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Research Article

Butyrylcholinesterase genetic variability in Guarani Amerindians from the Brazilian state of Mato Grosso do Sul

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Abstract

Human butyrylcholinesterase (BChE; EC 3.1.1.8) is a polymorphic enzyme coded by the *BCHE* gene (3q26.1-q26.2) while the *CHE2* gene (2q33-q35) determines a still not characterized substance that forms a complex with BChE (C_5), being the CHE2 C5+ and CHE2 C5- phenotypes detected in electrophoresis. The present study investigated *BCHE* and *CHE2* variability and the BChE activity of Brazilian Guarani Amerindians from the Kaiowá and Ñandeva sub-groups living in several indigenous territories in the Brazilian state of Mato Grosso do Sul. The frequency of the *BCHE* exon 2 *D70G* (*A*) allele was 0.60% ± 0.35% while that of the *BCHE* exon 2 *G390V* (*F-2*) allele, never before screened in Amerindians, was 8.82% ± 1.35%. This is the first time that the *BCHE* gene exon 4 *A539T* (*K*) allele has been surveyed in Brazilian Amerindians where it was found at a frequency of 3.69% ± 0.85%, similar to that found in Chilean Mapuche Amerindians. The *BCHE* gene variability seen in this survey differs from that of non-isolated populations in respect to both *A539T* and *G390V* allele frequency. The CHE2 C5+ phenotype frequency was 14.40% ± 2.22% and falls within the range of that found for other Brazilian Amerindian samples.

Key words: *BCHE g*ene, *A* allele, *F-2* and *K* alleles, Guarani-Kaiowá, Guarani-Ñandeva.

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Introduction

The amino acid sequence of human butyrylcholinesterase (BChE; EC 3.1.1.8) is encoded by the *BCHE* gene (3q26.1-q26.2). This gene variability is characterized by DNA analysis and some phenotypes are also characterized by enzymatic inhibition tests. The alleles usually detected in population studies are: *U* (usual; wild-type); *D70G* (atypical or *A*; 70Asp → Gly; 209A → G); *T243M* (fluoride resistant *F-1*; 243Thr → Met; 728C → T); *G390V* (fluoride resistant *F-2*; 390Gly → Val; 1169G → T) and *A539T* (*K*; 539Ala → Thr; 1615G → A). The N-terminal aminoacid of the mature BChE protein is Glu 1 with nt 1 corresponding to the first nt in the codon for the Glu 1 (Arpagaus *et al*., 1990). The genetic variability of BChE also depends on the interaction of the *BCHE* and *CHE2* (2q33-q35) gene products. Two alleles of the *CHE2* gene (*CHE2*C5-* and *CHE2*C5+*) determine the CHE2 C5- and CHE2 C5+ (dominant) phenotypes characterized by the absence and presence of the C_5 complex, respectively. This complex is formed by the BChE tetramer linked to a still unidentified molecule (Masson, 1991) conditioned by the *CHE2* gene.

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BChE variability has been studied in many Brazilian Amerindian groups (review in Alcântara *et al.*, 1995) using both enzyme inhibition tests and electrophoretic methods. It is well-known that, compared to other groups, Amerindians present a restricted number of alleles in many of the genes so far investigated.

During the present study, two Guarani Amerindian sub-groups (Guarani-Kaiowá and Guarani-Ñandeva) from the Brazilian state of Mato Grosso do Sul were investigated for BChE variability, using DNA analysis for the detection of *BCHE* gene variants. The aim of the present study was to gather additional information on the genetic variability of butyrylcholinesterase in these Amerindians by examining the *CHE2* phenotype frequencies and searching for *BCHE* variants, some of which (*e.g. T243M* and *A539T*) having been previously uninvestigated in Brazilian Amerindians. The additional information on the variability of these loci was used for testing two alternatives of the hypothesis related to the origin of each BChE variant, namely that they were already present in paleo-Amerindians or were introduced due to European gene flow.

Materials and Methods

Sample populations

DNA and plasma were obtained from peripheral blood samples from Brazilian Amerindians of the Guarani-Kaiowá (N = 150) and Guarani-Ñandeva (N = 84) sub-groups along with 22 of both Kaiowá and Ñandeva ancestry. These Amerindians live in 18 municipalities of the Brazilian state of Mato Grosso do Sul, principally the Amambai, Porto Lindo and Limão Verde municipalities but also from the Campanaria, Campestre, Caraapo, Cerrito, Dourados, Guaimbé, Jacaré, Lagoa Bonita, Miranda, Panambi, Pirajuí, Sapucaia, Sossoro, Tacuru, Taquapiri municipalities. All the communities were within an area extending from 20°24'S to 23°93'S and from 54°58'W to 56°55' W. This study was approved by the "Ethics Committee for Research with Human Beings of the State University of Maringá, Paraná".

Enzyme inhibition

Enzyme inhibition tests (Alcântara *et al*., 1991, as modified by Picheth *et al.*, 1994) were used for the detection of the atypical variant. The phenotype screening of 250 individuals was performed using alpha-naphthyl-acetate as substrate and DL-propranolol as inhibitor. The non-usual phenotypes, as well as those with borderline values, were confirmed using DL-propranolol and Ro2-0683 [dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide] as inhibitors.

Enzyme activity

The method of Dietz *et al.* (1973) was used to determine BChE activity using propionylthiocholine as substrate at 25 °C.

Phenotypyng of the *CHE2* locus

The *CHE2* phenotypes were identified by acid agar gel electrophoresis with subsequent application of alpha-naphthyl-acetate as substrate (Van Ros and Vervoort (1973), as modified by Alcântara *et al.* (2001)). This procedure separates the C_5 complex from all other BChE forms which constitute only one band. The phenotypes were classified as CHE2 C5- when only one band was seen at the negative pole or CHE2 C5+ when one band was seen at the negative pole and another band (C_5 complex) at the positive pole.

Extraction of DNA and analysis of segments of the *BCHE* gene

DNA was extracted from peripheral leukocytes according to the method of Lahiri and Nurnberger (1991) and amplified by PCR (35 cycles with temperatures of 94 °C, 48 °C and 72 °C for 1 min each; final extension at 72 °C for 10 min): 1 μL of DNA (about 100 ng), 19 μL of PCR super MIX (Invitrogen) and 10 pmol of each primer. The primers used were N25 and N23, N75 and N73 (Souza *et al*., 2005), P35 and P33 (Höler *et al*., 1995), AP5 (Bartels *et al*. 1992b) and P43 (Höler *et al*., 1995), for the amplification of DNA sequences containing the *D70G* and *G390V* mutation sites (exon 2), exons 3 and 4 (*A539T* mutation), respectively.

SSCA (Single Strand Conformation Analysis) was used for variant detection. The mixture containing the PCR product (5 μL) was added to 6 μL of the denaturation solution (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanole, 10 mM EDTA and 10 mM NaOH), submitted to 94 °C (5 min) and kept on ice until being run on a 170 mm x 160 mm x 0.8 mm polyacrylamide gel (see Table 1 for the electrophoresis conditions used for each allele) and stained with silver nitrate according to the method of Budowle *et al.* (1991). Putative *G390V* (F-2) alleles were subsequently confirmed by PCR-RFLP, using the N75 and N73 primers and the *Hph*I restriction enzyme (Nogueira *et al.*, 1992). The digested DNA was subjected to polyacrylamide gel electrophoresis (proportion of bisacrylamide in relation to the total of polyacrylamyde = 3.3%; total concentration of acrylamyde plus bisacrylamyde = 8%) and stained with silver nitrate. A control for the *D70G* (*A*) allele was applied in the gel when necessary.

Statistical analysis

Frequency distributions, means ± standard error (SE), the *t*- test and χ^2 test (using Yates' correction when necessary) were calculated using Statistica for Windows (Statsoft, Tulsa, USA).

Results and Discussion

The enzyme inhibition tests showed two BCHE UA phenotypes in the 145 Kaiowá, one in the 83 Ñandeva and none in the 22 Kaiowá-Ñandeva, giving similar *D70G* (*A*)

Table 1 - Electrophoresis conditions for the amplified products of the human butyrylcholinesterase *BCHE* gene. The electrophoresis buffer was 1x tris-borate-EDTA (TBE).

Conditions	Exon 2 (*D70G*, *G390V*)	Exon 3	Exon 4 (*A539T*)
Gel's buffer	1x TBE	Tris-HCl, pH 4.6	1x TBE
Acrylamide[1]	T = 9%; C = 3.3%	T = 8%; C = 3.3%	T = 10%; C = 2%
Current and time	40 mA for 4 h	10 mA for 19 h followed by 2 h at 240 V	40 mA for 50 min

[1]T = total concentration of acrylamyde plus bisacrylamyde in the gel and C = proportion of bisacrylamide in relation to the total of polyacrylamyde.

allele frequencies for the Kaiowá (0.69% ± 0.49%) and the Ñandeva (0.60% ± 0.60%), the overall frequency for this allele being estimated as 0.60% ± 0.35%. A total of 68 individuals (43 Kaiowá, 20 Ñandeva and 5 Kaiowá-Ñandeva) had their DNA examined by PCR-SSCA for the region of the *D70G* mutation and the 3 heterozygotes detected by enzyme inhibition tests were confirmed as having the *U/D70G* genotype when compared to controls of this genotype.

The degree of non-Amerindian admixture was estimated as 17% in the Guarani-Ñandeva and 3% in the Guarani-Kaiowá sub-groups (Tsuneto, 2003) on the basis of the frequency of HLA haplotypes present in Europeans and Africans and only found in Amerindians as due to admixture. Considering the ethnic composition of the 250 Amerindians examined for the *D70G* variant (64% Guarani-Kaiowá and 36% Guarani-Ñandeva), the respective degrees of admixture of these sub-groups and the frequency of 1.5% of this variant in Euro-Brazilians (Chautard-Freire-Maia *et al*., 1984), the expected frequency for the *D70G* variant in this Guarani sample (0.12%) due only to European admixture is statistically lower than the one found ($\chi^2 = 9.61$; $p < 0.01$). African admixture was not considered as a possible source of the *D70G* allele because it has not been found in non-admixed Africans (Whittaker, 1986), pointing to a relatively recent European origin for this allele.

Alcântara *et al.* (1995) used enzyme inhibition to examine nine Brazilian Amerindian groups and detected the *D70G* allele only in one of these groups, the Mura group from the Guapenu indigenous territory with a frequency of 1.4% ± 1.0% that does not significantly ($\chi^2 = 0.21$; $p > 0.60$) differ from the overall frequency for this allele found in the Guarani from Mato Grosso do Sul. Alcântara *et al.* (1995) revised previous studies on 21 Brazilian Amerindian samples comprising 2,334 individuals in which the *D70G* allele was not found. In other South American Amerindian groups, the *D70G* allele has been found at a frequency ranging from 0.2% ± 0.2% in the Mataco-Chorote from Argentina (Vergnes and Quilici, 1970) to 3.1% ± 0.6% in the Makiritare from the Venezuela-Brazil border (Arends *et al*., 1970). Although the *D70G* allele has also been found in Mexican Amerindians at frequencies of 0.9% ± 0.4% (Lisker *et al.*, 1964), 0.5% ± 0.1% (Lisker *et al.*, 1967) and 3.8% ± 1.0% (Vergnes and Quilici, 1970) it was not found in the only two North-American groups so far studied, the Apache (Lubin *et al*., 1971) and the Navajo (Garry, 1977). Acuña *et al*. (2003) reported the *D70G* allele frequencies in the Chilean Mapuche people: 1.1% ± 0.8% in the Huilliche; 0.9% ± 0.6% in the Cunco and 0.0% in the Pehuenche. The present data appear to be the first obtained for Amerindians at the DNA level, and show that the frequency of the *D70G* allele is within the range previously observed in the few Amerindian samples in which it has been found. The absence of this *BCHE* variant allele in more than 2,300 South American Amerindians supports the hypothesis that its occurrence in the Guarani from Mato Grosso do Sul is due to gene flow from European populations. The finding that the present frequency of the *D70G* allele in the Guarani is higher than the expected frequency may be explained by random genetic drift.

The genotype and allele frequencies for the *BCHE* gene and phenotype frequencies for the *CHE2* gene are given in Table 2 and the reproductions of polyacrylamide gels showing the amplified DNA products of two segments of exon 2, exon 3 and exon 4 submitted to SSCA are shown in Figure 1.

Since the frequencies of the *G390V* (*F-2*) allele in the Guarani-Kaiowá (9.54% ± 1.82%) and Guarani-Ñandeva (7.25% ± 2.21%) did not differ statistically ($\chi^2 = 0.60$, $p > 40\%$), an overalll frequency for the Guarani can be used (8.82% ± 1.35%), including individuals with mixed Guarani origin. The *G390V* frequencies previously obtained in our laboratory (Alcântara *et al.*, 1995) based on enzyme inhibition tests of individuals from 13 Brazilian Amerindian groups showed the occurrence of a fluoride resistant allele in only four groups (7.1% in the Urubu Kaapor, 2.9% in the Asurini, 0.7% in the Mura and 0.5% in the Sateré-Mawé). Assuming that the fluoride resistant allele in these groups is the *G390V* allele, we compared these allelic frequencies with the present *G390V* frequency (8.82% ± 1.35%), using χ^2 tests and found statistically significant differences with: the Asurini ($p < 0.05$), Mura ($p = 0.001$) and Sateré-Mawé ($p < 0.0001\%$).

The fluoride resistant phenotype presents a wide geographic distribution characterized by low frequencies in non-isolated populations. Concerning the present data, this phenotype was found in 28% (5/18) of the Brazilian Amerindians studied, and relatively high frequencies were observed among groups of the Tupi-Guarani linguistic group (Urubu-Kaapor, Asurini and Guarani from Mato Grosso do Sul) that inhabit very distinct geographic areas. Mikami (2005) examined the DNA of Euro-Brazilians and found the same and very low frequency (0.28% ± 0.19%) for each of the *T243M* and *G390V* fluoride resistant alleles. Considering the ethnic composition (65% Guarani-Kaiowá and 35% Guarani-Ñandeva) of the present sub-groups examined for the *G390V* allele and the respective degrees of admixture of these sub-groups along with the 0.28% frequency of this allele in Euro- and Afro-Brazilians (Mikami, 2005), the expected *G390V* frequency (0.022%) in this Guarani sample due only to admixture is lower statistically ($\chi^2 = 15135.52$, $p < 0.0001$) than the frequency found, *i.e.* less than one expected allele for 39 alleles detected. Taken together, these observations do not seem to support the hypothesis raised in a previous study (Alcântara *et al.*, 1995) that Brazilian Amerindians have received the *G390V* allele by gene flow from Europeans, although this type of admixture has been detected in the groups presenting this allele. In place of the previous hypothesis, we suggest that

Table 2 - Genotype and allele frequencies (% ± SE) of the human butyrylcholinesterase *BCHE* gene and phenotype frequencies (% ± SE) of the *CHE2* gene in Guarani Amerindian sub-groups from the Brazilian state of Mato Grosso do Sul.

BCHE genotype or allele	Guarani Amerindian sub-groups					
	Kaiowá		Ñandeva		Kaiowá-Ñandeva	
	N	% ± SE	N	% ± SE	N	% ± SE
Exon 2						
U/U	106	80.92 ± 3.43	59	85.51 ± 4.24	17	80.95 ± 8.57
U/G390V	25	19.08 ± 3.43	10	14.49 ± 4.24	4	19.05 ± 8.57
G390V	262	9.54 ± 1.82	138	7.25 ± 2.21	42	9.52 ± 4.53
Exon 3						
U/U	149	100.00 ± 0.00	83	100.00 ± 0.00	22	100.00 ± 0.00
U	298	100.00 ± 0.00	166	100.00 ± 0.00	44	100.00 ± 0.00
Exon 4						
U/U	137	93.84 ± 1.99	70	92.11 ± 3.09	21	95.45 ± 4.44
U/A539T	7	4.79 ± 1.77	6	7.89 ± 3.09	1	4.55 ± 4.44
A539T/A539T	2	1.37 ± 0.96	0	0.00 ± 0.00	0	0.00 ± 0.00
A539T	292	3.77 ± 1.11	152	3.95 ± 1.58	44	2.27 ± 2.25
CHE2 phenotype						
CHE2 C5-	124	85.52 ± 2.92	68	81.93 ± 4.22	22	100.00 ± 0.00
CHE2 C5+	21	14.48 ± 2.92	15	18.07 ± 4.22	0	0.00 ± 0.00

N = number of individuals for genotypes and phenotypes and number of genes for alleles.

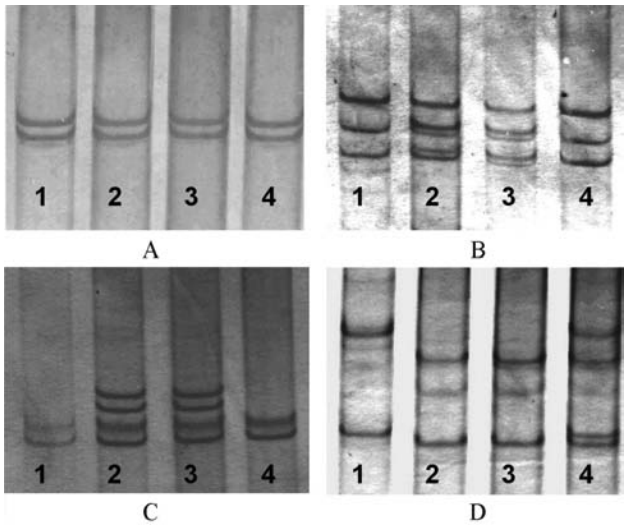

Figure 1 - Polyacrylamide gels showing amplified products of the human butyrylcholinesterase *BCHE* gene. A: absence of variability in exon 3. B: exon 2 (*G390V* region) - *U/U* (1, 4); *U/G390V* (2, 3). C: exon 2 (*D70G* region) - *U/U* (1, 4); *U/D70G* (2, 3). D: exon 4 - *A539T/A539T* (1); *U/U* (2, 3); *U/A539T* (4).

the *G390V* allele was already present in the paleo-Amerindians.

Exon 3 and the flanking regions of introns 2 and 3 (237 pb) did not show molecular variability (Figure 1), owing to the probable idiomorphic character of the mutations already described in this exon and in intron 2: J (*E497V*, Bartels *et al.*, 1992a); and four mutations responsible for silent enzymes (*IVS2-8T* → G, Y500X, *Q518L* - Primo-Parmo *et al.*, 1996; *R515C* - Maekawa *et al.*, 1995). There is an approximately 90% probability of SSCA detecting a variation in a segment of this length (Hayashi and Yandell, 1993), and since we did not use positive controls for these five rare mutations, the absence of variability could be due to the 10% lack of resolution of SSCA.

Since no significant difference ($\chi^2 = 0.01$, $p > 0.90$) was detected in the frequency of the *A539T* allele at exon 4 in the Guarani-Kaiowá and Guarani-Ñandeva sub-groups, the overall frequency of this allele is 3.69% ± 0.85%, including the total sample of 244 individuals. This was the first time Brazilian Amerindians have been screened for the *A539T* allele, which occurred with a similar frequency to that found by Acuña *et al.* (2003) in the Chilean Mapuche (5.76% ± 2.29%; $\chi^2 = 0.49$, $p > 0.45$). The frequency of the *A539T* allele in the Guarani of Mato Grosso do Sul is significantly lower ($p < 0.0001$) than that found in Euro- Brazilians (18.4% ± 2.8%, $\chi^2 = 41.19$) and Afro-Brazilians (17.1% ± 2.9%, $\chi^2 = 33.53$) by Souza *et al.* (1998) and is also lower ($p < 0.001$ at least) than in other non-isolated populations already studied, including populations from North America (12.8% ± 3.4%, $\chi^2 = 11.49$, Bartels *et al.*, 1992b), Japan (16.4% ± 2.4%, $\chi^2 = 35.31$, Shibuta *et al.*, 1994 and 17.5% ± 2.3%, $\chi^2 = 42.62$, Izumi *et al.*, 1994), Denmark (18.0% ± 5.4%, $\chi^2 = 16.60$, Jensen *et al.*, 1996) and Scotland (19.6% ± 3.9%, $\chi^2 = 35.48$, Gaffney and Campbell, 1994).

The hypothesis of absence of the *A539T* allele in the ancient South American Amerindians and of its present occurrence due to admixture with individuals of European and African origin was tested using the degree of admixture

(17% in the Guarani-Ñandeva and 3% in the Guarani-Kaiowá sub-groups) reported by Tsuneto (2003). Considering that the *A539T* allele frequency is 17.80% in Euro- and Afro-Brazilians (Souza *et al.*, 1998), the expected frequency due only to admixture for the *A539T* variant in this Guarani sample would be 1.38% and is statistically lower ($\chi^2 = 19.14$, $p < 0.001$) than the 3.69% found in the present study. The hypothesis that the *A539T* allele only occurred in Amerindians due to admixture was formulated by Acuña *et al.* (2003) to explain the frequency of this variant in the Chilean Mapuche but the present data can only be explained by European admixture if it was followed by genetic drift. Considering that the *A539T* allele has a wide ethnical distribution and could also have been present in paleo-Amerindians, an alternative hypothesis is that the low frequency found in this Guarani group in relation to other ethnic groups could be due only to the role played by genetic drift in a small and isolated population.

The frequencies of the CHE2 C5+ phenotype was 14.48% ± 2.92% in the Guarani-Kaiowá and 18.07% ± 4.22% in the Guarani-Ñandeva sub-groups (Table 2) and did not differ statistically ($\chi^2 = 0.51$, $p > 45\%$), indicating that these sub-groups can be considered as one population sample in relation to *CHE2* gene variability with the overall CHE2 C5+ frequency in the 250 examined individuals being 14.40% ± 2.22% (not shown in Table 2). The CHE2 C5+ phenotype has been found in many other Amerindian populations (Primo-Parmo *et al.*, 1986; Guerreiro *et al.*, 1987; Alcântara *et al.*, 1995), indicating a wide distribution among Brazilian Amerindians from what was inferred the occurrence of the *CHE2*C5+* allele in the paleo-Amerindians. Alcântara *et al.* (1995) have reported that the Guarani-M'byá sub-group from Rio das Cobras, in the Brazilian state of Paraná, has a CHE2 C5+ frequency of 45.93% ± 3.80% and when this was compared with the present data (14.40% ± 2.22%), a statistically significant difference was found ($\chi^2 = 51.10$; $p < 0.0001\%$). The present data on CHE2 C5+ phenotype frequencies are in the same direction as data previously obtained on the basis of *HLA-DRB1* allele frequencies which led to estimates of genetic distances between the M'byá and Kaiowá (0.0410), M'byá and Ñandeva (0.0307) and Kaiowá and Ñandeva (0.0165) (Tsuneto *et al.*, 2003; Petzl-Erler, 2005, pers. comm.). Considering the CHE2 C5+ present data and the frequency distribution of this phenotype in the 29 different samples of Brazilian Amerindian populations studied by Alcântara *et al.* (1995) in which the frequency of this phenotype ranged from 0 to 50.5% without showing any gradient, it seems that genetic drift is the main evolutionary factor responsible for the *CHE2* variability distribution in these Amerindian groups. This may also be the reason for the difference shown by the Guarani sub-groups from quite different Brazilian geographic areas. As far as we can ascertain from the available data, natural selection does not seem to have played any relevant role in the present distribution of CHE2 C5+ frequencies in the Brazilian Amerindians so far studied.

The mean BChE activity of the Kaiowá sample (N = 49; 3.89 ± 0.20 KU/L) and of the Ñandeva sample (N = 27; 4.28 ± 0.29 KU/L) do not differ ($t = 1.13$). The overall mean BChE activity was 4.01 ± 0.15 KU/L for 86 individuals, including mixed descendants from the two groups. This overall mean was significantly higher ($p < 0.001$) than the mean values reported for 189 individuals from the Pacaás Novos (2.5 ± 0.07 KU/L, $t = 9.12$), 193 individuals from the Sateré Mawé (3.0 ± 0.08 KU/L, $t = 5.82$) and 23 individuals from the Tenharim (2.9 ± 0.1 KU/L, $t = 6.10$), all Brazilian Amerindian groups studied by Primo-Parmo *et al.* (1986). The differences found in these mean activities may be due to differences in eating habits, BChE variant frequencies and plasma storage time.

A well known fact documented by many studies is that Amerindians show less genetic variability than non-isolated populations. The *BCHE* gene variability found in present study in the Guarani sub-groups differs from that found in non-isolated populations in terms of the frequencies of the *G390V* and *A539T* alleles. When the total heterozygote frequency (24.6%: *U/D70G* = 1.20%; *U/G390V* = 17.65%; *U/A539T* = 5.74%) found for the *BCHE* gene in the present study was compared to the respective expected (33.4%: *U/A539T* = 29.26% - Souza *et al.,* 1998; *U/D70G* = 3.53% and *U/G390V* = 0.56% - Mikami, 2005) for the general population of Curitiba (South Brazil) it was shown that the Guarani presented significantly lower heterozygote frequency ($\chi^2 = 8.00$; $p < 0.01$) on what refers to these variants considered for the *BCHE* gene.

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