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HLA class II diversity in seven Amerindian populations. Clues about the origins of the Aché

Key words:
Amerindians; genetic polymorphism; HLA; human evolution; MHC

Acknowledgments:
Thanks are due to the Brazilian Fundação Nacional do Índio for authorizing this study and for logistic assistance. In Paraguay, the Fundación Moisés Bertoni provided logistic support. The subjects of this investigation were informed about the aims of the study and gave their consent, which is gratefully acknowledged. We thank Dr Alberto Prioli for assistance. Financial support was provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa de Apoio a Núcleos de Excelência (PRONEX), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação da Universidade Federal do Paraná (FUNPAR), and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

Abstract: The study of the HLA variability of Native American populations revealed several alleles specific to one or more of the Latin American indigenous populations. The analysis of Amerindian groups distributed all over the continent might inform about the area of origin and the dispersal of these alleles and shed light on the evolution of this remarkable polymorphism. Moreover, HLA alleles and haplotypes are excellent markers to understand the genetic relationships between populations. For these reasons, we characterized the HLA class II polymorphism in seven South American Amerindian populations and compared the results with those previously reported for other Amerindian groups. The Guarani-Kaiowá ($n = 160$) and Guarani-Ñandeva ($n = 87$) were from the Brazilian state of Mato Grosso do Sul, the Guarani-M'byá ($n = 93$) and Kaingang ($n = 235$) from Paraná state, the Aché ($n = 89$) from eastern Paraguay, the Quechua ($n = 44$) from Andean Peru. From Amazonia, a heterogeneous group was analyzed ($n = 45$). The most frequent alleles and haplotypes are common also in other Amerindian populations. Each *HLA-DRB1* allele was typically found in combination with just one *DQA1-DQB1* haplotype, most likely as a result of some form of random genetic drift and reduced gene flow from non-Amerindians. The frequency distribution differed significantly among all populations, although differences were less pronounced between the Guarani subgroups. Marker alleles allowed an estimate of European and sub-Saharan African gene flow into these populations: Quechua 23%, Guarani-Ñandeva 14%, Kaingang 7%, Guarani-M'byá 4%, Guarani-Kaiowá, Amazonia, and Aché 0%. Interestingly, the *DRB1*1413* allele, previously found only among the Guarani-M'byá (frequency 15%), appeared in the Aché (8%). The relationship of the Aché to other Amerindian populations is unclear, and this finding reveals a link with the Guarani. On the basis of genetic distance and the HLA allele/haplotype set, we propose that the Aché are differentiated Tupi-Guarani group, most closely related to the Guarani-M'byá.

Amerindians living in Brazil are very diverse. Nowadays, they are 217 ethnic groups, speaking more than 180 different languages and

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Received 6 April 2003, revised 1 July 2003, accepted for publication 22 July 2003

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Tissue Antigens.

Tissue Antigens 2003: **62:** 512–526
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adding up to approximately 350,000 individuals (1). The Guarani language belongs to the Tupi-Guarani linguistic family, which is part of the Tupi branch. The Guarani were amongst the first Amerindians contacted by the European conquerors, at the beginning of the 16th century. Part of the population was decimated, part incorporated by intermarriage in the Brazilian-admixed population, and part remained isolated. The contemporary Guarani population in Brazil has been estimated as 35,000 people, living in the Southern, South-eastern, and Central regions (2). In Argentina, Bolivia, and especially Paraguay, there are also Guarani villages. Guarani-M'byá, Guarani-Kaiowá, and Guarani-Ñandeva are three subgroups, differing in several aspects of their culture. In spite of intense contact with neo-Brazilians and with other indigenous groups, the Guarani preserve much of their cultural identity, the M'byá being the most traditional.

The Aché constitute a relatively small population (about 700 individuals) of native inhabitants of eastern Paraguay who differ from other indigenous Paraguayan groups regarding several aspects, including language and culture. Their language is listed under the Tupi-Guarani linguistic family, and individuals belong to the two subgroups Ypety and Yvytyruzu (3). The Aché, referred to also as Guayaki, live in small groups, generally composed of 15–70 individuals and move camp frequently. They avoid interethnic marriages and meetings and also generally do not visit any of the Guarani groups.

The Kaingang (or Caingang, formerly named also Coroado and Guaianá) are Gê-speaking Amerindians. Approximately 25,000 Kaingang live in southern Brazil, distributed over more than 30 localities (4). Agriculture is their principal subsistence activity, although hunting and fruit gathering are also important. Individuals may belong to one of the two subgroups Kamé and Kairu.

Although most Peruvians speak Spanish, some 30% of them speak the Quechua- or Aymara-native dialects. Quechua-speaking population groups of Peru live in the Andean highlands, while the Aymara live mostly around the Lake Titicaca. Those who speak Quechua as their first language are called Quechua Indians by their dominant Spanish-speaking neighbors. However, most Quechua speakers, who live in numerous culturally distinct populations, prefer to identify themselves with their Inca heritage. They refer to themselves as Runa, 'the people'. The official language of the ancient Inca Empire was Quechua, but the Quechua culture originated in central Peru at least 1000 years before the rise of the Inca Empire in the early 1400 's. Actually, it is spoken by 13 million people of Peru, Ecuador, Bolivia, Brazil, Argentina, Colombia, and Chile (5).

Indigenous groups of eastern Amazonia belong to different linguistic families. The Guajá (Awá) speak a language of the Tupi branch, the Katuena (Wai-Wai) and Arara (Ukarãngmã) speak a Karib language, whereas the Xikrin are a subgroup of the Kayapó and speak a Gê language (1).

The human major histocompatibility complex (MHC) comprises more than 200 genes, including those of the human leukocyte antigen (HLA) system. The classical HLA genes are the most polymorphic among all known genes. Several HLA alleles are widely distributed in populations all over the world, probably being the most ancient among extant alleles. Others have a more restricted distribution, being nevertheless shared by populations autochthonous to one or more than one continent. These may have originated after the first human migrations out of Africa. Still others seem to be confined to a few populations or even to just one population and probably have a much more recent origin. Analyses of gene frequency data and of allele and haplotype structure in isolated populations are contributing to the understanding of the mechanisms generating new alleles, the evolutionary factors that result in genetic polymorphism, the system dynamics in populations, and the biological relationships among populations.

Each of the genetically isolated Amerindian populations has a comparatively restricted number of HLA alleles, and the allele sets differ markedly among populations, especially between the North and South American Native populations. Moreover, pronounced differences are seen between Native Americans and other ethnic groups (6). Even so, the nucleotide sequences of the HLA alleles seen in Amerindian populations reinforce the hypothesis of their Asian origin: all alleles pertain to lineages shared with Eastern Asians, including the specific alleles. DNA sequencing of the HLA alleles in Amerindian populations revealed many variants restricted to one or more Latin American indigenous populations and not found in other regions of the world, including North American Natives. The proportion of these alleles is highest in the *HLA-B* locus, followed by *HLA-DRB1*. They probably originated after the arrival of the founder populations in the American continent (7).

The turnover model proposed to explain the small number of HLA alleles seen in Latin American Native populations, and the high proportion of new alleles postulates the concerted action of random genetic drift and balancing, pathogen-driven selection, resulting in preferential maintenance of new alleles (generated mainly by gene conversion) (7). Conversely, the high diversity found in exogamic groups, as exemplified by most of the urban populations, is the product of gene flow (7, 8).

Several questions concerning the origins of the alleles and haplotypes characteristic of Southern Amerindians and the genetic relationships between populations remain unanswered. Where and when did the alleles originate? What does the distribution of alleles and haplotypes tell about the relationships among populations and about the evolutionary factors, which would influence HLA variability? To

approach these questions, we characterized the *HLA-DRB*, *-DQA1*, and *-DQB1* polymorphism in seven South American Indian populations. Genotype, allele, and haplotype frequencies and the degree of linkage disequilibrium were estimated. Data were used to compare populations, to estimate the gene flow from non-Amerindians into these seven populations, to test for evidences of natural selection or selective neutrality, and to calculate the genetic distance between diverse Amerindian and non-Amerindian populations.

Materials and methods

Population samples

The Guarani-Kaiowá ($n = 160$) were contacted in the indigenous reservation areas named Amambai (23°06′S, 55°12′W; population of approximately 4500 people amongst Guarani-Kaiowá and Guarani-Ñandeva) and Limão Verde (23°12′S, 55°06′W; 460 people). The Guarani-Ñandeva ($n = 87$) are from Porto Lindo (23°48′S, 54°30′W; 1600 people) and Amambai. These villages are located in southern Mato Grosso do Sul, in Central Brazil. The Guarani-M'byá ($n = 93$) are from the indigenous reservation Rio das Cobras, municipality of Nova Laranjeiras (25°18′S, 52°32′W; 1600 people, including Kaingang and Guarani-M'byá. Approximately two-thirds are Kaingang), Paraná state. The Kaingang ($n = 235$) are also from Rio das Cobras and from Ivaí, municipality of Manoel Ribas (24°30′S, 51°40′W; 700 people), Paraná state. The Aché ($n = 89$) live in eastern Paraguay, in the villages of Arroyo Bandera (23°30′S, 55°50′W; 110 people) and Chupa Pou (24°10′S, 56°30′W; 500 people). The Quechua ($n = 44$) are from the mountainous regions of Peru. The Amerindians from the Amazon region ($n = 45$) are a heterogeneous group, formed by individuals pertaining to different groups and subgroups: Awá-Guajá-Awá ($n = 16$), Awá-Guajá-Turiaçu ($n = 1$); Awá-Guajá-Guajá ($n = 1$); Katuena of the Mapuera village ($n = 12$); Arara of Laranjal ($n = 11$), and Xikrin ($n = 4$). The Guajá inhabit the Alto Turiaçú, Carú, and Awá reservation areas located in the valleys of the Gurupi, Turiaçú, and Pindaré rivers (2–6°S, 46°W) in the State of Maranhão. Today, their population is estimated at 280 persons. The Katuena are of the Mapuera village, located on the left margin of the Mapuera River (0°40′S, 57°55′W), in the state of Pará. The population is estimated at 152 people. In the history of the Arara, there are records of several conflicts with non-Indians, as a result, the Arara's population was drastically reduced. The Arara of Laranjal village is located in the state of Pará (3°30′S, 53°21′W; 195 people). Xikrin is a Kayapó subgroup; their village is located on the left bank of the middle Bacajá River, a right-hand tributary of the Xingú River, in the state of Pará (4°55′S, 51°20′W; population estimated at 555 people).

Biological material

DNA was obtained from peripheral blood, collected with anti-coagulant ethylenediaminetetraacetic acid, and extracted by standard salting-out and phenol/chloroform/isoamyl alcohol methods. Class II HLA genotypes were determined by polymerase chain reaction-sequence-specific oligonucleotide probes (PCR-SSOP). The products of locus- or group-specific PCR were applied on positively charged nylon membranes, in the form of dot-blots, denatured and fixed, and then allowed to hybridize sequentially with a set of biotin-labeled allele- or group-specific SSOP. Procedures followed the protocols and reagents of the XII International Histocompatibility Workshop (9). Initially, all *DRB* genes were coamplified, and a limited set of 30 SSOP was used. This low-resolution typing allowed assigning the alleles of each individual to groups. After, group-specific PCR was performed for the *DRB1* locus. Dot-blots were prepared and different subsets of the 91 *DRB* probes were used to identify the alleles of groups *DRB1*01*, *DRB1*03*, *DRB1*04*, *DRB1*07*, *DRB1*08*, *DRB1*09*, *DRB1*10*, *DRB1*11*, *DRB1*12*, *DRB1*13*, *DRB1*14*, *DRB1*15*, and *DRB1*16*. In addition, the pertinent *DRB3* and/or *DRB5* gene-specific PCR-SSOP were performed. The *HLA-DQA1* and *HLA-DQB1* genes were also typed by PCR-SSOP, using 19 and 35 probes, respectively. DNA samples with known genotypes were used as positive and negative controls for the probes.

Data analysis

Genotype, allele, and haplotype frequencies for loci *HLA-DRB1*, *-DRB3*, *-DRB5*, *-DQA1*, and *-DQB1* were estimated directly by counting. The significance of the differences between allele frequencies of populations was evaluated with the RXC software (10) that employs the metropolis algorithm to obtain an estimate of the *P*-value.

The significance of deviations of genotype frequencies from values expected under Hardy–Weinberg equilibrium was evaluated by Guo & Thompson's (11) exact test, using the ARLEQUIN software package, version 2.000 (12). The CONVERT software (13) was used to generate the input files for the ARLEQUIN package.

Haplotypes were assigned to each individual on the basis of well-established linkage disequilibria among alleles of these loci, considering previous reports for isolated Amerindian populations (6, 14–16). Absolute linkage disequilibrium (Δ) for two and three loci was calculated by the difference between the observed and the expected

Populations whose *HLA-DRB1* allele frequencies were used for the genetic distance analyses

Population	Language	Country	Reference
Guarani-M'byá	Tupi-guarani	Brazil	This study
Guarani-Kaiowá	Tupi-guarani	Brazil	This study
Guarani-Ñandeva	Tupi-guarani	Brazil	This study
Kaingang	Gê	Brazil	This study
Aché	Tupi-guarani	Paraguay	This study
Quechua	Quechua	Peru	This study
Amazonian Indian	various	Brazil	This study
Terena	Aruak	Brazil	(62)
Ticuna	Macro-tucanoan	Brazil	(45)
Xavante	Gê	Brazil	(42)
Toba	Guaicuru	Argentina	(63)
Wichi	Mataco	Argentina	(38)
Chiriguano	Tupi-guarani	Argentina	(6)
Chilenean Indian	unknown	Chile	(64)
Cayapa	Chibchan-paezan	Ecuador	(46)
Arsario	Arhuaco	Colombia	(34)
Kogui	Arhuaco	Colombia	(34)
Arhuaco	Arhuaco	Colombia	(34)
Wayú	Arawak	Colombia	(34)
Ingano	Quechua	Colombia	(15)
Paez	Chibcha	Colombia	(15)
Guambiano	Chibcha	Colombia	(15)
Ijka	Arhuaco	Colombia	(31)
Guahibo	Arawak	Colombia	(31)
Nukak	Makú	Colombia	(31)
Sikuani	Arawak	Colombia	(31)
Wauana	Chocó	Colombia	(31)
Embera	Chocó	Colombia	(31)
Tule	Cuna	Colombia	(31)
Bari	Chibchan-paezan	Venezuela	(30)
Yucpa	Carib	Venezuela	(14)
Venezuelan Mestizo	Spanish	Venezuela	(65)
Seri	Hakano-coahuitleca	Mexico	(16)
Lacandon	Mayanse	Mexico	(35)
Mazatecan	Macro-mindteco	Mexico	(66)
Zapotec	Otomangue	Mexico	(67)
Mixtec	Otomangue	Mexico	(57)
Mixe	Penutian	Mexico	(67)
Mexican Mestizo	Spanish	México	(68)
North American Indian	Unknown	USA	(42)
Eskimo	Inuit	Alaska	(50)
Tsimshiam	Penutian	Canada	(51)
Carrier-Sekani	Athabaskan	Canada	(51)
Brazilian Caucasoid 1	Portuguese	Brazil	(69)
Brazilian Caucasoid 2	Portuguese	Brazil	(49)
Brazilian Negroid	Portuguese	Brazil	(49)
Japanese	Japanese	Japan	(52)
Wailbri	Adynyamathanha	Australia	(70)

Table 1

haplotype frequencies. The expected haplotype frequency corresponds to the product of the allele frequencies. Relative linkage disequilibrium (Δ') was calculated according to Imanishi et al. (17). The significance of the deviations was estimated using the RXC software (10).

The Ewens–Watterson test of selective neutrality (18, 19) was applied for each locus and population, using the ARLEQUIN package.

To estimate the degree of gene flow from non-Amerindians (genetic admixture), the cumulated frequency of marker alleles/haplotypes of non-Amerindian origin was used (20).

Genetic distances were calculated using Cavalli–Sforza chord measure (21). The populations whose *DRB1* locus allele frequencies were used and the corresponding references are summarized in Table 1. A dendrogram was constructed by the neighbor joining method (22). Analyses were performed with the PHYLIP software (programs GENDIST and NEIGHBOR) (23). The tree was drawn with the TREEVIEW program (24).

Results

HLA-DRB1, *-DQA1*, and *-DQB1* distributions

In comparison to exogamic populations, all Amerindian populations analyzed have low allelic diversity (Tables 2–4). In agreement with the results of previous studies of Amerindian populations, the common alleles of locus *HLA-DRB1* belong to the *DRB1*04*, *DRB1*08*, *DRB1*09*, *DRB1*14*, and *DRB1*16* lineages/groups (Table 2). Alleles pertaining to lineages *DRB1*01*, *DRB1*03*, *DRB1*07*, *DRB1*10*, *DRB1*11*, *DRB1*12*, *DRB1*13*, and *DRB1*15* occur at comparatively low frequencies in the populations analyzed. They are considered markers of gene flow from populations of other continents.

Of the *DRB1*16* lineage, the only allele found, *DRB1*1602*, is the most frequent in this locus among the Guarani and does not occur in the Aché (Table 2). Among the populations analyzed,

allele *DRB1*0411* is the most widespread of lineage *DRB1*04*, with a mean frequency of 25%. The frequency in the Aché (74.1%) is uncommonly high for a HLA allele. However, it does not occur in the Quechua and the only Kaingang individual having allele *DRB1*0411* carries a haplotype commonly found among the Guarani. Gene flow from the Guarani-M'byá into the Kaingang population has been estimated as 0.5% (20), and this individual is probably of mixed Kaingang and Guarani ancestry. The next most common *DRB1*04* allele is *DRB1*0407*, occurring in three of the seven populations, with the highest frequency in the Quechua (14.8%). Notice the frequency difference of this allele among the three Guarani subgroups. It does not occur in the Kaingang, which instead have *DRB1*0404* at a high frequency (25.5%).

***HLA-DRB1* allele frequencies (%)**

		Population						
Lineage	Alelles	Guarani-M'bya ($n=93$)	Guarani-Kaiowá ($n=160$)	Guarani-Ñandeva ($n=87$)	Aché ($n=87$)	Kaingang ($n=235$)	Amazonia ($n=41$)	Quechua ($n=44$)
*DRB1*01*	*0101*			1.1				
	0102			0.6		0.8		1.1
	0103			1.1				
*DRB1*15*	*15*							2.3
*DRB1*16*	*1602**	37.1	33.4	29.3		12.5	7.3	5.7
*DRB1*03*	*0301*	1.6		0.6				3.4
	0302			0.6				
*DRB1*04*	*04*			1.8		0.8		2.3
	*0403**				0.6			
	*0404**	0.5	0.3			25.5		
	*0407**		8.1	1.1				14.8
	0408							2.3
	*0411**	26.9	4.1	6.9	74.1	0.6	63.4	
*DRB1*11*	*11*	1.1		0.6		1.5		4.6
	1101			3.4				
	1102					2.6		
	1104			3.4				
*DRB1*12*	*12*							1.1
*DRB1*13*	*1301*			0.6		0.2		3.4
	1303			0.6				
*DRB1*14*	*14*						1.3	4.5
	*1402**	0.5	20.3	20.1	5.2	1.5	8.5	13.6
	*1413**	15.0	0.3	0.6	8.0			
*DRB1*07*	*07*	0.5		1.7		0.2		4.5
*DRB1*08*	*0802**	2.7	10.0	9.8	3.4	49.6	11.0	12.5
	*0804**	8.1	0.3	4.6			8.5	
	*0807**	2.1	11.6	6.3	8.0			1.1
*DRB1*09*	*090102**	2.7	10.6	4.6		0.4		18.2
*DRB1*10*	*10*	0.5				1.9		
	ND	0.7	1.0	0.6	0.7	1.9		4.6

*Alleles previously found in genetically isolated South American Indian populations.
n, sample size; ND, not defined.

Table 2

***HLA-DQA1* allele frequencies (%)**

	Population					
Alleles	Guarani-M'byá ($n=93$)	Guarani-Kaiowá ($n=160$)	Guarani-Ñandeva ($n=87$)	Aché ($n=87$)	Kaingang ($n=235$)	Quechua ($n=44$)
01	0.5		3.4		2.8	1.2
0102						4.5
0103	1.1				0.2	
0104						1.2
0201	0.5		1.7		0.2	4.5
*03**	30.1	23.4	14.4	74.7	27.1	36.4
*0401**	12.9	21.9	21.3	11.5	51.2	13.6
*0501**	54.8	54.1	58.6	13.8	18.0	28.4
ND		0.6	0.6		0.5	10.2

*Alleles previously found in genetically isolated South American Indian populations.
n, sample size; ND, not defined.

Table 3

Two alleles of the *DRB1*14* lineage were found, *DRB1*1402* and **1413*. The first has a wide distribution but presents a very low frequency in the Guarani-M'byá. In contrast, *DRB1*1413* is seen only in two of the Guarani subgroups (0.3–15.0%) and in the Aché (8.0%). Its virtual absence in the Guarani-Kaiowá and the Guarani-Ñandeva is remarkable.

Lineage *DRB1*08* has three alleles in these populations. Allele *DRB1*0802* appears in all of them, with the highest frequency occurring in the Kaingang (49.6%) and the lowest in the Guarani-M'byá (2.7%). *DRB1*0807* was not seen either in the Amazonian or in the Kaingang populations, whereas *DRB1*0804* is absent in the Aché, Kaingang, and Quechua populations.

Allele *DRB1*090102* is the only allele of lineage *DRB1*09* found in the present study. The highest frequency was seen in the Quechua (18.2%). This allele was not seen in the Aché and Amazonian Indians.

All *DRB1*1602* and *DRB1*14* individuals had, respectively, alleles *DRB5*0202* and *DRB3*0101* too, an evidence that linkage

***HLA-DQB1* allele frequencies in Amerindian populations (%)**

	Population					
Alleles	Guarani-M'byá ($n=93$)	Guarani-Kaiowá ($n=160$)	Guarani-Ñandeva ($n=87$)	Aché ($n=87$)	Kaingang ($n=209$)	Quechua ($n=44$)
0501	0.5		3.4		2.9	1.2
06			0.6			5.7
0602	1.1					
0603					0.2	
02	2.1		1.2		0.5	
0201			1.2			7.9
*0301**	53.2	54.1	57.5	13.8	18.7	31.8
*0302**	26.9	16.9	10.3	74.7	26.1	18.2
*030302**	2.7	10.9	4.6		0.4	18.2
*0402**	12.9	18.1	20.7	11.5	51.0	13.6
ND	0.6		0.5		0.2	3.4

*Alleles previously found in genetically isolated South American Indian populations.
n, sample size; ND, not defined.

Table 4

disequilibrium is absolute for these DRB haplotypes (data not shown).

As for locus *HLA-DQA1*, the sum of the frequencies of the alleles *DQA1*03*, *DQA1*0401*, and *DQA1*0501* exceeds 90% in all the seven population samples (Table 3). The most frequent alleles are *DQA1*0401* in the Kaingang (51.2%), *DQA1*0501* in the Guarani (54.1 to 58.6%), and *DQA1*03* in the Aché (74.7%) and Quechua (36.4%). The Amazonian Indians were not tested for *HLA-DQA1* or *HLA-DQB1*.

In the *HLA-DQB1* locus, alleles *DQB1*0301*, *DQB1*0302*, and *DQB1*0402* were found in all populations. The Aché lack allele *DQB1*030302*, which is seen at low frequency in the Kaingang (0.4%; Table 4). Allele *DQB1*0302* is the most frequent (74.7%) in the Aché, *DQB1*0301* in the Guarani (53.2–57.5%), and Quechua (31.8%) and *DQB1*0402* in the Kaingang (51.0%).

Hardy–Weinberg equilibrium and selective neutrality

Genotype frequencies are in Hardy–Weinberg equilibrium (Table 5). The Amazonian population sample was not tested, because it is heterogeneous. Distribution of allele frequencies is consistent with selective neutrality, i.e., there is no evidence of the action of balancing selection for these genes, even though the observed homozygosity is, in general, lower than the expected homozygosity (Table 5).

Haplotype frequencies and linkage disequilibrium

The most frequent haplotypes are *DRB1*0411-DQA1*03-DQB1*0302* in the Aché (74.1%), *DRB1*0802-DQA1*0401-DQB1*0402* in the Kaingang (49.6%), and *DRB1*1602-DQA1*0501-DQB1*0301* among the Guarani (26.3–37.1%; Table 6). Usually, each *HLA-DRB1* allele is found in just one DR-DQ haplotype. The haplotypes have relative delta values (Δ') close to 1, and significant positive linkage disequilibrium was observed for all the most frequent haplotypes in at least one of the populations (Table 6). For less common haplotypes, often the Δ' value is close to 1 but $P > 0.05$, due to the small sample size.

Gene flow

The degree of genetic admixture was estimated on the basis of the HLA allele frequencies. Non-Amerindian alleles are identified due to their absence from nonadmixed Amerindian populations and to the extended MHC haplotypes bearing these alleles, which include also other alleles characteristic of populations from other continents. On the basis of the frequencies of *HLA-DRB1* alleles of probable non-indigenous origin (Table 2), the Quechua present the highest estimated admixture rate (22.7%), followed by the Guarani-Ñandeva (14.3%), Kaingang (7.2%), and Guarani-M'byá (3.7%). No evidence of gene flow from non-Amerindians was detected for the Guarani-Kaiowá, Aché, and the Amazonian Indians.

Population comparisons and genetic affinities

The *HLA-DRB1*, *-DQA1*, and *-DQB1* allele frequencies were statistically compared between the seven populations. *HLA-DRB1* allele frequencies were used to estimate genetic distances (results not shown; some are commented below). Allele frequencies differ significantly ($P < 10^{-6}$) among the populations studied, regardless of them being of the same geographic region or linguistic group. Among the seven populations studied, the genetic distance is lowest between the

Results of the Hardy–Weinberg equilibrium test and the Ewens-Watterson test of selective neutrality

	HLA-DRB1					HLA-DQA1					HLA-DQB1				
	Fo (%)	Fe (HW) (%)	Fe (EW) (%)	*P* (HW) (%)	*P* (EW) (%)	Fo (%)	Fe (HW) (%)	Fe (EW) (%)	*P* (HW) (%)	*P* (EW) (%)	Fo (%)	Fe (HW) (%)	Fe (EW) (%)	*P* (HW) (%)	*P* (EW) (%)
M'byá	15.0	24.2	26.4	26.8	53.4	36.6	40.5	46.0	98.7	44.9	33.3	38.5	41.2	95.1	52.7
Kaiowá	16.4	19.3	31.3	25.9	10.8	35.2	39.3	54.5	25.3	23.7	35.2	36.1	63.4	9.7	7.0
Ñandeva	11.5	14.7	12.0	20.8	86.0	41.4	40.8	46.7	8.8	44.2	36.8	38.4	33.0	8.2	75.3
Aché	56.3	56.4	40.9	53.8	85.0	57.5	58.8	70.0	63.9	35.0	57.5	58.8	69.8	63.9	35.1
Kaingang	33.6	23.9	23.6	25.5	88.0	35.6	26.6	46.1	16.8	35.9	36.4	26.4	42.5	19.6	41.5
Quechua	6.8	9.5	14.6	30.2	11.4	22.7	20.8	32.1	19.4	29.3	20.5	15.3	29.5	21.9	10.1

Fe (EW), homozygosity expected under selective neutrality; Fe (HW), homozygosity expected under Hardy–Weinberg equilibrium; Fo, observed homozygosity; *P*, probability.

Table 5

Haplotype frequencies (%) and linkage disequilibrium

Haplotype (DRB1-DQA1-DQB1)	Guarani–M'byá ($n=93$)				Guarani–Kaiowá ($n=160$)				Guarani–Ñandeva ($n=87$)				Aché ($n=87$)				Kaingang ($n=235$)				Quechua ($n=44$)			
	Hfo	Δ	Δ′	*p*	Hfo	Δ	Δ′	*p*	Hfo	Δ	Δ′	*p*	Hfo	Δ	Δ′	*p*	Hfo	Δ	Δ′	*p*	Hfo	Δ	Δ′	*p*
01-01-0501									2.9	2.9	1	NS					0.8	0.8	1	NS				
03-0102-0201																					3.4	3.4	1	NS
03-0501-02	1.6	1.6	1	NS					0.6	0.2	0.274	NS												
1602-0501-0301	37.1	26.3	1	$<10^{-6}$	33.4	23.6	1	$<10^{-6}$	29.3	19.4	1	$<10^{-6}$					12.5	12.1	1	$<10^{-6}$	4.5	4.0	0.769	NS
0403-03-0302													0.7	0.3	1	NS								
0404-03-0302	0.5	0.5	1	NS													25.5	23.7	1	$<10^{-6}$				
0407-03-0302					8.1	7.8	1	$<10^{-6}$	1.1	1.1	1	NS									12.5	11.5	0.835	5.3×10^{-3}
0408-03-0302																					2.3	1.8	1	NS
0411-03-0302	26.9	24.7	1	$<10^{-6}$	4.1	3.7	1	$<1.2\times10^{-3}$	6.9	6.8	1	3.4×10^{-3}	74.1	32.7	1	$<10^{-6}$	0.6	0.6	1	NS				
11-0102-0602	1.1	1.1	1	NS																				
11-0501-0301									7.5	5.0	1	4.3×10^{-2}					4.1	4.0	1	4×10^{-5}	3.4	3.4	0.755	NS
13-0501-0301									0.6	0.2	0.513	NS												
1402-0501-0301	0.5	0.4	1	NS	20.3	14.4	1	$<10^{-6}$	19.5	12.7	0.955	6.4×10^{-4}	5.2	5.1	1	2.0×10^{-2}	1.5	1.4	1	NS	13.6	12.4	1	2.6×10^{-3}
1413-0501-0301	15.0	10.6	1	3.8×10^{-4}	0.3	0.2	1	NS					8.0	7.9	1	9.4×10^{-4}								
07-0201-0201									0.6	0.6	0.545	NS									3.4	3.4	0.755	NS
0802-0401-0402	2.7	2.6	1	NS	6.6	6.2	0.646	$<10^{-6}$	9.2	8.8	0.936	1.0×10^{-4}	3.4	3.4	1	NS	49.6	37.7	1	$<10^{-6}$	12.5	12.3	1	5.3×10^{-3}
0802-0401-0302									0.6	0.6	0.064	NS												
0804-0401-0402	8.1	8.0	1	2.4×10^{-4}	0.3	0.3	1	NS	4.6	4.4	1	3.5×10^{-2}												
0807-0401-0402	2.1	1.7	1	NS	11.3	11.2	0.971	$<10^{-6}$	6.3	6.0	1	4.6×10^{-3}	8.0	8.0	1	1.2×10^{-3}								
090102-03-030302	2.7	2.6	1	NS	10.6	10.6	1	$<10^{-6}$	4.6	4.6	1	3.5×10^{-2}					0.7	0.5	1	NS	18.2	17.0	1	4×10^{-5}
1001-0101-0501	0.5	0.5	1	NS													1.9	1.9	1	2.2×10^{-2}				

Δ, absolute linkage disequilibrium; Δ′, relative linkage disequilibrium; Hfo, observed haplotype frequency; *n*, sample size; *P*, probability.

Table 6

two Guarani subgroups of Mato Grosso do Sul (0.0165) and highest between the Aché and the Quechua (0.1071) and between the Aché and the Kaingang (0.1019). The genetic distance between the Aché and the three Guarani subgroups is of 0.0466–0.0728. Within the Guarani group, the Aché are closer to the Guarani-M'byá. Cultural and linguistic distinctiveness correlate with distinctiveness at the *DRB1* locus, even for populations residing in the same region (e.g., the Kaingang and the Guarani-M'byá, genetic distance of 0.0704), as reported previously (20).

Discussion

The pioneering studies about the HLA polymorphism in isolated human groups were those of Rubinstein et al. (25) on the Mapuche of Chile, of Amos et al. (26), who analyzed the Maya, and of Bodmer and Bodmer (27), who investigated the African pygmies. The major purpose of the 1972 International Histocompatibility Workshop was the study of a large number of isolated populations, which included the Amerindian populations Maya, Zuni, Papago, Warao, Quechua, and Aymara (28). By the end of the 1980 s and with the introduction of high-resolution DNA-based typing methods, population genetic studies experienced a revival, and new insights into the evolution of the HLA polymorphism have been achieved. However, several questions remained unanswered, namely: Where and when did the supposedly new HLA alleles seen in Amerindians originate? What do their distributions tell about the relationships among populations and about the evolutionary factors, which would influence the HLA polymorphism? To approach these questions, we analyzed the HLA class II polymorphism of seven Amerindian populations. The results add information on the origin of some *HLA-DRB1* alleles and the relationships among these and other Amerindian populations.

HLA-DRB1 alleles

Only five of the 13 *HLA-DRB1* allelic lineages described thus far occur in Amerindian populations putatively without non-Indian gene flow (*DRB1*04*, *DRB1*08*, *DRB1*09*, *DRB1*14*, and *DRB1*16*). Not all of them are present in all the populations analyzed. *DRB1*16* does not occur in the Aché, and the Aché and the Amazonian Indians lack the lineage *DRB1*09*. Further, several *DRB1* alleles found in other Amerindian populations are not seen in the populations analyzed, especially alleles of the *DRB1*04* lineage, which is the most diversified among Amerindians. On the other hand, *DRB1*1413*, found in the Guarani and in the Aché, has never been reported in other Amerindian or non-Amerindian populations.

The only allele of lineage *DRB1*16* thus far observed in Amerindians is *DRB1*1602*. It is widely distributed in this ethnic group, but is absent in the Aché. Two *DRB1*1602* alleles exist, whose products do not differ in their amino acid sequence. *DRB1*160201* (previously named *DRB1*16021*) is the one found in Amerindians, always on a haplotype bearing *DQA1*0501-DQB1*0301*. It was first described in the Warao of Venezuela (29). The highest frequencies have been reported for the Bari of Venezuela (40.3%) (30), the Guahibo of Colombia (39.0%) (31), and the Guarani-M'byá (37.1%) (this study). In Asian populations, allele *DRB1*160201* is associated with *DQA1*0102-DQB1*0502* or *DQB1*0602* (32). Thus, it is possible that this allele had originated twice, by independent mutation events. A more detailed analysis of its intronic and flanking non-coding regions in several ethnic groups might clarify this point.

Lineage *DRB1*04* is very diversified in Amerindians and also in populations of other continents. Most Amerindian populations have one or two of the alleles *DRB1*0403*, *DRB1*0404*, *DRB1*0407*, *DRB1*0411*, and *DRB1*0417*. Allele *DRB1*0403* has a worldwide distribution (33) and is frequent in Colombian, Venezuelan, and Mexican Amerindians. The highest frequency (22.0%) was observed in the Tule of Colombia (31). In this study, *DRB1*0403* was found in only one individual of the Aché population. Likewise, allele *DRB1*0404* is present in populations of all continents (33). It is seen at high frequency (25.5%) in the Kaingang population. The highest frequencies thus far reported (21.0–32.0%) occurred in the Colombian Sikuani, Ingano, and Guambiano Amerindians (15, 31). Allele *DRB1*0407* is also widespread in the indigenous populations of South and North America, reaching very high frequencies (45–60%) in the Kogui and Arsario populations in Colombia and the Seri in Mexico (16, 31, 34). In this study, *DRB1*0407* was found to be common in the Quechua (14.8%). Allele *DRB1*0407* has been observed in European and Eastern Asian, but not in Sub-Saharan African populations (33). On the contrary, allele *DRB1*0411* is common in Amerindians in Latin America but not North America, apart from Mexico. High frequency was observed for the Yucpa in Venezuela (61.4%) (14), the Lacandon in Mexico (56.5%) (35), and the Amazonian Indians and the Aché (63.4 and 74.1%, respectively). The high frequency in the Aché is unusual for the polymorphic HLA genes and most likely resulted from stochastic evolutionary factors, such as some form of random genetic drift, founder, or bottleneck effect. Apart from Amerindians, it seemingly occurs only in the Australian Aborigines (36). Allele *DRB1*0417* did not occur in the populations of the present study. It most likely originated in South America, because it has thus

far been found exclusively in the Toba and Wichi populations of Argentina (37, 38).

Of lineage *DRB1*14*, only the allele *DRB1*1402* is widely distributed in Amerindian populations, being uncommon in the other continents, except East Asia and especially North-east Asia (39). It occurs at high frequency in the Guarani-Kaiowá and Guarani-Ñandeva, but not in the Guarani-M'byá, among whom it is replaced by *DRB1*1413*. In fact, allele *DRB1*1413* has been first identified in the Guarani-M'byá (40, 41) and since then was not found in any other population. Its restricted distribution is compatible with the hypothesis of a recent origin. The results of the present study support this hypothesis, as *DRB1*1413* was found to be common (8%) among the Aché, who are geographically close to the Guarani and thought to be related to them (see below). A third *DRB1*14* allele, *DRB1*1406*, occurs at highest frequency in the Toba and Wichi populations from Argentina (17.1–27.2%) (42). It is common in indigenous North-east Asian populations and occurs at lower frequencies in other Eastern Asians (39). *DRB1*1406* was not seen in the populations analyzed.

At least one of the *DRB1*08* lineage alleles *DRB1*0802*, *DRB1*0804*, and *DRB1*0807* is present in all Amerindian populations. Of these, only *DRB1*0802* (**080201*) is widely distributed and has been found also in all the study populations, with a maximum frequency of 49.6% in the Kaingang. The Ijka of Colombia present the highest of the reported frequencies (62.0%) (31). The *DRB1*0807* allele was originally identified in admixed Brazilians (43) and in the Yucpa population of Colombia and Venezuela (44). In the Amazonian Ticuna population, the frequency is high (22.5%) (45). Because this allele is not seen in populations of other continents, it probably originated in America. In this study, it is seen among the Guarani and, at low frequency, in the Quechua. In turn, allele *DRB1*0804* is present in the Amazonian Indians and the Guarani (being, however, rare in the Guarani-Kaiowá). Two *DRB1*0804* alleles have been reported to occur in South American Indians. *DRB1*080402* has been identified in the Cayapa population of Ecuador (46), while *DRB1*080401* is found in African populations (47) and in populations which received gene flow from sub-Saharan Africans. The typing method allowed to distinguish between these two alleles in the Guarani-M'byá, the allele observed being *DRB1*080401*. The presence of *DRB1*080401* in Amerindians has mostly been explained by gene flow from Africans, due to its low frequency in this ethnic group and its apparent absence in Eastern Asians. In Africans, **080401* is found in a haplotype bearing allele *DQB1*0301*. The same combination was also seen in some Amerindians (48), reinforcing the hypothesis of gene flow. However, among the populations studied here, the haplotype found is *DRB1*0804-DQA1*0401-DQB1*0402* and its frequency (4.6–8.1%) is higher than the estimated admixture with Africans and Europeans. In the Sikuani population of Colombia, this allele is associated with *DQA1*0401-DQB1*0402* as in the present study, leading the authors to postulate that it originated in America (31). In fact, in the Guarani-M'byá, the extended MHC haplotype of loci *DPB1*, *DPA1*, *DMA*, *DMB*, *LMP2*, *TAP1*, *LMP7*, *TAP2*, *DQB1*, *DQA1*, *DRB1*, *HLA-B*, *-C,* and *-A* is unique and includes alleles not found in non-South American Indian populations, as *HLA-B*5104* and *Cw*1503* (49). Altogether, these observations strongly indicate that *DRB1*080401* indeed originated *de novo* in America.

Only two *DRB1*09* alleles are known. Allele *DRB1*090102* is the one seen among Amerindians, with highest observed frequency of 20% in the Cayapa (46). In this study, the allele was found to be common in Quechua (18.2%) and in Guarani-Kaiowá (10.6%). *DRB1*090102* occurs also in North American Native populations (17) including the Tsimshiam, Na-Dene, and Eskimo (50, 51) and among Eastern Asians (52), being one marker of the Asian ancestry of Native American populations.

Haplotypes and gametic association

In Amerindians, even more than in populations of other continents, DR-DQ haplotypic variability is reduced, and each *HLA-DRB1* allele is typically found in combination with just one *DQA1-DQB1* haplotype. This was observed also in the study populations. The strong associations may result from (a) the reduced physical distance among loci, which, in turn, results in very low recombination; (b) recent origin of at least one of the alleles; (c) founder effects or random genetic drift; (d) natural selection. These causes are not mutually exclusive, and their relative importance remains to be demonstrated. Nevertheless, the extreme DR-DQ disequilibrium in Amerindians reveals the effects of stochastic evolutive forces (some form of random genetic drift and, for some haplotypes, recent origin of the *DRB1* allele) and of the reduced gene flow from non-Amerindians.

Haplotypes *DRB1*1602-DQA1*0501-DQB1*0301* and *DRB1*14-DQA1*0501-DQB1*0301* may be taken as ethnic markers, because they are unique to the indigenous populations of the Americas.

Selective neutrality

No significant deviation was found for any of the genes analyzed, although homozygosity was usually lower than expected under selective neutrality. This trend is consistently observed for the classical HLA genes, being an evidence of balancing selection. It

should be noticed that very large population samples are needed to obtain statistical significance if selection pressure is low, as estimated for the HLA genes (53). Apart from this, there are evidences that the evolution of the HLA polymorphism is complex, with multiple stochastic and deterministic evolutive factors acting in concert (7, 54), so that the demonstration of each of these factors still is a difficult task.

Gene flow

All Amerindian populations have significantly reduced allelic and haplotypic HLA diversity, when compared to most European, African, and Asian populations or their descendants in other continents (33, 55–57). Amerindian populations which did not receive gene flow from populations originated in other continents, lack alleles of lineages *DRB1*01*, *DRB1*03*, *DRB1*07*, *DRB1*10*, *DRB1*11*, *DRB1*12*, *DRB1*13*, and *DRB1*15*. In populations which are experiencing isolate breakdown, alleles of these lineages may occur at low frequencies, in gametic association with European and/or African marker alleles in other MHC loci. They allow the quantification of gene flow. Among populations analyzed, the Quechua have the highest admixture rate, with 22.7% non-Amerindian *DRB1* alleles, followed by the Guarani-Ñandeva (14.3%), the Kaingang (7.2%), and the Guarani-M'byá (3.7%). For the Guarani-Kaiowá, the Aché, and the Amazonian populations, there is no evidence of admixture with non-Amerindians. A similar admixture rate has been reported previously for the Peruvian Quechua population (22%) (58). Also the Chilean Huilliche, the Argentinian Chiriguano, the Colombian Wayú and the Mexican Zapoteco, and Mixtec populations have a high proportion of non-Amerindian alleles (12–19%) (6, 34). On the other hand, some urban populations, such as Laitec in Chile and Tlaxcala in Mexico, have a high Amerindian component, of 80 and 76%, respectively (58).

Genetic affinities and the origin of the Aché population

Genetic distances based on the *HLA-DRB1* allele frequencies allowed to construct a dendrogram summarizing the affinities between the populations studied and other 41 populations (Fig. 1). Although there is no simple grouping of the population by geographic or linguistic closeness, the power of this single HLA locus as a marker of affinities is amazing. For instance, the Colombian populations pertaining to the Arhuaco linguistic group (Arsario, Kogui, Arhuaco, and Ijka) cluster together, as those of the Chibcha group do (Paez and Guambiano). A linguistically more heterogeneous group of Colombian populations includes the Guahibo, Nukak, Wauana, Embera, and Tule populations. On basis of the *HLA-DRB1* allele frequencies, the Kaingang and the Xavante, the only Gê populations analyzed, also are more related to each other than to all the other populations. Excepting the Seri and the Lacandon, the Mexican Amerindians form a cluster, as the Argentinean populations do. The Guarani-Kaiowá and the Guarani-Ñandeva are closer to each other than to all the other populations. The linguistically and geographically most heterogeneous group is the one including the Aché, the Guarani-M'byá, and the Amazonian Indians. Their grouping is explained especially by the high frequency of allele *DRB1*0411*. The Amerindian populations cluster apart from all others, including the Na-Dene and the Eskimo. Populations presenting a higher degree of admixture with non-Amerindians tend to be less differentiated (e.g., the Quechua, the Chilenean Indians, the North-American Indians, and the Wayú).

The origin of the Aché is much debated, because they present distinct morphological characteristics and have been quite isolated. The Aché language has been recognized as belonging to the Tupi-Guarani linguistic group, since the 17th century. Some authors consider that the Aché are a differentiated Guarani group, while others claim that they descend from a Gê group that preceded the Guarani colonization of Paraguay. Oral traditions of both the Aché and the Guarani populations suggest that they might have been once related (3). Battilana et al. (59) examined the variability of 32 loci, including 22 blood group and protein systems and 12 *Alu* insertions, and compared the Aché with the Xavante, a population of the Gê linguistic group, and with the Guarani. Because of the marked genetic peculiarities of the Aché, they could not decide which of the two hypotheses concerning their classification was correct, but considered more likely the view that they are a differentiated Guarani group. On the other hand, analysis of the polymorphism of two cytochrome P-450 (*CYP*), and two glutathione S-transferase (GST) genes and of the *TP53* gene (60), and of 15 short tandem repeat polymorphisms (61) indicated a closer relationship of the Aché with Gê than with Tupi-Guarani groups. The authors considered that the Aché might descend from a Gê group, but had also assimilated some amount of the Guarani gene pool, maybe through intertribal admixture. In the present study also, the Aché clearly differed from all the other populations. However, the *HLA-DRB1* genetic distance from the Guarani, especially from the M'byá subgroup is less than that from the Kaingang (0.0466, 0.0685, 0.0728, and 0.1019 for Guarani-M'byá, Guarani-Ñandeva, Guarani-Kaiowá, and Kaingang, respectively). Interestingly, allele *DRB1*1413*, that had been found exclusively in the Guarani-M'byá since its discovery more than 10 years ago (40, 41), occurs at a relatively high frequency (8%) in the Aché, revealing a genetic relationship between the two populations. Furthermore, all the five

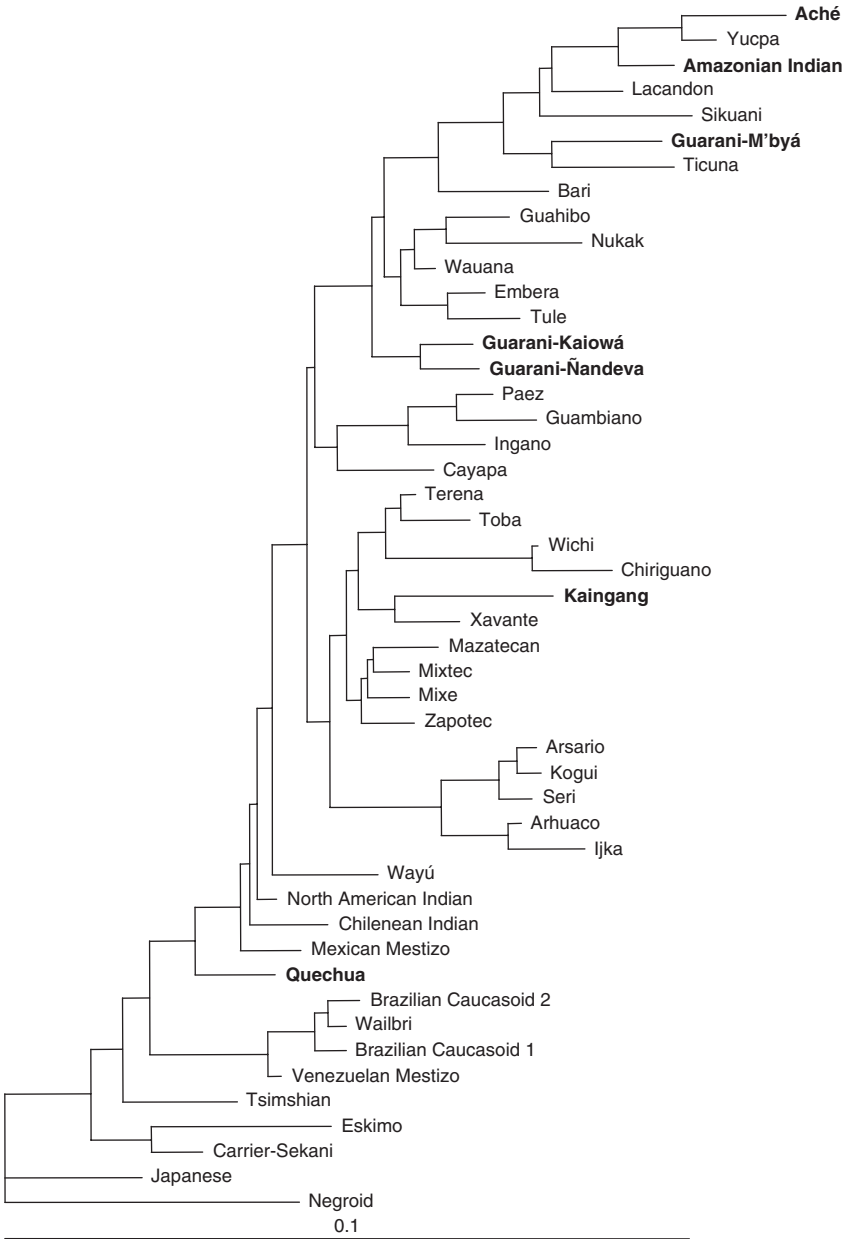

Fig. 1. NJ (neighbor joining) tree generated from a genetic distance matrix based on *HLA-DRB1* allele frequencies. Genetic distances were obtained by Cavalli–Sforza chord measure. Populations of this study are given in bold. The references for the populations are shown in Table 1.

HLA-DRB1 alleles frequent in the Aché (*DRB1*0411*, *DRB1*1402*, *DRB1*1413*, *DRB1*0802*, and *DRB1*0807*) are shared with the Guarani, while only *DRB1*0802* and *DRB1*1402*, which are alleles common in numerous Amerindian groups, are shared with the Kaingang (allele *DRB1*0411* is seen in one individual of the Kaingang population due to admixture with Guarani, see above). Altogether, these results lead us to suggest that the Aché may be a differentiated Tupi-Guarani group, most closely related to the Guarani.

Conclusions

The populations studied present limited allele and haplotype diversity and differ markedly for the HLA class II polymorphism. The most frequent alleles belong to a subset of lineages found in Eastern Asians, thereby supporting the Asian origin hypothesis. Diversity is highest in the Quechua and the Guarani-Ñandeva, which received the most intense gene flow from non-Amerindians and lowest in the Aché, Guarani-Kaiowá, and Amazonian populations.

Populations of the same linguistic group and residing in the same geographic area (Guarani-Kaiowá and Guarani-Ñandeva) differ less than the others. This might be due to a recent divergence and/or to continuing gene flow. Interchange among geographically distant and/or culturally distinct populations (Guarani-Kaingang-Aché) seems to be comparatively small.

Allele *DRB1*1413*, detected several years ago in the Guarani-M'byá and never seen in other populations, has been found among the Aché. This suggests a genetic relationship between the two, which is supported by the low genetic distance and their relative proximity in the *HLA-DRB1* dendrogram. The limited geographic and ethnic distribution of allele *DRB1*1413* is compatible with the hypothesis of recent origin of this allele.

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