S/16S gene (*N. laticaudatus*) when the 107 fragments for RAG2 gene segment and the 155 fragments for 12S/16S fragments were considered.

Despite the low values for gene variation and polymorphisms, they were different among species. The species with the highest gene variation for RAG2 and most polymorphic were *E. perotis*, *A. lituratus*, and *C. perspicillata*, whereas the most polymorphic species for 12S/16S were not necessarily the ones that showed the highest gene variation. *N. laticaudatus* was the most polymorphic and with the highest variation. *C. perspicillata* and *C. abrasus* were the second most polymorphic species; however, *C. abrasus* had the third highest value for gene variation.

Still, concerning the values of genetic identity within the species and considering the RAG2 gene, *C. perspicillata* had the highest variation, with identity indexes ranging from 74 to 97%, followed by *E. perotis*, ranging from 74 to 90%. The same was observed for the 12S/16S gene, in which *N. laticaudatus* had the highest variation in identity index, ranging from 70 to 97%. *M. nigricans* had the second highest variation, ranging from 74 to 95%.

These results indicate the occurrence of intraspecific variations that do not occur in the same manner among different species of bats. Apparently, the variations are not related to the origin of the specimens, since five of the six *C. perspicillata* specimens used in our study were collected in the same colony, in São José do Rio Preto, different from *E. perotis*, where specimens were captured in different colonies, located in municipalities surrounding São José do Rio Preto.

In the present study, the small variations observed can be interpreted as a natural variability within the species and genus, which exhibited a conserved pattern in the two gene sequences in different species and families analyzed.

The conservation observed in the gene segments evaluated in the present study is shared by cytogenetic, morphologic and molecular data reported for some of the species analyzed here (Eger, 1977; Patton and Baker, 1978; Freeman, 1981; Morielle-Versute et al., 1996; Ditchfield, 2000; Leite-Silva et al., 2003; Rivers et al., 2005).

The study of Ditchfield (2000), which compared the phylogeographic patterns of 17 species of bats from the Phyllostomidae family (including *A. lituratus*, *C. perspicillata*, *Sturnira lilium*, and *Glossophaga soricina)* and other small terrestrial mammals, using cytochrome *b* sequence revealed that the genetic variation in bats is 10 times lower than in other small mammals in the geographic variation feature and that the rate of molecular evolution is slower for bats, similar to what is described for birds.

In the present study, the highest variability was demonstrated by the Molossidae family, when only species and genus were considered for RAG2 and 12S/16S sequences. This variability can be attributed to the variation that is characteristic of some genera and species of the family, especially *E. perotis* and *N. laticaudatus.*

Eger (1977) observed the occurrence of morphological variation among different geographic populations of species of *Eumops*, especially of *E. bonariensis*, and Freeman (1981) after evaluating a morphological character set in 78 species of molossids, singled out *Eumops* as

a morphologically distinct group among the molossids, despite recognizing the genus as being monophyletic based on derived traces and similarities of the skull form. These variations in *Eumops* are also observed in cytogenetic data, which have pointed out that the karyotypes of the *Eumops* are the most variable among the molossid species (Warner et al., 1974; Morielle-Versute et al., 1996; Finato et al., 2000).

The species of Vespertilionidae analyzed were similar to Molossidae species, and the differences must be related to the variations in the species of the three genera, especially in *Lasiurus* species. *L. ega* had lower indexes of genetic identity when compared to other species.

Despite the little information for the *Lasiurus* genus, the number of species recognized for *Lasiurus* is highly variable, and reports on their evolutionary history have used cytogenetic and molecular analyses for the characterization of different species (Morales and Bickham, 1995; Marchesin and Morielle-Versute, 2004). The genus has been divided into three subgroups in accordance with fur color: hoary (*L. cinereus*); red (*L. blossevillii*, *L. borealis*, *L. pfeifferi*), and yellow (*L. xanthinus*, *L. ega*, and *L. intermedius*). The yellow bats were considered so morphologically divergent that some authors proposed placing these species in the new genus *Dasypterus* (Tate, 1942; Hill and Harison, 1987), where this group includes *L. ega*.

The situation observed by the Phyllostomidae, in which the four species of three different genera had little variation, is certainly related to the small number of taxa included in our study, since the family is taxonomically diverse and, despite being karyotypically conserved, displays morphological and molecular variability among and within different genera (Van Den Bussche, 1992; Baker et al., 1979, 2000, 2003; de Faria and Morielle-Versute, 2006; Martins et al., 2007).

Carollia perspicillata, had the second largest index of genetic variability, and can be representive of the complexity of the group. Although only four species of *Carollia* had been recognized until the research by Koopman (1993), eight species are currently recognized (Wright et al., 1999; Solari and Baker, 2006), and the recognition of these species has been possible by combined analysis of different sets of data, especially molecular (mtDNA) sequences and cytogenetic data (Baker et al., 2002; Solari and Baker, 2006).

The analyses in the present study indicate a higher genetic homogeneity for the nuclear gene. The polymorphisms generated by the RAG2 gene seem to be more informative than the ones generated by 12S/16S, at least for the taxa studied. These results are in accordance with Springer et al. (2001) who compared the performance of mitochondrial and nuclear datasets and observed that nuclear genes are more efficient in clade reconstruction than are mitochondrial genes. The authors claimed that nuclear genes are less affected by nucleotide substitutions.

Our data reinforce the idea that the RAG2 is a useful gene in Chiroptera. It has role in immunologic response and because this it is not probably linked with morphologic features, which have been frequently used in bat classification and, due to their complexity, has hidden the basal condition of the phylogenetic relationships of the group (Baker et al., 2000, 2003). Taken together, the results obtained from the analyses of the mitochondrial and nuclear genes contribute to our knowledge of the diversity of New World bats. The genetic variability found in different taxa suggests that an additional diversity, previously unnoticed, can be revealed through the use of different taxa and molecular

strategies, reinforcing the importance of this methodology in the evolutionary analysis of the group.

ACKNOWLEDGMENTS

The authors are grateful to CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de Ensino Superior), FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and FUNDUNESP (Fundação para o Desenvolvimento da UNESP) for financial support.

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