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Brain-derived HIV-1 *tat* sequences from AIDS patients with dementia show increased molecular heterogeneity

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HIV-1 infection results in a dementing illness affecting 20% of patients with AIDS. Several HIV-1 genes have been implicated in the pathogenesis of HIV-induced neurological disease. To search for distinct HIV-1 sequences associated with the development of dementia, brain-derived *tat*, *env*, and *pol* sequences were examined from AIDS patients defined pre-mortem as demented (HIV-D)[*n*=5] or non-demented (HIV-ND)[*n*=5]. Estimations of evolutionary distances and frequency of non-synonymous mutation rates revealed significant differences between brain-derived *tat*, *env*, and *pol*-encoded reverse transcriptase sequences. However, established zidovudine-associated resistance mutations in reverse transcriptase sequences were identified in only one HIV-D and one HIV-ND patient despite prolonged treatment of some patients. Non-synonymous/synonymous substitution rates among the *tat* sequences derived from patients with HIV-D were significantly higher compared to the HIV-ND group ($P<0.001$). The ratios of transversions to transitions were also significantly higher among the HIV-D *tat* sequences ($P<0.01$). Phylogenetic analyses showed clustering of sequences from each clinical group among the brain-derived *tat* and *env* sequences. These studies indicated that differing selective forces act on individual HIV-1 genes in the brain which may influence the development of dementia.

Keywords: HIV-1; dementia; tat; reverse transcriptase

Introduction

Human immunodeficiency virus type-1 (HIV-1) infection results in a dementing illness, HIV-associated dementia (HIV-D), in 20% of patients with AIDS (Lipton and Gendelman, 1995). Productive HIV-1 infection in the brain is limited to perivascular macrophages and microglia, and to a lesser extent, astrocytes in AIDS patients with and without dementia (Wiley *et al*, 1986; Glass *et al*, 1995; Takahashi *et al*, 1996). Infection of brain macrophages and microglia is influenced by specific amino acids within and adjacent to the V3 hypervariable region of the HIV-1 envelope (Sharpless *et al*, 1992; Power *et al*, 1995). Phylogenetic and sequence comparisons of HIV-1 *gag*, *pol*, and *env* sequences derived from brain and other organs indicate that compartmentalization of virus occurs in the brain, suggesting that brain-adapted HIV-1 quasispecies can evolve (Gartner *et al*, 1997; Wong *et al*, 1997; Hughes *et al*, 1997). Among other neurotropic RNA viruses, including influenza A (Ward, 1996) and animal retroviruses, multiple viral genes (Mankowski *et al*, 1997) or different domains within the same viral gene (Hasenkrug *et al*, 1996) contribute to disease in the brain. Several HIV-1 genes have been implicated in the pathogenesis of HIV-induced neurological disease including the LTR (Corboy *et al*, 1992), *tat* (Magnuson *et al*, 1995), and *env* including both gp120 (Dreyer *et al*, 1990) and gp41 (Adamson *et al*, 1996). We have identified specific mutations in the V3 region of gp120 that are associated with the development and severity of HIV-D (Power *et al*, 1994). The HIV-1 *pol*-encoded reverse transcriptase (RT) is implicated in HIV-1 neurotropism (Wong *et al*,

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1997) and is critical for viral replication (Green, 1991). HIV-1 Tat has been shown to be neurotoxic (Nath *et al*, 1996), can induce expression of proinflammatory cytokines in brain cells (Chen *et al*, 1997), is a transactivator of viral and host genes (Gaynor, 1995), and recently we have found that *tat* sequences from matched brain and spleen samples differ significantly (Mayne *et al*, 1998). To test the hypothesis that mutations within several HIV-1 genes are associated with the development of HIV-D, we examined brain-derived *tat*, RT, and previously reported *env* sequences (Power *et al*, 1994) from AIDS patients with and without HIV-D. These studies suggested that individual HIV-1 genes are subject to differing selective pressures which may predict the neurological status of the patient depending on the gene examined.

Results

Clinical features

Ten AIDS patients were studied in whom the premortem neurological diagnosis was confirmed as non-demented (HIV-ND; n=5) or demented (HIV-D; n=5). The severity of HIV-D was scored by the Memorial Sloan-Kettering [MSK] scale (Figure 1A) (Price and Brew, 1988). The HIV-D and HIV-ND groups did not differ significantly in ages, CD4 counts, histopathological findings, and duration of zidovudine (ZDV) prior to death (Figure 1A), or daily doses of ZDV (Table 1). These patients were not exposed to any other antiretrovirals (ARV) except for patient 34 who received didanosine (ddI) for 6 months (Figure 1).

Molecular variation of brain-derived sequences

Both RT (Figure 1A) and *tat* (Figure 1B) brain-derived sequences showed multiple residues differing from the B clade consensus sequence (Myers *et al*, 1995). Moreover, many amino acids (AA) observed in the brain-derived sequences (AAs indicated by squares, Figure 1A and B) have not been identified previously in reported databases (Myers *et al*, 1995). AAs differing from the consensus sequences were clustered in specific regions. However, residues critical for gene function, such as the cysteines in codons 22–37 and the core regions (codons 38–48) of

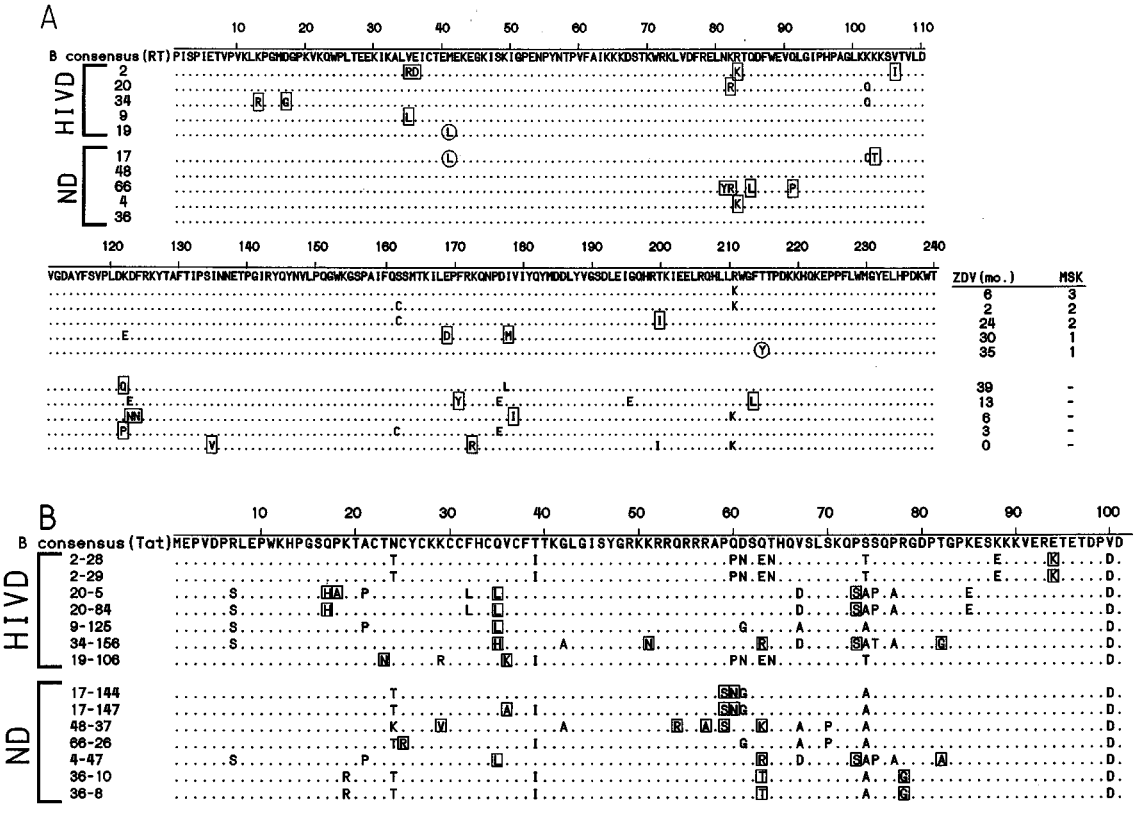

Figure 1 Brain-derived HIV-1 reverse transcriptase [RT] (**A**) and *tat* [Tat](**B**) sequences aligned with the B clade consensus sequence (Myers *et al*, 1995) from HIV-D [HIVD] (n=5) and HIV-ND [ND](n=5) patients in order of severity of HIVD, measured by MSK score. Patient number and duration of AZT therapy, and HIV-D severity (MSK) are shown for each patient with RT sequences but only patient and clone numbers are shown with *tat* sequences. Residues in circles and squares indicate amino acids that are established ZRAM [circles] or not previously reported in other data bases [squares] (Myers *et al*, 1995).

tat (Gaynor, 1995) and the active sites in RT at codons 100 and 184 (Moyle, 1996) were highly conserved in all sequences. All brain-derived *tat* sequences differed from the *tat* B clade consensus sequence at positions 74 and 100 but a similar consistent difference between RT brain-derived and the consensus sequences was not observed.

Given the molecular diversity observed among the RT sequences above, zidovudine-resistance associated mutations (ZRAM) might be expected to occur in brain-derived RT sequences from patients treated with ZDV. Previously reported ZRAM at codons 41 and 215 of RT were identified in only two patients (patients 19 [HIV-D] and 17 [HIV-ND]) who had been treated for 35 months and 39 months prior to death, respectively (Figure 1A, AAs in circles). Patient 19 demonstrated mutations at codons 41 and 215 and patient 17 displayed a mutation at codon 41 only. In none of the other patients were ZRAM observed, despite therapy ranging in duration from 2–30 months. Patient 34 was also treated with ddI for 6 months but did not display previously reported ddI resistance-associated mutations. To ensure that a subpopulation of sequences displaying ZRAM was not overlooked, multiple clones (n=4) were selected from two patients not showing ZRAM (patients 2 and 9) who were treated with ZDV for 6 and 30 months respectively (Figure 1). Although sequence diversity among clones from the same individual ranged from 0 to 2%, ZRAM were not detected in any clones from patients 2 or 9 (data not shown).

Table 1 Clinical features of AIDS patients with and without HIV dementia[a]

Group[b]	*Age (yr)*±*s.e.*	*CD4 (cells/mm^3)* ±*s.e.*	*ZDV duration (mo)*±*s.e.*	*Daily ZDV dose (mg)*±*s.e.*
HIV-D (n=5)	31±3.2	15±14	19±14.6	520±408
HIV-ND (n=5)	34±6.5	47±53	12±15.7	300±173

[a]Cryptococcal meningitis was identified at autopsy in two patients (patients 2 and 17). Gliosis was identified in HIV-D (n=5) and ND (n=3) and diffuse myelin pallor was observed in three HIV-D patients. Multi-nucleated giant cells were not observed in any of the patients. Repeated clinical evaluations indicated that the opportunistic infections developed between the last clinical assessment and death and did not contribute to signs and symptoms of HIV-D.
[b]Ages, CD4 levels, ZDV doses and duration did not differ significantly between clinical groups (Mann Whitney U test, $P>0.05$).

Phylogenetic analyses of brain-derived sequences
Construction of phylogenetic trees by maximum likelihood, maximal parsimony, and neighbor-joining methods with bootstrapping was performed. This analysis included the present brain-derived sequences, the HIV-1 D clade consensus sequence (Myers *et al*, 1995) as an outgroup, and previously reported B clade viruses (CAM1, SF2, D31, HAN, MN) for which RT, *tat*, and *env* sequences were available (Figure 2). The topology of trees constructed by each method was similar and high bootstrap values (>80) were obtained for comparisons of clones from the same patient but not for sequences from different patients. For the *tat* and *env* sequences, clustering of sequences from three or more patients belonging to the same clinical group (Figure 2, shown with brackets) was observed but no clustering within groups occurred among RT

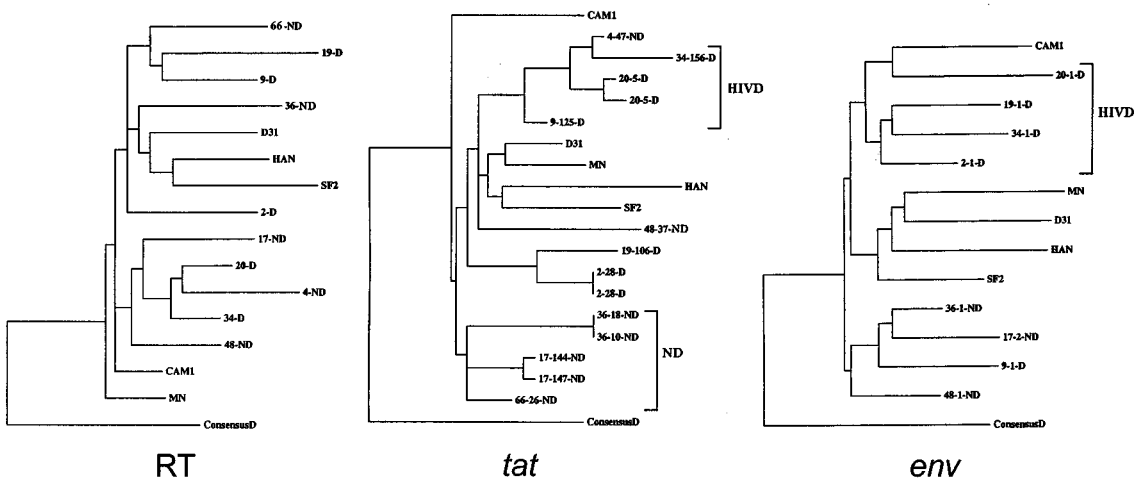

Figure 2 Phylogenetic trees based on maximum parsimony analysis of brain-derived, previously reported B clade viruses, and the D clade consensus sequence from RT, *tat* and *env* sequences. Trees showed *tat* and *env* sequences from HIV-D [HIVD] or HIV-ND [ND] groups clustering together for some patients in HIV-D or HIV-ND groups as shown by parentheses but clustering among groups was not observed among the RT sequences.

sequences. Similarly, the previously reported *env* and *tat* B clade sequences tended to cluster together and not with the brain-derived sequences, underlining the evolutionary differences between blood- and brain-derived viruses.

Analysis of molecular variation by comparisons of mean distance (*d*), non-synonymous (*Ka*), and synonymous (*Ks*) values (Kumar *et al*, 1993) for brain-derived *tat*, RT, and *env* sequences was performed including all patients for each gene (Table 2). When the three genes were compared (Table 2), significant differences were observed among *d* and *Ka* values respectively (ANOVA, $P<0.001$ and $P<0.0001$), but not among the *Ks* values. Comparison of *Ka* and *Ks* values for each gene revealed that RT-derived values from both HIV-D and HIV-ND groups clustered together with lower *Ka* values compared to *tat* and *env* sequences (Figure 3A). Mean *d*, *Ka*, and *Ks* values did not differ significantly between clinical groups for each gene. To test for distinguishing selective pressures between clinical groups (Li, 1997), the mean *Ka:Ks* was calculated for each gene. The *Ka:Ks* values for the *tat* sequences derived from the HIV-D group (Figure 3B) were significantly higher than the HIV-ND derived *tat* sequences (Student's *t*, $P<0.001$) and were highest among all groups of sequences examined. To define the nucleotide changes underlying the increased *Ka:Ks* in the brain-derived *tat* sequences from the HIV-D group, nucleotide substitution rates were calculated for both clinical groups' *tat* sequences. Transversions predominated among the *tat* sequences from the HIV-D group (G to C and T to A) compared to HIV-ND sequences (data not shown). The ratio of transversions to transitions among the *tat* sequences was significantly higher in the HIV-D group compared to the HIV-ND group (Mann-Whitney *U*, $P<0.01$) but did not differ between groups for RT and *env* sequences.

Table 2 Comparison of mean distance (*d*), non-synonymous (*Ka*) and synonymous (*Ks*) substitution rates (±s.e.m.) per patient among brain-derived RT, *tat*, and *env* sequences from ten AIDS patients[a]

	RT	*tat*	*env*	P[b]
d	0.028±0.002	0.067±0.015	0.083±0.015	0.001
Ka	0.015±0.003	0.063±0.009	0.079±0.008	0.0001
Ks	0.071±0.013	0.082±0.018	0.094±0.016	ns

[a]Values were pooled for HIV-D and HIV-ND patients because significant differences were not observed between groups.
[b]ANOVA.

Discussion

The present studies indicate that extensive sequence heterogeneity exists among brain-derived HIV-1 genes that are critical for viral replication and there appear to be selective pressures acting on *tat* and *env* but not RT sequences, distinguishing HIV-D from HIV-ND sequences. These findings imply that the *tat* and *env* genes may be important in the pathogenesis of HIV-D and that *tat* sequence variation may account, in part, for the variation in course and severity of HIV-D, as suggested previously for *env* sequences (Power *et al*, 1994). A similar finding of increased heterogeneity among human T-cell leukemia virus type I (HTLV-I) *tax* sequences has been reported (Renjifo *et al*, 1995). *tax* sequences from patients with HTLV-1 associated myelopathy (HAM) showed more nucleotides differing from the consensus sequence than corre-

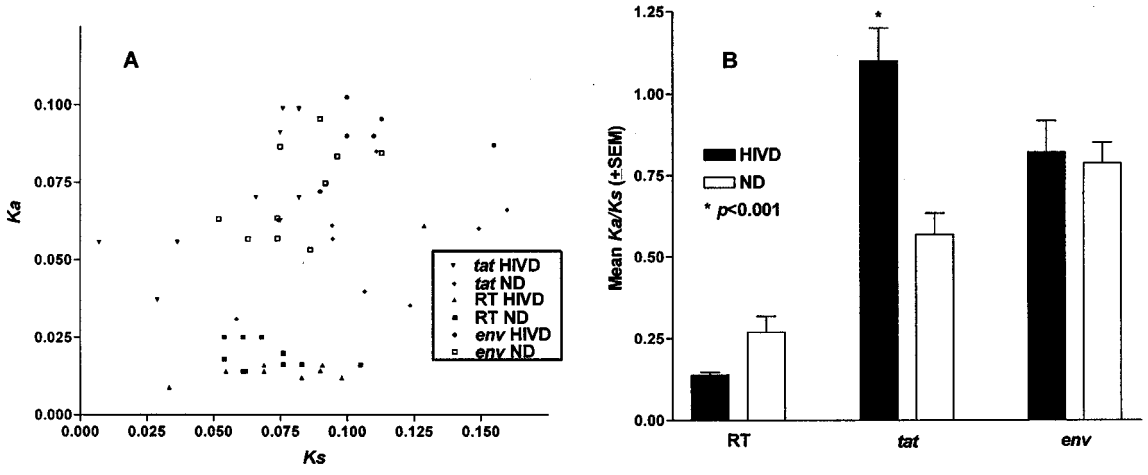

Figure 3 Comparison of synonymous [*Ks*] and non-synonymous [*Ka*] substitution rates (**A**) and *Ka:Ks* (**B**) based on RT, *tat* and *env* sequences from HIV-D [HIVD] and HIV-ND [ND] patients. *Ka* and *Ks* values (**A**) did not differ significantly between groups for each gene but *Ka* values were lower for the RT sequences. However, mean *Ka:Ks* values (**B**) were highest for *tat* sequences from HIV-D patients and were significantly increased compared to sequences from HIV-ND patients (Student's *t*-test, $P<0.001$).

sponding sequences from patients with adult T cell leukemia or healthy carriers. Most of the mutations in the present *tat* sequences from the HIV-D group were located in the augmenting region of the first exon (codons 57–72) or in the 5′ region of the second exon (codons 73–78). Mutations in both domains could contribute to the development of neurological disease. For example, the augmenting region influences viral replication (Gaynor, 1995) and thus may determine viral load in the brain. Alternatively, the second exon appears to be important for intracellular transport (Ma *et al*, 1997) and thus mutations in this region may affect the extracellular quantity of *tat*, influencing its potential immune activating or neurotoxic properties. Although the *tat* sequences differed between the HIV-D and HIV-ND groups in some respects, unlike the *env* sequences (Power *et al*, 1994) from the same patients, *tat* sequences from HIV-D and HIV-ND did not differ at specific positions. An explanation for this finding is that selection pressures acting on HIV-1 variants in the brain may differ depending on the viral gene being examined. For example, significant differences in *d* and *Ka* values were observed between genes in the present study (Table 2) and earlier studies of lentiviruses (Van Hemert and Berkhout, 1995). However, the individual selection pressures acting on the sequences remain uncertain.

The finding of decreased *Ka:Ks* values among the HIV-ND *tat* sequences is interesting. It suggests that purifying selection is acting on the HIV-ND sequences to maintain relative uniformity and preserved function (Chao, 1994). The emergence of mutant *tat* sequences among the HIV-D group may be occurring in the absence of purifying selective influences which is supported by the predominance of T to A or G to C mutations. These mutational patterns are unlike other reported retroviral sequences which are primarily A to G (Kim *et al*, 1996). Hence, it is conceivable that a rapidly mutating virus in which several genes mutate at different rates, reflected by the significant differences in *Ka* and *d* values, could selectively mutate in one gene in a different manner from other genes. Our finding of phylogenetic clustering within groups among the *tat* and *env* genes suggests two possibilities. The first is these patients were initially infected with a similar virus which is unlikely because the patients were ascertained from a large university clinic. Alternatively, that brain-derived *tat* and *env* sequences from the same clinical group have undergone parallel sequence evolution with the independent development of the same motifs due to similar selective pressures. These pressures could be common host immune response genes leading to selection for or against certain viral variants. In any case, the finding of clustering among both *tat* and *env* sequences from demented patients implies that whatever viral or host factor(s) accounts for this cluster may have the capability to influence the occurrence of clinical dementia.

Despite the sequence variation observed in the brain-derived *tat* and RT sequences and prolonged ARV treatment of some patients, the frequency of drug resistance associated mutations including ZRAM was low in the present study. Earlier studies of RT codons 74 and 215 in brain-derived HIV-1, primarily from children with HIV encephalopathy, indicate that ZRAM occur frequently at these positions (Sei *et al*, 1995). However, recent studies (Wong *et al*, 1997) of brain-derived RT sequences showed that in four adult patients treated with ZDV and ddI, ZRAM were detected in two of four patients with a lower frequency of detection in brain compared to spleen or lymph node. The explanation for this dichotomy in findings is likely better tissue penetration of ZDV achieved in children treated with continuous infusion in the Sei *et al* (1995) study. An important implication of the present findings is that ZRAM are relatively infrequent in brain-derived HIV-1 in adults with previous ZDV exposure and not associated with HIV-D occurrence. Therefore, the continued use of high dose ZDV in combination with other antiretroviral drugs may be beneficial in the treatment of HIV-D patients with systemic ZDV resistance.

Our studies indicated that brain-derived *tat* sequences differed from the B clade *tat* consensus sequences at many positions. Many of the reported functional assays of *tat* activity, including neurotoxicity and cytokine induction in brain cells have used peptide homologous to non-brain-derived *tat* sequences. Thus, future studies using *in vitro* and *in vivo* assays may benefit from including brain-derived *tat* sequences to gain a clearer insight into *tat*-mediated action(s) in the brain.

Methods

The AIDS Brain Bank (ABB) at Johns Hopkins University contains brain tissue from autopsied patients with AIDS from the Baltimore area who were prospectively characterized by the AIDS Neurology Group, prior to death (Tyor *et al*, 1992; Power *et al*, 1994; Wesselingh *et al*, 1993; Glass *et al*, 1995). Subcortical white matter from the mid-frontal gyrus of patients with AIDS was selected from the ABB, based on clinical features including presence or absence of HIV-D, duration of zidovudine (ZDV) therapy prior to death, and neuropathological findings. To avoid selection bias by *in vitro* viral isolation, RNA was extracted directly from HIV-infected brains from which cDNA was synthesized (Wesselingh, 1993). The HIV-1 *tat* and *pol* regions were amplified by nested PCR protocols (Power *et al*, 1994); the first (30 cycles) and second

PCR (30 cycles) amplifications were performed with *pol* primers, P1 (5′-GTA CAG TAT TAG TAG GAC CT-3′)/2851C (5′-TGA CGT CGA CTC ATT GAC AGT CCA GCT-3′) and P2 (5′-CAC CTG TCA ACA TAA TTG GGA AGA-3′)/P4C (5′-ACT GTC CAT TTA TCA GGA TG-3′) primers respectively which amplified the first 780 base pairs of the reverse transcriptase (RT) encoding region of *pol*. The *tat* fragment, including the first and second exon, was amplified using primers 5767 (5′-AGC TGC TGT TTA TTC ATT TCA-3′)/8433 (5′-ATC GTC CGG ATC TGT CTC TGT-3′) in the first reaction and 5792 (5′-TGG GTG TCG CAG AAT AGG-3′)/8433 in the second reaction yielding a product of 303 base pairs. Each step outlined above was performed in separate rooms to avoid contamination. The PCR products were cloned (pCR II, Invitrogen, San Diego CA) and sequenced using the dideoxy method from which amino acid (AA) sequences were inferred and sequences were aligned by Clustal (DNASTAR). Analysis of DNA distance (*d*), synonymous (*Ks*),and non-synonymous (*Ka*) nucleotide changes, and construction of phylogenetic trees was performed using MEGA (Kumar *et al*, 1993). Statistical analysis including parametric (one way ANOVA or Student's *t*) and non-parametric (Mann-Whitney *U*) tests were used (INSTAT2, Graphpad).

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