Analysis of the genetic diversity and phylogenetic relationship of Italian isolates of feline immunodeficiency virus indicates a high prevalence and heterogeneity of subtype B

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The genetic diversity of 32 Italian isolates of feline immunodeficiency virus (FIV) was studied. Isolates were obtained from domestic cats living in different areas. Sequence data were obtained from a 308 bp fragment of the p25 region of the gag gene. Phylogenetic relationships among these sequences and previously published sequences were determined. All the Italian isolates could be assigned to subtype B; however, four isolates formed two separate clusters and may represent genetic outliers. The reliability of classification results was confirmed by repeating the phylogenetic analysis with DNA sequences from the entire gag genes of two isolates and from the surface glycoprotein domain of the env gene of four isolates. It is concluded that the short segment of gag used permits reliable genotyping of FIV isolates. The study also shows that subtype B is largely prevalent in Italy.

Introduction

Feline immunodeficiency virus (FIV), a major pathogen of domestic cats throughout the world, shares many biological, immunobiological and pathogenic properties with HIV (Bendinelli et al., 1995; Willett & Jarrett, 1995). As these similarities have progressively become evident, the significance of FIV infection as a model for AIDS has steadily increased, especially in matters such as the development of vaccine strategies where formidable difficulties are encountered (Hilleman, 1995).

Vaccination studies have induced protection against homologous (Hosie et al., 1995; Matteucci et al., 1996; Yamamoto et al., 1991) and slightly heterologous FIV isolates (Yamamoto et al., 1993). However, there was no protection against more distant isolates (Johnson et al., 1994), thus suggesting that candidate vaccines should be tailored to the virus strain(s) likely to be encountered.

One of the advantages of FIV as a model for HIV vaccination studies is the large availability of naturally sick hosts. In countries such as Italy and Japan, where domestic cats are numerous and free to roam, FIV is especially abundant and, consequently, the frequency of transmission high (Bandecchi et al., 1992; Ishida et al., 1989). These regions can therefore be viewed as ideal locations for performing field trials of candidate anti-FIV vaccines. Thus, a prerequisite to any FIV vaccine field trial is an accurate understanding of the strains that circulate where the trial is planned.

Previous studies based on phylogenetic analysis of env gene sequences defined five, unevenly geographically distributed FIV subtypes: A (found in California and Europe), B (USA, not California, and Japan), C (British Columbia), D (Japan) and E (Argentina) (Kakinuma et al., 1995; Pecoraro et al., 1996; Sodora et al., 1994). Similar results were also obtained when the nucleotide (nt) sequences of the gag gene were analysed, indicating that this gene has the prerequisites needed for classifying virus isolates (Kakinuma et al., 1995; Rigby et al., 1993).

To investigate the genetic diversity of Italian isolates of FIV, we evaluated sequences of 32 isolates from different regions and determined their evolutionary relationships by comparison with previously characterized isolates. As a framework for analysis, we used sequences from a short segment of the p25 region of the gag gene that permits reliable genotyping of FIV isolates. The results show that all the
isolates studied belong to subtype B though some were quite divergent. Phylogenetic analysis in the \textit{env} region of two such divergent isolates indicated a lower degree of separation. By determining the proportion of synonymous and non-synonymous substitutions in the \textit{gag} and \textit{env} regions, this was attributed to an underestimate of phylogenetic distances by \textit{env}-based analysis.

**Methods**

**Virus isolates.** Table 1 summarizes information on the cats from which the FIV isolates were obtained. All the animals were naturally infected and were FIV positive and feline leukaemia virus negative as determined by ELISA (Pet Check; IDEXX). FIV seropositivity was further confirmed either by seroneutralization tests or Western blot analysis (Poli et al., 1992; Tozzini et al., 1992). All cats originated from urban areas and nine were housed together in the same open shelter. Peripheral blood samples were collected into citrate- or EDTA-coated tubes and transported to our laboratories within 48 h. Plasma was separated by low-speed centrifugation, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. PBMC were either used directly or cultured for FIV isolation with lymphoid MBM cells as described (Giannecchini et al., 1996). Cultures were considered virus positive when reverse transcriptase activity or p25 antigen was detectable in the supernatant on two consecutive occasions.

**DNA extraction.** High-molecular-mass DNA was extracted from uncultured PBMC or virus-positive cultures under PCR cleanroom conditions.

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* The first two capital letters indicate the country of origin; the next two define the region of origin (ER, Emilia Romagna; LI, Liguria; TO, Tuscany; VE, Veneto; SI, Sicily); the numbers indicate the reference number of the isolate; the three last letters indicate the study source (Pisa University).
† The capital letters L and M indicate that sequences were obtained directly from uncultured PBMC or from virus isolation positive cultures, respectively.
‡ Cats living in the same open shelter.
conditions either by overnight digestion with SDS–proteinase K followed by extraction with a phenol–chloroform mixture (1:1) and ethanol precipitation, or using the QIAamp blood kit (Qiagen) according to the manufacturer’s recommendations as described (Camarota et al., 1996). Each DNA sample was stored in aliquots at —80 °C until used.

**PCR.** Extracted DNA was subjected to single or nested PCR amplification, respectively, by using primer pairs designed from the most conserved portions of the gag and env genes, as defined by alignment of nt sequences from several FIV isolates with the CLUSTAL version V program (Higgins et al., 1992) of PC/Gene software (IntelliGenetics). PCR was carried out in a total volume of 50 μl containing 0.2 to 1 μg of genomic DNA, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 0.1% Triton X-100, 40 pmol each primer, 200 mM each of the four deoxy-nucleotide triphosphates and 2 U of Taq DNA polymerase (Perkin Elmer Cetus). Samples were denatured at 94 °C for 2 min, then subjected to 35 amplification cycles. Second round amplifications were carried out by using 2 μl of first PCR product with inner primer sets in 50 μl of fresh reaction mixture for 25 amplification cycles. Negative controls (DNA from uninfected cats and H₂O) were included in each assay, while positive controls (DNA from FIV-infected cells) were included in the second step only. Cycling profiles were designed according to the following guidelines: denaturation at 94 °C for 25 s, 2 °C below the lowest melting temperature of the primer set used for 30 s, and extension at 72 °C for 1 min/kb; amplification was completed by a final incubation at 72 °C for 10 min. Nt sequences and localization of primers used for amplification, sequencing and restriction fragment length polymorphism analysis (RFLP) are available by e-mail.

**DNA sequence analysis.** PCR products were sequenced with an automated DNA sequencer (ALF DNA Sequencer, Pharmacia) using cycle sequencing methods. Briefly, 250 and 500 fmol of amplicons were sequenced in parallel reactions with 1 pmol of fluorescein-labelled primer (synthesized by Pharmacia) and the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham) under the following cycling conditions: initial denaturation step 2 min, 94 °C; cycling 20 s, 94 °C and 45 s at the annealing temperature of the fluorescein-labelled primer used for 28 cycles. Predicted amino acid sequences and restriction endonuclease digestion patterns were obtained from nt sequences by using TRANSL and RESTR programs; finally, glycosylation sites in endonuclease digestion patterns were obtained from nt sequences by using the GCG PRETTY program with a minimal plurality sequence and average similarity calculations. Consensus sequences were calculated by using the GCG PILEUP program with the above settings. Phylogenetic relationships were also analysed by using the maximum-likelihood method included in the PHYLIP program DNAML with the following settings: transition/transversion ratio of 2:000, randomize data input and global rearrangements. Both FITCH, NEIGHBOR and DNAML unrooted trees were drawn by using the DRAWTREE program. Synonymous and nonsynonymous distances were calculated by the method of Nei & Gojobori (1986) in MEGA version 1.01 (Kumar et al., 1993).

Predicted protein sequences of the env gene were aligned by using the GCG PILEUP program with minor manual adjustments because of the gaps in the V5 region of the gene. Editing was done with the LINEUP program, referring to the consensus sequence predicted by using the PRETTY program with the above settings.

**Nucleotide sequence accession numbers.** The V3 through V5 env gene sequences of M2 and M3 isolates reported in this paper were used to replace the original sequences deposited in GenBank under accession numbers X69500 and X69501, respectively. The other sequences obtained in this study were also submitted. Reference sequences used (sequence type, GenBank accession numbers, geographical origin and references in parentheses): Petaluma clone FIV-14 (proviral clone, M25381, Petaluma, CA, USA, Olmsted, et al., 1989; Talbott, et al., 1989; PPR (proviral clone, M36968, San Diego, CA, USA, Phillips et al., 1990); Dixon (env gene, L00608, Northern CA, USA, Yamamoto et al., 1993); Dutch-4 (env gene, X69498 Amsterdam, NL, Rigby et al., 1993); Dutch-113 (proviral clone, X60725, NL); UK-8 (gag gene, M. Rigby, personal communication, and env gene, X69496, Portsmouth, England, UK, Rigby et al., 1993); UK-14 (gag gene, M. Rigby, personal communication and env gene, X69497, Colwyn Bay, Wales, UK, Rigby et al., 1993); France-Wo (env gene, L06135 and L06136 gag gene, FR, Pancino et al., 1993); Sendai-1 (gag gene, D37820 and env gene, D37813, Sendai, JP, Kakinuma et al., 1995); USCAtt 10A (env gene, U02412, Oakland, CA, USA, Sodora et al., 1994); TM2 (proviral clone, M59418, Tokyo, JP, Miyazawa et al., 1991); Aomori-1 (gag gene, D37823 and env gene, D37816, Aomori, JP, Kakinuma et al., 1995); Aomori-2 (gag gene, D37824 and env gene, D37817, Aomori, JP, Kakinuma et al., 1995); Sendai-2 (gag gene, D37821 and env gene, D37814, Sendai, JP, Kakinuma et al., 1995); Yokohama (gag gene, D37819 and env gene, D37812, Yokohama, JP, Kakinuma et al., 1995); USMASboy03B (env gene, U02420, Salem, MA, USA, Sodora et al., 1994); CABCpbar02C (env gene, U02394, Vancouver, British Columbia, CA, USA, Sodora et al., 1994); CABCpbar07C (env gene, U02397, Vancouver, British Columbia, CA, Sodora et al., 1994); Fukuoka (gag gene, D37822 and env gene, D37815, Fukuoka, JP, Kakinuma et al., 1995); Shizuoka (gag gene, D37818 and env gene, D37811, Shizuoka, JP, Kakinuma et al., 1995). Finally, the gag gene sequence of an FIV isolate of subtype C (cat 3270) was kindly provided by Jim I. Mullins, Department of Microbiology, University of Washington, Seattle, USA.

**Results**

**Nucleotide sequence and phylogenetic analysis of the gag p25 region**

We had previously examined the gag p25 region of FIV isolates to develop an RFLP analysis to distinguish the FIV

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strains Petaluma (subtype A) and M2 (subtype B) in singly and dually infected cats (Cammarota et al., 1996). We had also suggested that the gag p25 region had the prerequisite qualities for classifying new isolates in general. To test this hypothesis, multiple nt sequence alignments were created by using the reference isolates of subtype A, B, and D listed in the legend to Fig. 1. Alignments were then analysed to calculate the average sequence similarities among the sequences aligned. Plots of subtype A and B revealed high intrasubtype similarity (average above 97%, in Fig. 1A and 1B, respectively), and the existence of two highly conserved inner motifs in both subtypes (Y and W in Fig. 1A and X and Z in Fig. 1B). These portions were subtype-specific as demonstrated by their disappearance from consensus sequences created with both subtype A and B isolates, where intersubtype similarity was 83% (Fig. 1A + B).

A similar analysis, for subtype D isolates also showed large portions of conserved sequences (Fig. 1D), but the limited number of sequences available did not allow firm conclusions.

To verify whether these motifs contain enough information to classify FIV isolates, we sequenced the entire gag gene of the isolates M2 and M3 and a 308 bp fragment, encompassing the subtype-specific conserved motifs, was used for phylogenetic analysis. Pairwise genetic distances as estimated from multiple nt alignments of these sequences together with those of other reference isolates allowed us to readily distinguish the different subtypes, indicating that the 308 bp fragment contains sufficient information to characterize FIV isolates (data not shown). The two Italian isolates were clearly related, since their genetic distance was 0.0264 and nt identity 97.4%, while identity between these isolates and the others ranged from 79.7% (France-Wo) to 83% (Dutch-113) for subtype A isolates; 95.1% (Sendai-1) to 96.4% (TM2) for subtype B isolates; and 85.3% and 86.0% with the Shizuoka and Fukuoka isolates of subtype D. These observations indicated a close distance between the Italian isolates considered here and the known subtype B isolates.

The reliability of genetic distances calculated with the 308 bp fragment was confirmed by distance values obtained from analysis of the nt sequences of the entire p25 regions. Similar results were also found when the nt sequence of a subtype C isolate (courtesy of J. I. Mullins) was included in a DNADIST analysis (data not shown).

Phylogenetic relationships were inferred from the genetic distances by constructing unrooted phylogenetic trees using the distance-based Fitch–Margoliash method. To determine the heterogeneity of FIV strains circulating in Italy, we obtained blood samples from 30 additional FIV-seropositive cats and characterized the infecting viruses by analysing the nt sequence of the 308 bp segment. Although the samples were not obtained as part of a systematic epidemiology survey, they were from different geographical locations (Table 1), at different stages of disease, and most were from free-roaming cats. Nine were from animals living in the same open shelter.

Sequencing was carried out on PCR products obtained from DNA extracted from uncultured PBMC or isolation-positive cultures and from at least two independent amplification reactions. Similarities within isolates were first examined by pairwise nt sequence alignments. Overall nt divergence between the isolates ranged from 0–32% (M2 versus L105) to 10–06% (M88 versus M56), average 5±02%. The highest values of divergence were observed for the M88, L152, L153, and L155 isolates. A matrix of pairwise genetic distances showed that all Italian isolates were closely related to subtype B but isolates M88, L152, L153 and L155 clearly differed from the others, as they exhibited high distance values with the other Italian isolates and with reference subtype B isolates (data not shown).

A sequence distance matrix was then used to build unrooted phylogenetic trees by using Fitch–Margoliash and bootstrap resampling methods. To appraise the sensitivity of Fitch–Margoliash to the input order of species, a pilot study was done in which the program was run by using either a fixed or multiple randomized order of sequences and by repeating the process 10 times. The resulting trees differed slightly but phylogenetic relations were identical (trees not shown).

Since the randomizing option was time consuming and did not influence segregation, Fitch–Margoliash trees were produced from a fixed order of bootstrapped sequence only and by using the multiple data set option (100 reiterations). As shown in Fig. 2(A), subtypes A, B and D were clearly discernible. All the Italian isolates clustered together with subtype B isolates,
suggesting a common phylogenetic origin. Although bootstrap data were not highly significant, seven clusters were distinguishable within subtype B, five of which almost equally related to each other and two clearly independent. All the Japanese isolates grouped in one of the former clusters, indicating a distinct genetic relatedness, while the Italian
Fig. 2. Phylogenetic trees of all the FIV isolates studied based on the 308 bp segment of the p25 region. (A) Fitch–Margoliash tree. Branch lengths represent percentage sequence divergence, calculated by using Kimura’s two-parameter model with a transition/transversion of 2:00. Bootstrap values above 70 out of 100 are shown at the branch points. (B) Maximum Likelihood tree generated by using a transition/transversion ratio of 2; ln likelihood = -2141.31284; 6992 trees examined. Branch lengths estimate the expected number of substitutions per site x 1000. (C) Fitch–Margoliash tree based on the V3 through V4 region of the env gene of four Italian isolates and all the reference isolates. Bootstrap values above 70 out of 100 are shown at the branch points.
isolates were distributed in all the branches independently of their geographical origin. The two outlier clusters comprised three isolates from Genoa (L152, L153 and L155) that segregated together and isolate M88, which clustered by itself, suggesting that these isolates may have undergone partly separate evolution.

An additional point of interest was that most of the nine isolates obtained from cats living in the same shelter grouped together (isolates M8, M9, M17, M19, M56 and M62), but three (L115, M64 and M14) grouped in two separate branches, thus implying that at least three distinct FIV lineages were present in the shelter.

The reliability of the above results was validated by further examining the sequences by using the DNAML program. The resulting tree was very similar to that obtained with the distance-based method (Fig. 2B). Although some of the sequences changed place, again all the Italian isolates grouped together with subtype B. Interestingly, as compared with the Fitch–Margoliash tree, the positions of the branches defining isolate M88 and the cluster of isolates from Genoa were reversed relative to the branch defining subtype D, while isolates from cats living in the same shelter grouped into two branches because L115, M14 and M64 grouped together with other isolates.

Finally, the nt sequences of these isolates were also examined for the restriction endonuclease pattern within the 308 bp fragment. The KpnI site was present in all the Italian isolates, thus confirming the validity of the RFLP analysis proposed for recognition of subtype B isolates.

**Nucleotide and amino acid analysis of the env gene of selected Italian isolates**

A 1006 bp fragment of the env gene of the M2 and M3 isolates was sequenced following amplification by PCR. Nt sequences from a 513 bp fragment of env encompassing the V3 through V4 region of the M88 and L152 isolates were also obtained. Predicted Env amino acid sequences of all these isolates were aligned with the sequences of 21 isolates previously characterized (eleven subtype A, six subtype B, and two each of subtypes C and D) and further analysed to calculate and display consensus sequences. Because of the uneven length of database sequences, nt and amino acid sequence analysis was limited to a 675 bp fragment encompassing the V3 through V5 region (343 amino acids from the start of the leader).

Fig. 3 shows amino acid variations across the three hypervariable regions as defined by Pancino et al. (1993). As previously reported (Rigby et al., 1993), multiple amino acid sequence alignment revealed a high degree of heterogeneity and substantial length variation in the V5 region; M2 and M3 contain a deletion of three amino acids (position 568 to 570), similar to what was observed for the other subtype B isolates. Contrary to what was observed in the M2 and M3 env sequences published previously (Rigby et al., 1993), the cysteine residues were perfectly conserved in the Italian isolates. Of the potential N-linked glycosylation sites, most were conserved among the isolates examined.

The nt sequences of V3 through V4 were used to calculate genetic distances and infer phylogenetic relationships by using Fitch–Margoliash and bootstrapping methods. The four FIV subtypes were clearly evident in the unrooted tree (Fig. 2C) and, although no clear-cut phylogenetic linkages could be determined, all Italian isolates still clustered into phylogroup B. Isolates M88 and L152 did not group with the ordinary isolates of subtype B but the degree of separation was considerably lower than observed in gag trees.

Among the reference strains, the Japanese Yokohama isolate behaved differently as compared to a previous study where it was classified within subtype B (Kakinuma et al., 1995), because it clustered within this subtype in the gag trees and as a genetic outlier in the env tree. This discrepancy was not simply due to the different distance-based methods since it was observed also by analysing env sequences with the method used by Kakinuma et al. (Neighbour-Joining; data not shown). In the original report, the positions of the Yokohama isolate in the env trees built with the entire gene sequence or with the V3 through V5 segment were slightly different. In fact, this isolate clustered within subtype B only with the V3 to V5 segment, which contains a highly homoplastic site in the V5 region where most sequences were gapped, similarities uncertain and the phylogenetic information carried therefore modest (Stewart, 1993; Learn et al., 1996). We used both the V3 through V4 and the V3 through V5 regions trimmed of the homoplastic region and obtained results similar to those obtained by Kakinuma et al. with the entire env sequence, where the influence of homoplaspy is greatly reduced. Thus, the different positions in different phylogenetic trees of the Yokohama isolate appear to depend on the sequence and/or length of the genomic region examined, similar to what was found for recombinant HIV-1 isolates (Robertson et al., 1995; Sabino et al., 1994).

**Synonymous versus nonsynonymous nucleotide changes**

Fig. 4 shows the proportion of synonymous and nonsynonymous sites showing nt changes (p distance) in the 308 bp gag and the 513 bp env segments sequenced, evaluated by intra- and intersubtype pairwise comparisons. The sequences are grouped according to degree of evolutionary relatedness as inferred from env and gag phylogenetic trees. Furthermore, to better understand the evolution of the Italian genetic outliers (M88, L152, L153 and L155), their sequences are considered separately from other subtype B sequences. As expected, the gag gene showed the least accumulated variation (Fig. 4A) and the env gene the most (Fig. 4B). For either gene the amounts of variation in both synonymous and non-
Fig. 3. Alignment of the predicted translation products of the V3 through V5 region of the env gene of isolates M2, M3, M88 and L152 and 21 previously sequenced isolates, beginning 343 amino acids from the start of the leader. Sequences were aligned and then manually adjusted by using PILEUP and LINEUP programs, respectively. Consensus was calculated by using the PRETTY program. Residues differing from the consensus sequence are shown. Dots denote identity with the consensus sequence. Black and hatched boxes under the alignment indicate the location of potential N-linked glycosylation sites present and absent in M2 and M3 isolates, respectively. Numbers under each box indicate the ratio of the number of glycosylation sites present to the total number of sequences examined; since two such sites were unevenly present in the Italian isolates, the numbers in parentheses under the two corresponding boxes indicate the isolate where the glycosylation site is present. Asterisks indicate the position of cysteine residues. Sites which distinguish subgroups A and B from the others are marked by shaded boxes. Other amino acid sites which distinguish subtype C and D from the others are boxed, but the small number of sequences available for these subtypes does not allow firm conclusions. Lines above the alignment indicate the V3, V4 and V5 regions as defined by Pancino et al. (1993). FIV sequences are grouped according to subtype.
Fig. 4. Synonymous and nonsynonymous \( p \) distances between pairs of DNA sequences from the 308 bp \( gag \) fragment (A) and the 513 bp \( env \) fragment (B). Each point represents one pairwise comparison. The synonymous (or nonsynonymous) \( p \) distance is the proportion of the possible synonymous (or nonsynonymous) changes which have occurred.

-+, comparisons within subtypes; □, comparisons within \( gag \) genetic outliers (isolates L152, L153 and L155); ■, comparisons between all \( gag \) genetic outliers (M88, L152, L153 and L155); and other subtype B isolates; ○, comparisons between subtypes.

Discussion

The main purpose of this study was to investigate the extent of genetic diversity among FIV isolates circulating in Italy and examine their relationships to previously characterized strains. Because FIV vaccination experiments have shown that significant protective immunity can be achieved solely against virus strains homologous or closely related to those used for vaccine preparation (Hosie et al., 1995; Matteucci et al., 1996), in designing vaccine trials in the field it will be essential to precisely know the diversity of FIV strains present in the area and to tailor the immunogens accordingly.

Previous phylogenetic analysis of FIV strains based on the entire \( env \) gene or its V3 through V5 region have led to the recognition of at least five subtypes, designated A to E (Kakinuma et al., 1995; Pecoraro et al., 1996; Sodora et al., 1994). Similar phylogenetic lineages were also obtained when the nt sequences used were from the \( gag \) region, thus demonstrating that this genomic region also lends itself to FIV classification (Kakinuma et al., 1995). The latter observation, together with the availability of two primer sets capable of amplifying with high specificity and sensitivity the p25 region of FIV isolates belonging to different subtypes (Cammarota et al., 1996), prompted us to investigate more closely whether this region contains sufficient information for phylogenetic inference and to identify the subregion most informative in this regard.

Analysis of published \( gag \) sequences in multiple alignments revealed an elevated degree of nt identity within each FIV subtype and the presence of short non-overlapping segments that were 100% conserved among isolates belonging to a same subtype, indicating that they were highly significant for phylogenetic analysis. Examination of the alignments also permitted the identification of restriction endonuclease sites that, as we confirmed by analysing a limited number of isolates, might be usefully exploited for rapid genotyping of new isolates by RFLP analysis.

To assess whether the subtype-specific domains identified above were suitable for our purposes, we carried out a phylogenetic analysis of existing published sequences and of two newly sequenced Italian isolates (M2 and M3) using a 308 bp segment encompassing such domains. The results of this analysis and of similar analyses performed with the entire p25 region or the V3 through V4 segment of the \( env \) gene showed that the two isolates grouped within subtype B regardless of the genomic region examined, thus indicating...
that they belong to this phylogroup. Moreover, all trees were supported by high bootstrap values, indicating a high statistical significance, and gave grouping patterns that closely resembled those reported previously (Bachmann et al., 1996). Taken together, these findings confirmed the validity of sequencing the selected 308 bp stretch of the p25 region for classifying new FIV isolates.

The 308 bp gag segment was then used to characterize thirty additional isolates, mostly from central Italy but several also from the north and south. All were found to cluster within subtype B, thus showing that this subtype is highly prevalent in Italy. A similar conclusion was recently reached in a concomitant study by J. Mullins and his group using a heteroduplex mobility assay on isolates that only partially overlapped those studied here (Bachmann et al., 1997). Thus, the epidemiology of FIV in Italy appears to differ considerably from that in North European countries where only subtype A strains have hitherto been identified. It would, therefore, be of interest to perform similar epidemiological studies in other Southern European countries.

Interestingly, in the gag trees the majority of subtype B isolates were contained in five major clusters closely related to each other. This is compatible with the idea that they have diverged at approximately the same time from an ancient common ancestor and subsequently undergone independent evolution. The fact that all the Japanese strains fell into a unique phylogroup favours this interpretation. However, some Italian isolates exhibited a pronounced level of genetic heterogeneity. These were isolate M88, which clustered by itself, and isolates L152, L153 and L155, which segregated together in another cluster clearly distinct from the other subtype B isolates. Since this behaviour was observed in both the Fitch–Margoliash and the DNAML tree, despite an inversion relative to the subtype D branch (Fig. 2A, B), we are inclined to think that these isolates represent a real phylogenetic separation rather than differences in nt substitution rates. To address this issue further, we sequenced and analysed the V3 through V4 region of the env gene of two such outliers, M88 and L152, and of two typical subtype B isolates, M2 and M3. The results were inconclusive, since the genetic distances observed suggested that M88 and L152 were in some way ‘intermediate’ between the possible recombinant Yokohama isolate and the remaining subtype B isolates, but phylogenetic data and bootstrap values did not permit solid conclusions.

The discrepancy between env and gag trees could in part be due to a substantial underestimate of nt changes in env due to the accumulation of multiple substitutions in hypervariable sites. To explore this possibility we calculated the p distance values for synonymous and nonsynonymous substitutions, which provide information as to the selective forces which affect genetic evolution (Li et al., 1985). In agreement with previous studies (Rigby et al., 1993; Sodora et al., 1994), we found that the phylogenetic distances evaluated in env were progressively underestimated as the nt divergence of the pairs of sequence examined increased, possibly due to large numbers of back mutations partially counterbalancing forward mutations in hypervariable sites.

In conclusion, we have shown that sequencing a short, easily amplifiable segment of gag can be used for classifying new FIV isolates. The approach can be advantageous because it does not require the continuous design of new primers and amplification profiles needed for sequencing the more heterogeneous env sequence and might be considered as alternative or confirmatory to methods, such as the heteroduplex mobility assay and RFLP analysis, that have been proposed for rapid FIV subtyping. By sequencing this segment of gag of 32 isolates obtained from different regions of Italy and part of the env gene of two of four isolates that appeared to be outliers in gag trees, we showed that subtype B is highly prevalent in Italy. Subtype B FIV strains offering a sufficient breadth of intratypic cross-protection should therefore be major components of candidate FIV vaccines to be used in this geographical area.

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immunodeficiency virus envelope glycoproteins.


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