



## Genetic diversity and prevalence of *CCR2-CCR5* gene polymorphisms in the Omani population

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### Abstract

Polymorphisms in the regulatory region of the *CCR5* gene affect protein expression and modulate the progress of HIV-1 disease. Because of this prominent role, variations in this gene have been under differential pressure and their frequencies vary among human populations. The *CCR2V64I* mutation is tightly linked to certain polymorphisms in the *CCR5* gene. The current Omani population is genetically diverse, a reflection of their history as traders who ruled extensive regions around the Indian Ocean. In this study, we examined the *CCR2-CCR5* haplotypes in Omanis and compared the patterns of genetic diversity with those of other populations. Blood samples were collected from 115 Omani adults and genomic DNA was screened to identify the polymorphic sites in the *CCR5* gene and the *CCR2V64I* mutation. Four minor alleles were common: *CCR5-2554T* and *CCR5-2086G* showed frequencies of 49% and 46%, respectively, whereas *CCR5-2459A* and *CCR5-2135C* both had a frequency of 36%. These alleles showed moderate levels of heterozygosity, indicating that they were under balancing selection. However, the well-known allele *CCR5Δ32* was relatively rare. Eleven haplotypes were identified, four of which were common: HHC (46%), HHE (20%), HHA (14%) and HHF\*2 (12%).

**Key words:** *CCR2*, *CCR5*, divergence, haplotype, HIV-1, Oman.

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### Introduction

The C-C motif chemokine receptor 5 (*CCR5*) is expressed in memory/effector T cells, monocytes/macrophages and immature dendritic cells (Oppermann, 2004). *CCR5* plays a critical role in physiological and pathological conditions through its ability to bind chemokines and regulate the migration of leukocytes throughout the body (Guerignon and Combadière, 2012). *CCR5* is also a co-receptor for human immunodeficiency virus-1 (HIV-1) that facilitates virus entry into cells and mediates infection (Littman, 1998; Mummidi *et al.*, 1998).

The *CCR5* gene is located in the short arm of chromosome 3 and consists of four exons and two introns (Mum-

midi *et al.*, 1997). The gene was identified with two functional promoters: an upstream promoter known as P1 and a weak downstream promoter known as P2. The P1 promoter gives rise to two full-length transcript variants while the P2 promoter results in several truncated transcripts that lack exon 1, although all produce the same *CCR5* protein (Mummidi *et al.*, 2000; Wierda and van den Elsen, 2012).

A 32-bp deletion in the open reading frame (ORF) of the *CCR5* gene (*CCR5Δ32*) causes a premature termination of translation that results in a non-functional receptor (Dean *et al.*, 1996; Liu *et al.*, 1996). *CCR5Δ32* has a variable distribution among populations of different ethnic backgrounds, *e.g.*, it is common in Caucasians but rare among Asian and African populations (Martinson *et al.*, 1997; Novembre *et al.*, 2005). The *CCR5Δ32* allele has a frequency of 10-20% among European populations, with the highest frequency found in northern Europe, and decreases to 2-5% in the Middle East and the Indian subconti-

ment (Su *et al.*, 2000). Polymorphisms in the *CCR5* promoter region can influence expression of the corresponding protein in Asian and African populations, where carriers of the *CCR5Δ32* mutation are rare (McDermott *et al.*, 1998; Mummidi *et al.*, 2000; Picton *et al.*, 2012). In addition, there is a marked difference in allele frequencies among different ethnic groups (Gonzalez *et al.*, 1999; Catano *et al.*, 2011; Malhotra *et al.*, 2011).

Another chemokine receptor, *CCR2*, is located close to the *CCR5* gene region in chromosome 3 (Al-Abdulahadi and Al-Rabia, 2010). A valine to isoleucine amino acid substitution at position 64 of *CCR2* (*CCR2V64I*) is tightly linked to certain single nucleotide polymorphisms (SNPs) in the *CCR5* *cis*-regulatory region (Kostrikis *et al.*, 1998; Wichukchinda *et al.*, 2008). *CCR264I* and the promoter SNP, rs1799987, have a beneficial role during HIV-1 infection (Vieira *et al.*, 2011).

Historically, Oman played a central role as a gateway for the spice and frankincense trade that linked Yemen and India to Africa and Eurasian regions. Consequently, the human population of this region of the Middle East is expected to display a high degree of diversity that reflects its cosmopolitan past (Cinnioglu *et al.*, 2004; Semino *et al.*, 2004; Al-Abri *et al.*, 2012). This suggests that a high degree of diversity is likely to be found in the *CCR2-CCR5* genes in the Omani population. Although a few studies have examined the frequency of *CCR5Δ32* in the Arabian Peninsula (Salem *et al.*, 2009; Voevodin *et al.*, 1999), no studies have investigated the allele frequencies of other polymorphisms and the gene diversity of the *CCR2-CCR5* complex in this region. In this study, we examined the frequency of the variable sites (the *cis*-regulatory and coding regions) of the *CCR5* gene and estimated the allele frequency of the V64I mutation in the *CCR2* gene in the Omani population. We also explored the genetic diversity based on the *CCR2-CCR5* gene locus in the Omani population and compared it with other populations.

## Materials and Methods

### Study population

Samples were collected from 115 Omani adults (60 males, 55 females) between April 2010 and April 2012. The mean age of the subjects was 36.2 years (range: 19-72). Forty-four of the subjects were healthy blood donors and 71 were patients attending Sultan Qaboos University Hospital. The study population consisted predominantly of Arabs from various regions of the country: Muscat (the capital), north, south, west and the remote region of Musandam. The aim of the study was explained individually and informed written consent was obtained from all participants. This study was approved by the Medical Research and Ethics Committee of the College of Medicine and Health Sciences, Sultan Qaboos University.

### Genotyping and nucleotide sequence analysis

Genomic DNA from all participants was extracted from peripheral blood samples anti-coagulated with ethylenediaminetetraacetic acid (EDTA) using QIAmp DNA mini kits (Qiagen, Germany), according to the manufacturer's protocol. Polymerase chain reaction (PCR) amplification of genomic DNA followed by DNA sequencing was done to cover the *CCR5* polymorphic sites -2733, -2554, -2459, -2135, -2132, -2086 and -1835. This numbering system is based on the first nucleotide of the *CCR5* translational start site defined as 1 and the nucleotide upstream from that as -1, as originally described by Mummidi *et al.* (2000). All samples were also tested for the presence of the *CCR5Δ32* and *CCR2V64I* variants in the coding regions of these two genes. The sequences of the primers used are listed in Table 1.

PCR was done using *AmpliTaq* Gold enzyme and PCR buffer (Applied Biosystems, Foster City, CA, USA). Amplified fragments were purified using ExoSAP (USB, Affymetrix, Inc. USA) and sequenced bidirectionally using

**Table 1** - Primer sequences used for PCR and sequencing reactions.

dbSNP ID	Position	Forward primer	Reverse primer	Tm (°C)
rs1799864	CCR2 V64I	TTGTGGGCAACATGCTGG	GCAATGTGCAAATTATTCA	55
rs2856758	-2733	GCTGACAATACTTGAGATTT	TCCAGGATCCCCCTCTAC	
rs2734648	-2554			57
rs1799987	-2459	CCCGTGAGCCCATAGTTAAAACCTC	CCTTACTGTTGAAAAGCCCTGTGA	61
rs1799988	-2135			
rs41469251	-2132	ATGTGAAGTCCAGGA	TGAGACATCCGTTCCCC	55
rs1800023	-2086			
rs1800023	-2086	GCCTTACTGTTGAAAAGCCCTGTGA	TAGGGGCTTCTCTCAGCTGCCT	69
rs1800024	-1835			
rs333	CCR5Δ32	GGTGGTGACAAGTGTGA	TCCAAAAGCACATTGCC	55

Position of SNP in the promoter region of the *CCR5* gene is based on the first nucleotide of the *CCR5* translational start site being designated as 1. Tm: annealing temperature.

BigDye terminator v.3.1 chemistry (Applied Biosystems) and then run on a 3130 XL Genetic Analyzer (Applied Biosystems). All sequences were aligned with the reference sequence (GenBank accession number: NT\_22517.18) and analyzed for the presence of SNPs using Lasergene sequence analysis software package v.7.1 (DNASar, Madison, WI, USA).

### Statistical analysis and population divergence

GenAIEx6.41 software (Peakall and Smouse, 2006) was used to estimate the allele frequencies of each SNP in the study sample followed by testing for significant deviation from Hardy-Weinberg equilibrium (HWE). Heterozygosity (as a measure of genetic diversity in the sample group) was calculated. Arlequin v.3.5 package (Excoffier and Schneider, 2005) was used to estimate the pairwise linkage disequilibrium (LD) between all pairs of alleles at different loci based on the expectation-maximization (EM) algorithm. The statistical significance of the LD between pairs of SNPs was assessed by the chi-square test.

Haplotypes were constructed using DnaSP v.5 (Stephens and Donnelly, 2003). The phylogenetic relationships between the *CCR2-CCR5* haplotypes were re-constructed using the median joining (MJ) algorithm contained in Network 4.6 software (Bandelt *et al.*, 1999). POPTREE software was used to draw the unrooted neighbor-joining (NJ) tree using the corrected pairwise  $F_{ST}$  distances from *CCR2-CCR5* haplotype frequencies calculated in 1000 bootstrap replicates to assess the bootstrap values (Saitou and Nei, 1987).

## Results

### Allele frequencies of the *CCR2-CCR5* gene locus

One hundred and fifteen samples from Omani adults were successfully genotyped at nine variable sites: one in *CCR2* and eight in *CCR5*. The allele frequencies and genotypes are shown in Table 2. The overall genotype distribution did not deviate significantly from Hardy-Weinberg proportions ( $p > 0.05$ ). The commonly known *CCR5* $\Delta 32$  was found in only one individual. The minor allele frequency (MAF) of the *CCR264I* allele was 13% and most of the carriers of this allele were heterozygous (20.9%). The most common minor alleles were *CCR5-2554T* and *CCR5-2086G* that occurred at a frequency of 49.1% and 46.5%, respectively. Two alleles, *CCR5-2135C* and *CCR5-2459A*, were present at lower frequencies of 36.5% and 36.1%, respectively. To further examine the degree of diversity of the *CCR2-CCR5* gene locus in the study sample, we estimated the expected heterozygosity of each SNP ( $H_e$ ). The loci -2554, -2459, -2135 and -2086 had a high level of heterozygosity (0.500, 0.461, 0.464 and 0.498, respectively) (Table 2).

### Linkage disequilibrium between pairs of SNPs

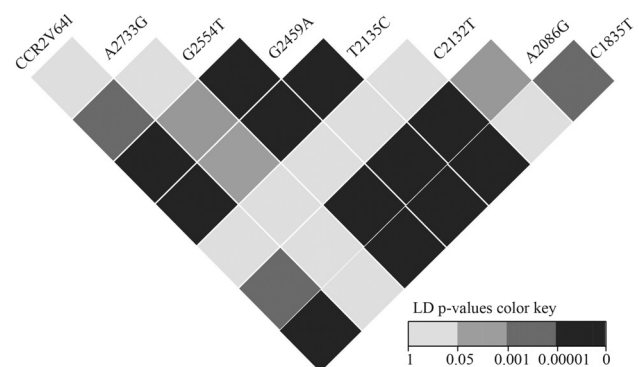
Figure 1 summarizes the results of the LD tests done for all pairwise combinations of SNPs. There was a significant LD between the distal *CCR2V64I* and the majority of *CCR5* SNPs in the promoter region. The internal SNPs at positions -2554, -2459 and -2135 were in strong LD between each other ( $p < 0.0001$ ) whereas the SNP at position -2132 was not linked to any of the tested positions.

### Haplotype frequencies and distribution

The network analysis divided all haplotypes into two main clusters. One cluster consisted of the human haplogroup (HH) - HHA, HHC and HHD, while the second cluster was composed of HHE, (HHG\*1 & HHG\*2) and (HHF\*1 & HHF\*2) (Figure 2). Three newly identified haplotypes found in three individuals in a heterozygote state could not be grouped in the previously published *CCR5* evolutionary-based classification (Mummidi *et al.*, 2000). This difficulty reflected the presence of a different combination of nucleotides at position -2459 that was not used for the classification (Figure 2). For example, haplotypes HHF\*1 and HHF\*2 had adenine at position -2459 whereas the newly identified haplotypes HHF\*1A and HHF\*2A had guanine instead. The new haplotypes were designated based on their position in relation to their descendant haplotype (Figure 2). The most common haplotype in the studied population was HHC (46.1%) followed by HHE (20.0%). Other haplotypes such as HHA (14.3%) and HHF\*2 (12.2%) were less common. Two haplotypes (HHD and HHG\*1) were both present at 2.6%. Minor haplotypes were HHF\*1 and HHG\*2 (the *CCR5* $\Delta 32$ -bearing haplotype), both present at 0.4%. The HHB haplotype was not detected in any of the subjects (Table 3).

### Population divergence

The frequency of the HHA haplotype varies among populations, with the highest frequencies in African populations (Pygmy, non-Pygmy and African-American). Two



**Figure 1** - Pairwise linkage disequilibrium results among SNPs. Colored boxes indicate significance levels of the chi-square test (light grey  $p > 0.05$ ; grey:  $0.001 < p < 0.05$ ; dark grey:  $0.0001 < p < 0.001$  and black:  $p < 0.0001$ ).

**Table 2** - Allele frequency (%), Hardy-Weinberg equilibrium (HWE), expected heterozygosity (He) and genotype distribution of all the tested loci in the sample population.

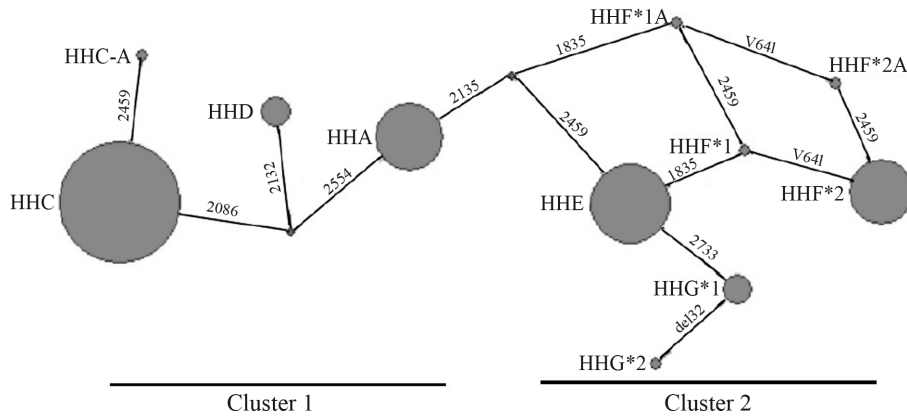
Allele	Position	Allele frequency (%) (N = 115)	HWE p value <sup>a</sup>	He <sup>b</sup>	Genotype	Genotype frequency (%)
<i>CCR2(V64I)</i>						
rs1799864	64V	87.0	0.391	0.227	VV	76.5
	64I	13.0			VI	20.9
					II	2.6
<i>CCR5 promoter</i>						
rs2856758	-2733 A	97.4	0.774	0.051	AA	94.8
	-2733 G	2.6			AG	5.2
					GG	0.0
rs2734648	-2554 G	50.9	0.928	0.500	GG	26.1
	-2554 T	49.1			GT	59.6
					TT	24.3
rs1799987	-2459 G	63.9	0.413	0.461	GG	42.6
	-2459 A	36.1			AG	42.6
					AA	14.8
rs1799988	-2135 T	63.5	0.790	0.464	TT	40.9
	-2135 C	36.5			CT	45.2
					CC	13.9
rs41469351	-2132 C	97.4	0.774	0.051	CC	94.8
	-2132 T	2.6			CT	5.2
					TT	0.0
rs1800023	-2086 A	53.5	0.429	0.498	AA	30.4
	-2086 G	46.5			AG	46.1
					GG	23.5
rs1800024	-1835 C	86.1	0.547	0.240	CC	74.7
	-1835 T	13.9			CT	22.6
					TT	2.6
<i>CCR5 coding region</i>						
rs333	Wild	99.6	0.963	0.009	W/W	99.1
	Δ32	0.4			W/Δ32	0.9
					Δ32/Δ32	0

<sup>a</sup>p values for tests of Hardy-Weinberg equilibrium (HWE) at each locus.

<sup>b</sup>He: expected heterozygosity =  $1 - \sum \pi_i^2$ , where  $\sum \pi_i^2$  is the sum of the squared population allele frequencies.

different haplotypes, HHC and HHE, are common in Asians and Europeans. The HHC haplotype is most common among Asian populations, including Thais (62%) (Nguyen *et al.*, 2004), Omanis (46%) and Indians (36%), followed by Europeans (35%). The HHE haplotype had the highest frequency among Europeans (32%) compared to other populations. The HHD haplotype was highly prevalent among African-Americans (20%) but only present at 2.6% in Omanis (Table 3). The HHG\*2 haplotype was less common as it was mostly limited to Europeans (8%) and relatively rare in Asians and Africans. HHB was a rare haplotype found mainly in Africans and was completely absent in other populations.

Corrected pairwise  $F_{ST}$  distances were calculated using the *CCR2-CCR5* haplotype frequency estimates (Table 3) and an unrooted NJ tree was constructed between Omanis and eight other populations. Figure 3 shows that the African populations were well-defined in one cluster that consisted of Pygmy and non-Pygmy populations while the second cluster (with bootstrap support of 82) involved the African-American population, which has a component of the African population mixed with Caucasian and Eurasian populations. These populations were further divided into two groups: the first group represented Indians (bootstrap support 88) and consisted of an isolated population composed mainly of southern Indians; the second group consisted of Ethiopian Jews, Europeans and Asians. Inter-

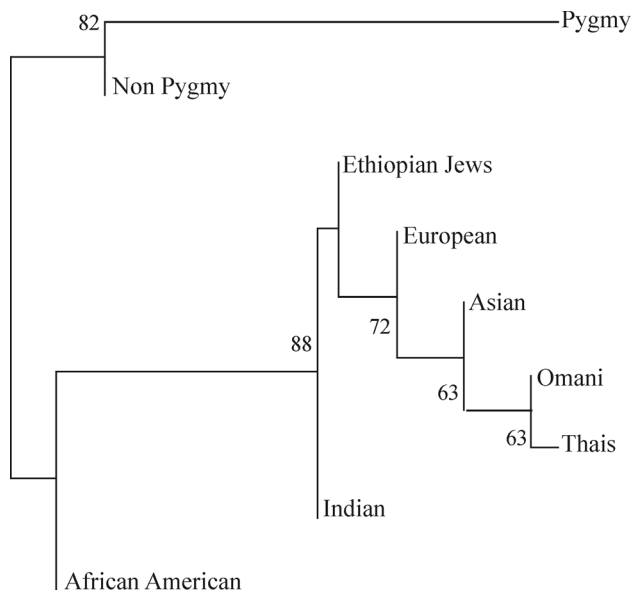


**Figure 2** - Median-joining network of the *CCR2-CCR5* haplotypes. The newly identified haplotypes are defined as HHC-A, HHF\*1A and HHF\*2A. The network was generated using Network 4.8 (Fluxus Engineering). The size of each node is proportional to the haplotype frequency. The numbers beside each line indicate the position of the nucleotide mutation.

**Table 3** - *CCR5* haplotype frequency distribution (%) among populations.

Haplotype	Pygmy <sup>1</sup>	Non-pygmy <sup>1</sup>	African American <sup>1</sup>	European <sup>1</sup>	Indian <sup>1</sup>	Asian <sup>1</sup>	Ethiopian Jews <sup>2</sup>	Thais <sup>3</sup>	Omanis <sup>4</sup>
HHA	71	24	21	10	18	5	14	6.1	14
HHB	3	5	1	0	0	0	0	0	0
HHC	1	9	15	35	36	42	33	62	46
HHD	0	17	20	1	1	0	0	0	2.6
HHE	13	20	19	32	29	25	30	16	20
HHF*1	6	8	4	1	2	3	0	0.5	0.4
HHF*2	6	16	15	8	18	5	24	15	12
HHG*1	1	2	4	4	1	0	0	1	2.6
HHG*2	0	0	3	8	0	1	0	0	0.4

References: 1. Gonzalez *et al.* (2001). 2. Korostishevsky *et al.* (2011). 3. Nguyen *et al.* (2004). 4. This study.



**Figure 3** - Unrooted neighbor-joining tree constructed based on pairwise *F<sub>ST</sub>*-corrected distances from *CCR2-CCR5* haplotype frequencies. Numbers at the nodes are bootstrap values.

estingly, the Omani population was closely grouped with Southeast Asian populations such as Thais.

### Discussion

The aim of this study was to obtain baseline data on *CCR2-CCR5* polymorphisms and to determine the haplotype profile of the Omani population. Our results confirmed the previous findings that the frequency of the *CCR5Δ32* allele is very low in Asian populations (Martinson *et al.*, 1997; Su *et al.*, 2000). Six major allele positions were identified (MAF > %): *CCR264I*, *CCR5-2554*, *CCR5-2459*, *CCR5-2135*, *CCR5-2086* and *CCR5-1835*. There was high LD between the SNPs in the *CCR2-CCR5* gene locus and this allowed assessment of the diversity found in these two regions in 11 haplotypes. The large number of haplotypes identified in this study, including three unique ones, indicated that the Omani population was genetically quite diverse when compared to other Asian populations such as Thais (Clark and Dean, 2004; Nguyen *et al.*, 2004).

Korostishevsky *et al.* (2011) investigated the LD between four SNP positions in the promoter region of *CCR5* and *CCR2V64I* among Ethiopian Jews. They observed significant LD between the three internal SNP positions (G2554T, T2135C and A2086G) while C1835T was found to be tightly linked only to *CCR2V64I*. In this study, we examined the LD at eight SNP positions and found that four internal SNP positions were tightly linked ( $p < 0.0001$ ). *CCR2V64I* was significantly linked to four promoter SNPs in the *CCR5* gene. These results demonstrate that the LD pattern between *CCR5* promoter SNPs and *CCR2V64I* in the Omani population differed from that of Ethiopian Jews.

The analysis of the *CCR2-CCR5* gene locus described here suggests that the Omani population is diverse and resembles the patterns reported in previous studies of Asian subpopulations. Four allele positions (-2554, -2459, -2135 and -2086) showed moderately high levels of heterozygosity. This heterozygosity reflects selection that favors the presence of these alleles to produce an excess of intermediate frequencies (Bamshad *et al.*, 2002; Ramalho *et al.*, 2010) that would be expected to reduce population differentiation. However, the other two tightly linked loci (*CCR264* and -1835) showed low heterozygosity, suggesting that there may be selection at these two positions. Moreover, these two linked loci have a greater global distribution, especially in Asia and Africa, and are therefore presumed to be older mutations compared to *CCR5Δ32*, which is restricted to Europe (Martinson *et al.*, 1997).

The diversity of the Omani population was confirmed by the identification of 11 haplotypes in the *CCR2-CCR5* gene locus. This finding agrees with results obtained from mitochondrial DNA (Luis *et al.*, 2004; Rowold *et al.*, 2007) that showed high intra-population diversity in Oman. The authors of these studies suggested that this diversity was a consequence of a series of demographic incidents that occurred in this region, partly because of Oman's strategic location as a passage between Africa and Eurasia. These conclusions agree with our phylogenetic analysis of the *CCR2-CCR5* haplotypes based on corrected  $F_{ST}$  distances that clustered the Omani population with other Asian populations but not far from the Europeans. In addition, the recent discovery of African Nubian fossils in the Dhofar region, the southern part of Oman, suggests the presence of a migratory route of early *Homo sapiens* from the Horn of Africa across the southern Red Sea into Asia (Rose *et al.*, 2011). This conclusion also agrees with previous genetic reports on Y-chromosome diversity (Semino *et al.*, 2004; Cadenas *et al.*, 2008) which showed that the diversity in Oman was partially explained by its position along the costal pathway associated with movements between large human conglomerates. Surprisingly, the legacy of the African haplotypes HHB and HHD is very low in the present Omani population, despite the fact that Oman had ruled a significant part of the East African coast for centuries. This may suggest a low degree of inter-marriage between the

Omanis and coastal East African populations or that the genetic influxes from Asian populations replaced those from Africa. Further investigations on a larger sample size would be necessary to confirm these findings.

This study is the first to describe the common *CCR2-CCR5* haplotypes in Oman. HHC and HHE were the most common haplotypes in the population studied. Further investigations are needed to delineate the functional relevance of the three newly identified haplotypes, particularly with regard to their role in the pathophysiology of HIV-1 infection and disease progression among HIV-1 infected Omani patients. Together, the results of this study show that the Omani population is genetically quite diverse. Future investigations should examine the pattern of genetic differences in the *CCR2-CCR5* gene loci between the northern and southern parts of the country.

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