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Association of SNPs and haplotypes in $GABA_A$ receptor β_2 gene with schizophrenia

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Disturbances in GABAergic system have been observed in schizophrenics.[1–3] In the present study, population association analysis was performed on 19 SNPs in the α_1, β_2, γ_2, ε and π subunit genes of $GABA_A$ receptor. Five SNPs in *GABRB2*, namely B2I7G1584T, rs1816071, rs194072, rs252944 and rs187269, were found to be significantly associated, and their haplotypes in linkage disequilibrium, with schizophrenia. This represents the first report on any disease association of SNPs in the human $GABA_A$ receptor genes, and focuses attention on the GABAergic hypothesis of schizophrenia etiology.[3,4]
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Schizophrenia is a debilitating mental illness that affects 1% of the world's population, with a heritability of 0.70–0.85 attributable to complex inheritance.[5] Despite extensive studies, its molecular causes have remained unidentified. Like many common diseases, it is believed to be multifactorial in origin, with both genetic and environmental contributions playing important roles in determining the symptoms. Several neurotransmitters, including dopamine, glutamate and serotonin are thought to be important to the disease, and significant associations with genes of the glutamate neurotransmitter and related systems have recently been reported.[6,7] Although a defect in neurotransmission involving γ-amino butyric acid (GABA) in schizophrenia was first proposed in the early 1970s, followed by extensive investigations on the GABAergic system in schizophrenic subjects,[1–4] the lack of molecular genetic evidence has left the GABAergic system out of the roster of leading candidate genes for schizophrenia.

The type A γ-amino butyric acid receptors ($GABA_A$) are the major inhibitory receptors in the CNS.[8,9] Among different subunit combinations, the $\alpha_1/\beta_2/\gamma_2$-containing heteropentamer is the dominant subtype in mammalian brains.[10,11] To test the potential involvement of $GABA_A$ receptor genetics in the molecular etiology of schizophrenia, single-nucleotide polymorphisms (SNPs) and SNP-based haplotypes in the genes of $GABA_A$ receptors have been examined in the present study. In all, 19 SNPs in the $GABA_A$ α_1, β_2, γ_2, ε and π subunit genes were assessed for possible association with schizophrenia (Table 1). Five of them were discovered, and the others confirmed, through sequencing of PCR-amplified DNA samples. None of the nine SNPs identified in the α_1, γ_2, ε and π subunit genes was associated with schizophrenia ($P > 0.05$; Table 1). On the other hand, five of the 10 SNPs in the β_2 subunit gene showed a significant association. Accordingly, a detailed investigation of the SNPs in the $GABA_A$ receptor β_2 subunit gene (*GABRB2*) was carried out.

Human *GABRB2* is widely expressed in different brain regions, especially the cerebellum.[12] It is located on chromosome 5 at 5q34 close to 5q33.2, which has been identified through genome-wide linkage analysis as one of the susceptibility loci for schizophrenia.[13,14] In the present study, population association and linkage disequilibrium (LD) analyses were carried out on schizophrenic patients and unaffected subjects of *Han* Chinese origin for 10 SNPs within *GABRB2*, spanning approximately 13 kb from ~1 kb upstream of exon 6 to ~200 bp downstream of exon 9. Five of them, all located in introns 7 and 8, showed significant association with susceptibility to schizophrenia both in an initial screening, and upon further testing with increased sample sizes of schizophrenics and controls (Table 1). These five positive SNPs include one novel SNP at base 1584 of intron 7 within the sequence TTGTATC(G/T)ATTACAG, which is designated B2I7G1584T, and four previously reported SNPs, rs1816071, rs194072, rs252944 and rs187269

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(dbSNP database). The genotype frequencies of all the five SNPs did not deviate from Hardy–Weinberg equilibrium for either the control or the patient group when tested with the program GENEPOP.[15]

At the allele level, the minor allele of each of the five positive SNPs was found to increase schizophrenia risk (Table 1). Strong associations with schizophrenia were found for rs252944 ($P=0.0003$; OR = 2.34, 95% CI 1.46–3.76), rs187269 ($P=0.0009$; OR = 1.93, 95% CI 1.31–4.85), rs1816071 ($P=0.0008$; OR = 2.05, 95% CI 1.34–3.13) and rs194072 ($P=0.0005$; OR = 2.50, 95% CI 1.48–4.23), and moderate association found for B2I7G1584T ($P=0.0046$; OR = 2.02, 95% CI 1.23–3.31).

Genotype frequency differences between the schizophrenic and control groups were also analyzed. As shown in Table 1, highly significant overall genotype association with schizophrenia was observed for rs252944 ($P=0.0018$) and rs187269 ($P=0.0031$), and significant association observed for B2I7G1584T ($P=0.0195$), rs1816071 ($P=0.0096$) and rs194072 ($P=0.0044$).

The effect of each of the five positive SNPs on schizophrenia susceptibility was further assessed in Table 2. The risk of schizophrenia for individuals having one or two minor alleles (m/m or m/M) at any of the five SNP sites is estimated to be about two times higher (OR = 1.89–2.61) than individuals with two copies of the major allele (M/M; $P=0.0010$–0.0079). A homozygous-minor-allele (m/m) genotype increases schizophrenia susceptibility over the major-allele-containing genotypes (M/m + M/M) in the cases of rs187269 ($P=0.0046$; OR = 4.60), rs1816071 ($P=0.0139$; OR = 2.86) and rs252944 ($P=0.0267$; OR = 5.12). These results thus point to a significant enhancement of the risk of schizophrenia by the

Table 1 Allele and overall genotype association analysis of SNPs in the α_1, β_2, γ_2, π and ε subunit genes

Gene for subunit	*SNP*[a] *(M/m)*	*Sample*[b] *size*		*Amino acid coded*	*Frequency (%)*						*Allele*[c]			*Overall genotype*[d]
					m		*M/m*		*m/m*					
		SCH	*CON*		*SCH*	*CON*	*SCH*	*CON*	*SCH*	*CON*	*P*	*OR*	*95% CI*	*P*
α_1	SA1-3 (C/T)	116	129	NA	25.43	20.16	35.34	32.56	7.76	3.88	0.1636	1.35	0.88–2.07	0.3327
	rs2279020 (G/A)	120	114	NA	46.67	42.54	48.33	44.74	22.50	20.18	0.3699	1.18	0.82–1.70	0.6220
β_2	rs2303055 (T/G)	115	113	NA	13.72	13.48	23.89	26.96	1.77	0.00	0.9408	1.02	0.60–1.74	0.3233
	rs967771 (G/A)	104	110	NA	9.62	7.73	19.23	13.64	0.00	0.91	0.4872	1.27	0.65–2.50	0.3483
	B2I7G1584T (G/T)	120	113	NA	22.92	12.83	34.17	20.35	5.83	2.66	*0.0046*	2.02	1.23–3.31	*0.0195*
	rs1816071 (A/G)	112	107	NA	36.61	21.96	35.71	28.97	18.75	7.48	*0.0008*	2.05	1.34–3.13	*0.0096*
	rs1816072 (T/C)	115	101	NA	45.65	35.15	44.35	42.57	23.48	13.86	*0.0266*	1.55	1.05–2.29	0.1044
	rs194072 (T/C)	120	106	NA	23.33	13.12	31.67	17.92	7.50	1.89	*0.0005*	2.50	1.48–4.23	*0.0044*
	rs252944 (G/C)	100	138	NA	26.00	13.04	38.00	23.19	7.00	1.45	*0.0003*	2.34	1.46–3.76	*0.0018*
	rs187269 (T/C)	131	171	NA	28.24	16.96	36.64	29.24	9.92	2.34	*0.0009*	1.93	1.31–4.85	*0.0031*
	rs1644522 (T/C)	120	122	NA	54.17	48.78	53.33	47.15	27.50	25.20	0.2349	1.24	0.87–1.77	0.2947
	rs1644436 (G/A)	112	110	NA	36.64	33.64	46.43	49.09	13.39	9.09	0.5121	1.14	0.77–1.68	0.5973
γ_2	SG2-3 (C/T)	113	105	N^{105}	19.47	21.91	31.56	38.10	3.54	2.86	0.5301	0.86	0.54–1.37	0.6196
	rs211037 (T/C)	137	133	N^{196}	45.62	42.86	46.00	49.62	22.63	18.05	0.4239	0.87	0.62–1.22	0.6088
	SG2-6 (G/C)	114	114	E^{216}/Q^{216}	6.58	6.14	13.16	12.28	0.00	0.00	0.8478	1.08	0.51–2.29	0.8424
	rs211014 (A/C)	117	120	NA	46.15	40.83	58.12	46.67	17.09	17.50	0.2427	1.24	0.86–1.79	0.1444
π	rs3805458 (C/T)	117	112	NA	29.49	27.23	40.17	36.61	9.40	8.93	0.5926	1.12	0.74–1.68	0.8265
	EP-1 (C/T)	126	113	F^{391}/L^{391}	27.38	28.32	38.89	38.94	7.94	8.85	0.8193	0.95	0.64–1.43	0.9657
ε	rs2256882 (C/T)	113	130	A^{193}/V^{193}	33.63	30.00	26.55	21.54	20.35	19.23	0.3912	1.18	0.81–1.73	0.5834

[a]Cluster ID of SNPs in dbSNP at NCBI (dbSNP Home Page) with prefix of rs, whereas SA1-3 (base 6 102 243 of Contig NT_023133.11 within *GABRA1*), B2I7G1584T (base 5 569 733 of Contig NT_023133.11 within *GABRB2*), SG2-3 (base 6 332 114 of Contig NT_023133.11 within *GABRG2*), SG2-6 (base 6 340 487 of Contig NT_023133.11 within *GABRG2*) and EP-1 (base 15 048 670 of Contig NT_023133.11 within *GABRP*) are SNPs discovered in this study. M = major allele; m = minor allele.

[b]SCH = schizophrenics; CON = normal control.

[c]*P*-values were calculated to test allele frequency differences between schizophrenic and control groups. OR = odds ratio; 95% CI = 95% confidence interval.

[d]*P*-values were tested to evaluate the overall genotype differences between the schizophrenic and control groups. *P*-values are given in italics for those <0.05 (significant), or <0.01 (very significant).

Table 2 Relative susceptibility to schizophrenia of different genotypes at the five positive SNP sites

SNP (M/m)	*m/m* vs *M/m + M/M*			*m/m + M/m* vs M/M		
	P	*OR*	*95% CI*	*P*	*OR*	*95% CI*
B2I7G1584T(G/T)	0.2315	2.27	0.57–9.01	*0.0054*	2.23	1.26–3.95
rs1816071 (A/G)	*0.0139*	2.86	1.21–6.77	*0.0075*	2.09	1.21–3.59
rs194072 (T/C)	0.0503	4.22	0.89–19.98	*0.0015*	2.61	1.43–4.76
rs252944 (G/C)	*0.0267*	5.12	1.04–25.20	*0.0010*	2.50	1.44–4.35
rs187269 (T/C)	*0.0046*	4.60	1.46–14.46	*0.0079*	1.89	1.18–3.02

Refer to Table 1 for abbreviations. *P*-values are given in italics for those <0.05 (significant), or <0.01 (very significant).

minor alleles. To compare the minor-allele homozygote and the heterozygote, association analysis was performed separately for m/m *vs* M/M and M/m *vs* M/M. The results suggest that the homozygous minor-allele genotypes tend to favor susceptibility to schizophrenia over the heterozygous genotypes, but an increased sample size would be needed before a firm conclusion can be drawn in this regard.

To further define the contribution of the five possible SNPs to schizophrenia susceptibility, haplotype associations with the disease were evaluated for all the possible two-SNP combinations of these five SNPs (Table 3). Among the two-loci haplotypes, rs1816071–rs187269, rs252944–rs187269 and rs194072–rs187269 yielded asymptotic *P*-values, derived from the model-free χ^2 statistics,[16,17] that were less than 0.00001 for χ^2 distribution with four degrees of freedom (Table 3). None of the 10 000 replicates in the permutations computed by the EHPLUS program produced any empirical *P*-value that exceeded the asymptotic *P*-values from the observed data, thus pointing to a strong association between these three two-loci haplotypes and schizophrenia. The remaining two-SNP combinations also showed significant association with schizophrenia, with *P*-value ranging from 0.0003 to 0.0149. In addition, pair-wise linkage disequilibrium analysis (Table 3) showed that the five positive SNPs were highly linked with D' value ranging between 0.6645 and 0.9687, further supporting the haplotype associations.

The frequencies of the three-SNP haplotypes were likewise found to be in LD with schizophrenia, especially for the haplotype rs194072–rs252944–rs187269 ($\chi^2_{8df} = 38.72$, $P < 1.0 \times 10^{-5}$). Upon the permutation of 10 000 replicates, the empirical *P*-value was smaller than the asymptotic *P*-value in this case. The empirical evidence thus further supported its disease association.

Four of the other five SNPs tested in *GABRB2*, namely rs2303055, rs967771, rs1644522 and rs1644436, were not significantly associated with the disease at either the allele or the genotype level ($P > 0.05$; Table 1). However, the association of rs1816072 with the disease, while insignificant ($P = 0.1044$) at the genotype level, was marginally significant ($P = 0.0266$; OR = 1.55, 95% CI 1.05–2.29; Table 1) at the allele level. Two haplotypes formed by the four negative SNPs were estimated and found not to be significantly associated with schizophrenia (rs2303055–rs967771, $P = 0.1741$; rs1644522–rs1644436, $P = 0.1557$).

The present study demonstrated that the five SNPs B2I7G1584T, rs1816071, rs194072, rs252944 and rs187269 in introns 7 and 8 of *GABRB2* (Figure 1) were significantly associated with susceptibility to schizophrenia in *Han* Chinese. Our results suggest that the *GABRB2* gene might be responsible for the previously reported positive linkage of chromosomal locus 5q33.2 with schizophrenia.[13] So far, no other gene has been found in the neighboring sequences up to 351 960 bp upstream (*GABRA6*) and 927 375 bp downstream (*LOC63920*) of the block of five positive SNPs (Figure 1). Although crossethnic multicenter studies are needed before the observed associations can be generalized, the fact that our association analysis and the earlier linkage study were based on samples from different ethnic groups increases the likelihood that the observed association might be multiethnic in nature.

The potential functional consequences of the minor alleles of the five positive SNPs, as well as interactions between *GABRB2* and other plausible candidate genes in determining schizophrenia susceptibility,[6,7,18,19] require further investigation in order to better understand the network of genetic factors underlying schizophrenia. It is known that there are at least two isoforms of *GABRB2* expression products,[20] and the possibility exists that the five positive SNPs may modulate transcriptional regulation, such as alternative splicing and mRNA expression, of *GABRB2*, thereby influencing schizophrenic phenotype expression,[21] as has been suggested for the γ_2 subunit of $GABA_A$ receptor.[22] While the minor alleles at the five loci increase disease susceptibility, they are neither necessary nor sufficient for the development of schizophrenia. The present study thus supports a multifactorial causation. By linking a $GABA_A$ receptor gene for the first time to schizophrenia, however, it has provided important evidence for the involvement of GABAergic processes in the etiological mechanism of the disease.

Table 3 Association of estimated two-SNP haplotype frequencies with schizophrenia and linkage disequilibrium analysis between pairs of positive SNPs in *GABRB2*

Haplotype[a]	*rs1816071*				*rs194072*				*rs252944*				*rs187269*			
	Association[b]		*LD*[c]		*Association*[b]		*LD*[c]		*Association*[b]		*LD*[c]		*Association*[b]		*LD*[c]	
	χ^2	*P*	*D'*	r^2	χ^2	*P*	*D'*	r^2	χ^2	*P*	*D'*	r^2	χ^2	*P*	*D'*	r^2
B2I7G1584T	20.84	0.0003	0.78	0.30	76.93	21.26	0.0003	0.87	0.72	164.10	15.38	0.0040	0.91	0.79	179.10	14.12	0.0069	0.79	0.41	87.47
rs1816071						15.74	0.0034	0.79	0.35	69.61	17.70	0.0014	0.80	0.36	74.60	29.46	<0.0001	0.66	0.33	61.56
rs194072											12.36	0.0149	0.97	0.92	275.44	25.56	<0.0001	0.77	0.49	119.11
rs252944																31.98	<0.0001	0.79	0.48	117.34

[a]Haplotype formed by two SNPs.
[b]Association analysis of each two-SNP haplotype with schizophrenia.
[c]Pairwise linkage disequilibrium in whole population was calculated by standard LD coefficient; D' = standardized LD coefficient; r^2 = Pearson correlation. Pairwise LD was calculated using a likelihood-ratio test, and followed a χ^2 distribution with one degree of freedom; $P<1.0\times10^{-5}$ in all cases, indicating that all five SNPs are significantly linked.

Materials and methods

Study subjects

Peripheral blood samples were collected from Shanghai Mental Health Center with informed consent from unrelated *Han* Chinese subjects. Schizophrenics (mean age of onset 26.7 years; mean age 47.9) were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV).[23] All patients displayed at least two of the following symptoms for a period of 1 month or more (except where successfully treated): delusion, hallucination, disorganized speech, grossly disorganized or catatonic behavior. Unrelated healthy *Han* Chinese (mean age 33.5 years) served as controls. All control individuals were interviewed to exclude any history of psychiatric disorders. All successfully genotyped results were included for disease association studies.

SNP discovery and genotyping

Genomic DNA was prepared from blood samples by using the DNA purification kit from Amersham, and diluted to 100 ng/ml. Primers were designed in an adjacent-primer-overlapping-200-base-pairs manner as described by Primer3 (Primer3 website). The specificity of each potential primer was checked through nBlast of the National Center for Biotechnology Information (Blastn Home Page). Only pairs of both forward and reverse primers with less than five hits to the human genome were accepted as specific primers. PCR was performed in a final volume of 20 μl containing 10 ng sample DNA, 75 nmol/l of each primer, 50 nmol/l of each dNTP, 2.5 mmol/l $MgCl_2$ and 1 U *Taq* DNA polymerase (Amersham). PCR amplification consisted of denaturation at 94°C for 5 min, followed by 40 cycles of 1 min at 94°C, 1 min at the annealing temperature optimum for each pair of primers, 90 s at 72°C, followed by a final extension step at 72°C for 5 min. PCR products were resolved on 1.5% agarose gel electrophoresis, and stained with ethidium bromide to confirm the specificity of the products generated. PCR products were purified by Montage™ PCR_{96} Purification kit (Millipore). Each sequencing reaction contained 2 μl ABI PRISM® BigDye™ Terminator (v.2.0), 5 μl purified PCR products and 1 mmol/l of primer. Sequencing cycling conditions consisted of 1 min denaturation at 96°C, followed by 34 cycles of 96°C for 30 s, 50°C for 30 s and 60°C for 3 min. Sequencing products were purified by AutoSeq96 Plates containing DNA Grade Sephadex G-50 (Amersham). The purified products were then denatured at 95°C for 5 min with addition of 5 μl hi-deionized formamide, and run on the ABI PRISM® model 3100 capillary DNA sequencer. Sequence chromatograms were aligned and analyzed for SNPs by the PolyPhred software.[24] The SNPs suggested by PolyPhred based on automated sequencer traces were double checked manually by two independent researchers to ensure the accuracy of the genotype calls. All of the SNPs analyzed were located within the high-quality region

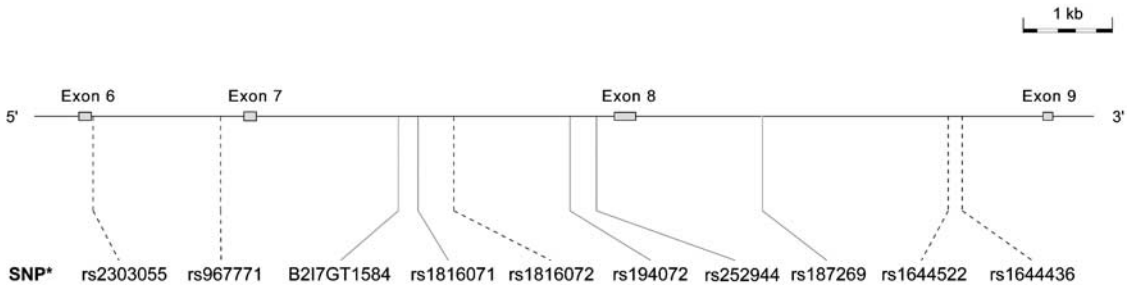

Figure 1 Positions of 10 SNPs in *GABRB2* gene. *Cluster ID numbers of SNPs are as designated by the dbSNP of NCBI except B2I7G1584T, which was discovered in this study.

of the chromatogram, from base 200 to base 400, and occasional low-quality sequencing passes were re-run. Samples from both patient and control groups were included in the same experimental batches to minimize batch-to-batch variations in genotyping.

Study design

The first stage of the present study was directed to the discovery of SNPs in the coding regions of various subunits of $GABA_A$ receptors by direct DNA sequencing of PCR-amplified exons and of intronic sequences flanking each exon, with about 48 samples from each of the two subject groups. When the SNP rs252944, about 500 bp upstream to exon 8 of *GABRB2* gene, showed promising association with schizophrenia, the study proceeded to the second stage. PCR was performed on genomic DNA flanking rs252944, spanning approximately 13 kb and consisting mostly of introns, on an expanded number of samples (Table 1). The PCR products were completely sequenced to: (a) discover any SNPs in this region and (b) genotype these SNPs for potential association with schizophrenia.

Statistical analysis

All the SNPs analyzed were tested for Hardy–Weinberg equilibrium by means of the program GENEPOP v. 1.2 (GENEPOP website).[15] The complete enumeration method was chosen as suggested by GENEPOP.

Possible SNP association with susceptibility to schizophrenia was assessed by comparing the allelic and genotypic frequencies of schizophrenics and controls, using the standard χ^2-test under normal approximation. The level of significance was indicated by *P*-values, as implemented by the CLUMP program.[25] A value of $P<0.05$ was considered to be indicative of a statistically significant effect.

The two-loci and three-loci haplotype associations were carried out using the permutation and model-free analysis (PMPLUS v.1.1) program[16,17] in the estimated haplotype-frequencies (EHPLUS) software package. In view of the incomplete penetrance and uncertain mode of inheritance of schizophrenia, model-free analysis and permutation test were performed. PMPLUS implemented the model-free statistics by maximizing log-likelihood ratio statistic over a range of parameter values, instead of fixed values as in Mendelian dominant or recessive models. A recessive model with disease allele frequency 0.1 and penetrances 0.005, 0.005, 0.5 was initially assumed and inputted as the user-specified model.[26] PMPLUS prepared input files based on user-inputted data with different inheritance models, including the user-specified, recessive, dominant, model-free and heterogeneity models for EH. Maximum likelihood estimates of all the 10 two-loci haplotype frequencies were then performed by employing the expectation-maximization (EM) algorithm,[27] as implemented in EH. The hypotheses concerning different disease-model χ^2-statistics were performed by EH. In order to resolve the potential inaccurate significance level caused by asymptotic theory in association analyses of two-loci haplotypes, additional permutation procedures of 10 000 replicates were followed to obtain empirical *P*-values by means of EHPLUS.[16]

LD between each pair of loci in the control group was tested using the standard LD coefficient[28] and the likelihood-ratio test, as implemented by the ARLEQUIN program (ARLEQUIN website). For the standard LD coefficient calculation, two-loci haplotype frequencies were estimated using a maximum-likelihood approach from unphased diploid genotype data, as computed in the ARLEQUIN program. All measures of LD were based on $D=x_{11}-p_1q_1$, where x_{11} is the estimated frequency of haplotype A_1B_1, and p_1 and q_1 are the frequencies of alleles A_1 and B_1 at loci *A* and *B*, respectively. Measures of complete association, D', and absolute association, r^2, were used in LD analyses. D' is given by $D/D_{\max}$, where $D_{\max}=\min[p_1q_1, p_2q_2]$ when $D<0$, or $D_{\max}=\min[p_1q_2, p_2q_1]$ when $D>0$. r^2 is given by $D/(p_1p_2q_1q_2)^{1/2}$. D' varies between 0 (no association) and 1 (maximum disequilibrium). For the likelihood-ratio tests, likelihood under the hypothesis of LD between loci is compared to the likelihood of the experimental data under LD.[29] Permutation procedure was performed in order to better approximate the underlying distribution of the likelihood ratio under the null hypothesis of linkage equilibrium. The significance level of LD was represented as *P*-value of the χ^2-test.

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Competing interests statement: We declare that we have no competing financial interests.

Electronic-database information

URLs for databases and program used herein are as follows:

- ARLEQUIN, http://lgb.unige.ch/arleguin/
- Blastn, http://www.ncbi.nlm.nih.gov/BLAST/
- dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
- GENEPOP, http://wbiomed.curtin.edu.au/genepop/
- Primer3, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

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