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Short Communication

Phylogenetic analysis of feline immunodeficiency virus in Central Europe: a prerequisite for vaccination and molecular diagnostics

Adolf Steinrigl and Dieter Klein

Institute of Virology, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria

Correspondence
Dieter Klein
dieter.klein@vu-wien.ac.at

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Feline immunodeficiency virus (FIV) is a worldwide-occurring lentivirus that severely impairs the immune function of infected domestic cats. Due to structural and biological similarities, FIV represents a promising model for human immunodeficiency virus (HIV) and AIDS. A major obstacle in developing vaccines against lentiviruses is their high mutation rate. Furthermore, mutations in target sequences provide a pitfall for molecular diagnostics. It is therefore important to determine the genetic diversity of lentiviruses in any region where vaccination or implementation of new diagnostic techniques are planned. This study presents a phylogenetic analysis of 30 FIV strains derived from Central Europe. In order to improve the reliability of genotyping, DNA from two different proviral genes was amplified and comparative phylogenetic trees were inferred. The highly coincident results point to the existence of extensive virus variation with the presence of at least two highly divergent subtypes of FIV in Austria and Germany.

Feline immunodeficiency virus (FIV) is a commonly occurring lentivirus able to establish persistent infections in domestic cats (Bendinelli *et al.*, 1995). As with human immunodeficiency virus (HIV), several genetically distinct subtypes (clades A–E) have been reported, revealing up to 26 % sequence diversity among parts of the *env* genes (Sodora *et al.*, 1994; Kakinuma *et al.*, 1995; Pecoraro *et al.*, 1996). Infection finally leads to a debilitating disease that resembles AIDS in humans: after a variably long clinical latency period, infected hosts become susceptible to secondary and opportunistic infections or develop tumours, which are rarely seen otherwise (Pedersen *et al.*, 1989; English *et al.*, 1994). Consequently, several attempts have been made to develop a vaccine against FIV infection using different strategies (Yamamoto *et al.*, 1993; Hosie *et al.*, 1995, 1998; Matteucci *et al.*, 1996; Lockridge *et al.*, 2000; Bigornia *et al.*, 2001; Pu *et al.*, 2001). Several single-subtype vaccines protected against challenge with homologous or slightly heterologous virus but failed to protect against more distantly related strains (Elyar *et al.*, 1997). In contrast, a double-subtype virus vaccine has been proven to elicit considerable protection against challenge with virus of a third subtype, not included in the vaccine (Pu *et al.*, 2001). This vaccine has now been approved by the USDA; however, its efficacy still remains to be shown under field conditions (Uhl *et al.*, 2002).

As the success of a vaccine could be hampered by the occurrence of highly divergent virus variants, the genetic diversity of FIV field strains circulating in all regions where vaccination is planned should be determined (Pistello *et al.*, 1997). This knowledge should also allow the adaptation of vaccines to regionally prevalent subtypes, if development of a 'global' FIV vaccine should be impossible.

The extensive genetic variation observed in FIV also has a direct impact on PCR-based methods which are increasingly used for diagnosis and monitoring of FIV infection (Leutenegger *et al.*, 1999; Hosie *et al.*, 2002). Furthermore, PCR assays to distinguish vaccinated from infected cats will gain importance with the advent of the first commercially available FIV vaccine (Uhl *et al.*, 2002). In general, these methods are highly influenced by variations in the target sequences, which usually increase with genetic distance (Klein *et al.*, 1999, 2001).

To gain insight into the genetic diversity of FIV in Central Europe, we sampled EDTA-blood from 30 FIV-positive domestic cats derived from Austria, Germany, Switzerland and Italy. FIV infection was initially determined by PCR and in 27 of 30 cases additionally by ELISA (Table 1). Partial proviral *gag* and *env* genes were amplified with primers FIV-1026F (5′-GGC ATA TCC TAT TCA AAC AG-3′) and FIV-1700R (5′-AAG AGT TGC ATT TTA TAT CC-3′) (Cammarota *et al.*, 1996) for *gag*, and FIV-7316F (5′-ATA CCA AAA TGT GGA TGG TG-3′) and FIV-7868R (5′-TGC AAG ACC AAT TTC CAG CA-3′) for *env* (sequences kindly provided by M. Pistello, University of Pisa, Italy). The *env*

Sequences reported in this study are deposited under the GenBank accession numbers: AF531031 – AF531076 and AY196330 – AY196343.

Table 1. Summary of all Central European FIV strains reported in this study

The countries of origin and provinces of FIV-infected cats are given. Results of serological testing and genotyping are indicated. The NCBI accession numbers are also given. ND, Not determined; +, positive ELISA result.

				Subtyping		GenBank	
Strain	**Country**	**Province**	**Serology**	***gag***	***env***	***gag***	***env***
ATVIa33	Austria	Vienna	ND	B	B	AF531056	AF531045
ATVIa85	Austria	Vienna	ND	B	B	AF531061	AF531040
ATVIa90	Austria	Vienna	+	B	B	AF531059	AF531036
ATESb20	Austria	Spain*/Vorarlberg	+	B	B	AF531049	AF531032
ATSTb30	Austria	Styria	+	B	B	AF531054	AF531046
ATVIb31	Austria	Vienna	ND	B	B	AF531063	AF531031
ATVIb97	Austria	Vienna	+	B	B	AF531055	AF531042
ATSTc01	Austria	Styria	+	B	B	AF531058	AY196341
ATNOc07	Austria	Lower Austria	+	B	B	AY196330	AY196336
ATNOd01	Austria	Lower Austria	+	B	B	AF531060	AY196332
ATVId02	Austria	Vienna	+	A	A	AF531075	AF531047
ATESd03	Austria	Teneriffa*/Vienna	+	B	B	AF531050	AY196331
ATVId05	Austria	Vienna	+	B	B	AF531062	AY196335
ATVId06	Austria	Vienna	+	B	B	AF531065	AY196334
ATNOd16	Austria	Lower Austria	+	B	B	AF531057	AY196342
ATVId20	Austria	Vienna	+	B	B	AF531064	AY196333
ATVId23	Austria	Vienna	+	B	B	AF531066	AY196337
DEBWa06	Germany	B.-Württemberg	+	B	B	AF531048	AF531044
DEBAb91	Germany	Bavaria	+	A	A	AF531069	AF531043
DEBAd58	Germany	Bavaria	+	A	A	AF531070	AF531037
DEBAd59	Germany	Bavaria	+	A	A	AF531074	AY196343
DENWd60	Germany	N.-Westfalen	+	A	A	AF531072	AY196340
DEBAd65	Germany	Bavaria	+	A	A	AF531068	AY196338
DEFRd68	Germany	France*/Bavaria	+	A	A	AF531071	AY196339
DENWd70	Germany	N.-Westfalen	+	A	A	AF531073	AF531038
DEBEd72	Germany	Berlin	+	B	B	AF531051	AF531039
CHTGa05	Switzerland	Thurgau	+	A	A	AF531076	AF531034
CHSHa10	Switzerland	Schaffhausen	+	A	A	AF531067	AF531033
ITROd76	Italy	Rome	+	B	B	AF531052	AF531035
ITROd78	Italy	Rome	+	B	B	AF531053	AF531041

*These strains were isolated from cats living in Austria and Germany, but coming originally from Spain or France.

sequences of samples DEBAd59 and ATNOd16 were amplified by nested PCR using FIV-7224F (5′-GTA CAG ACC CAT TAC AAA TC-3′) and FIV-8000R (5′-CTG CCA CTG GGT TAT ACC AA-3′) as outer and FIV-7316F/FIV-7868R as inner primers. After purification, the amplicons were directly sequenced on both strands, using PCR primers. In the case of strain DEFRd68, repeated PCR and sequencing experiments did not result in a clean sequence. Therefore, the PCR product was cloned into a commercial vector (TA Cloning kit; Invitrogen) and six positive clones were sequenced. The clonal sequences varied by a *P*-value of 0–1·3 % sequence diversity (for comparison, the sequence closest to these clones differed from them by 4·9–5·7 % sequence diversity). Finally, a consensus sequence for DEFRd68 was derived which was used for phylogenetic analysis.

Both *gag* and *env* data sets were compiled using reference strains from GenBank. In order to compare the *gag* and *env* topologies under the same conditions, we used only those reference strains from GenBank where both corresponding gene regions were available. Several Japanese strains reported as being likely recombinants of different subtypes within the *env* region investigated in this study (Carpenter *et al.*, 1998) were excluded from analysis. Sequences described as subtype E (Pecoraro *et al.*, 1996) overlapped with the Central European sequences only along a stretch of 288 nt in *gag* and were likewise excluded. Multiple alignments were created with CLUSTAL X (Thompson *et al.*, 1997) and edited with DAMBE (Xia & Xie, 2001), resulting in a 562 nt *gag* and a 504 nt *env* alignment.

To estimate the phylogenetic signal contained in the *gag*

and *env* alignments, likelihood mapping (Strimmer & von Haeseler, 1997) was performed using TREE-PUZZLE (Strimmer & von Haeseler, 1996). With this method, the percentage of completely resolved quartet trees from a representative fraction of all possible quartets in a data set can be taken as a measure for the phylogenetic signal contained in an alignment. As a result, both alignments appeared to be highly informative, revealing 87·0 % in *env* and 86·6 % in *gag* of all analysed quartets being completely resolved.

As none of the most commonly used models for phylogenetic inference is principally superior to the others (Graur & Li, 2000), distance (Saitou & Nei, 1987), parsimony (Fitch, 1977) and maximum-likelihood (Felsenstein, 1981; Strimmer & von Haeseler 1996) analyses were performed for phylogenetic tree construction. To determine the substitution models that would best fit our data sets for maximum-likelihood and neighbour-joining analyses, a likelihood ratio test (Swofford *et al.*, 1996) was performed using PAUP* (version 4.0b10; Swofford, 2002). In the *gag* alignment, the Tamura–Nei model (Tamura & Nei, 1993) with gamma-distributed rates (TN93 + Γ) resulted in a likelihood estimate not significantly worse than the general time reversible model with estimated gamma-distribution and proportion of invariant sites (GTR + Γ + I), which is the most complicated model implicated in the program. In contrast, the likelihood ratio test favoured a submodel of the GTR + Γ + I model in *env* with three different substitution rates: one for both possible transitions, one for three of the four possible transversions and one for A↔C changes. Consequently, the TN93 + Γ model was used to construct quartet puzzling trees (Strimmer & von Haeseler, 1996) as well as neighbour-joining trees (Saitou & Nei, 1987) based on the *gag* alignment, whereas the same tree-building algorithms were used, assuming the special GTR + Γ + I model for the *env* alignment. Parsimony analyses were performed on both data sets using the heuristic search algorithm. All described tree constructions were performed using PAUP*, and MEGA 2.1 (Kumar *et al.*, 2001) was used to calculate neighbour-joining trees.

The tree topologies resulting from the different calculations were highly similar within each data set. Representative maximum-likelihood trees (Fig. 1) and neighbour-joining trees (Fig. 2) are shown. Upon comparison, trees derived from the different proviral regions investigated showed highly concordant topologies (Figs 1 and 2). All Central European sequences reported in this paper grouped into either subtype A or B of FIV. Within clade B, several subclades were consistently observed among both data sets. According to the origin of the virus strains, we termed these subclades B-main, Austria-1, Austria-2, Austria-3 and Portugal. A single Austrian strain (ATVId23) did not fall into either of these subgroups and might represent a prototype of another subclade of subtype B, as is suggested by the long branch that separates it from the other strains. Only two Austrian strains (ATESb20 and ATESd03) cluster with B-main. Interestingly, both of them were isolated from cats which, according to the owners, were born in Spain but were taken to Austria later in their life (Table 1). The close proximity of these strains is especially intriguing, based on the fact that the samples were collected at different time points in distinct parts of Austria. Moreover, they displayed reasonable diversity from all other Austrian sequences, as they grouped with cluster B-main. Therefore, we consider it very likely that both cats had actually been infected in Spain. Unfortunately, there are no Spanish FIV-sequences available, and thus comparison with Spanish strains was not possible. Both Italian strains reported here (ITROd76 and ITROd78) were grouped together extremely closely as well, but in this case close epidemiological linkage is very likely as both samples are derived from a population of feral cats in Rome. The occurrence of subtype B strains of FIV in Germany has been suggested by heteroduplex mobility analysis (Bachmann *et al.*, 1997). Accordingly, two German strains (DEBWa06 and DEBEd72) fell into this subtype. All other German FIV strains belonged to subtype A, together with both Swiss sequences and a single Austrian strain. Formation of a highly supported subclade (Germany-1) consisting of two German strains was observed in subtype A. In *gag* but not in *env*, the single Austrian clade A strain ATVId02 also grouped to this cluster.

As stated above, the trees inferred from *gag* and *env* regions were highly similar. However, there were some interesting discrepancies: strain DEBEd72 and the US-Maryland strain both grouped to B-main in *gag*, but were clearly separated from this subclade in *env*. Surprisingly, the US-Maryland strain clustered close to or within subclade Austria-2 in *env*, supported by high quartet puzzling and bootstrap values (Figs 1 and 2).

Moreover, a separation of clade B from clades C and D was not supported in neighbour-joining analyses of the *env* data (Fig. 2b). To test whether inter-subtype recombination could be the reason for this observation as well as for the discordant grouping of DEBEd72 and Maryland strains, *gag* and *env* sequences were concatenated, and bootscanning (Salminen *et al.*, 1995) was performed using SIMPLOT (Lole *et al.*, 1999). USIL2489_7B, Petaluma, BM3070 and Shizuoka served as reference sequences for the four subtypes included. However, no significant signs of recombination were observed among our data. In the absence of recombination and significant differences in phylogenetic content, the observed discrepancies might be due to the different evolutionary constraints effective on the *gag* and *env* genes, respectively (Pistello *et al.*, 1997; Rigby *et al.*, 1993). In the presented data sets, roughly 90 % of substitutions occurring in *gag* were estimated to be synonymous in comparison to about 50 % synonymous substitutions in *env*. Synonymous substitutions are generally believed to better display the true evolutionary history, as they are theoretically not influenced by selective pressure (Graur & Li, 2000). The positive selection pressure on *env* might account for the observed differences in our data. However, a detailed description of FIV phylogeny

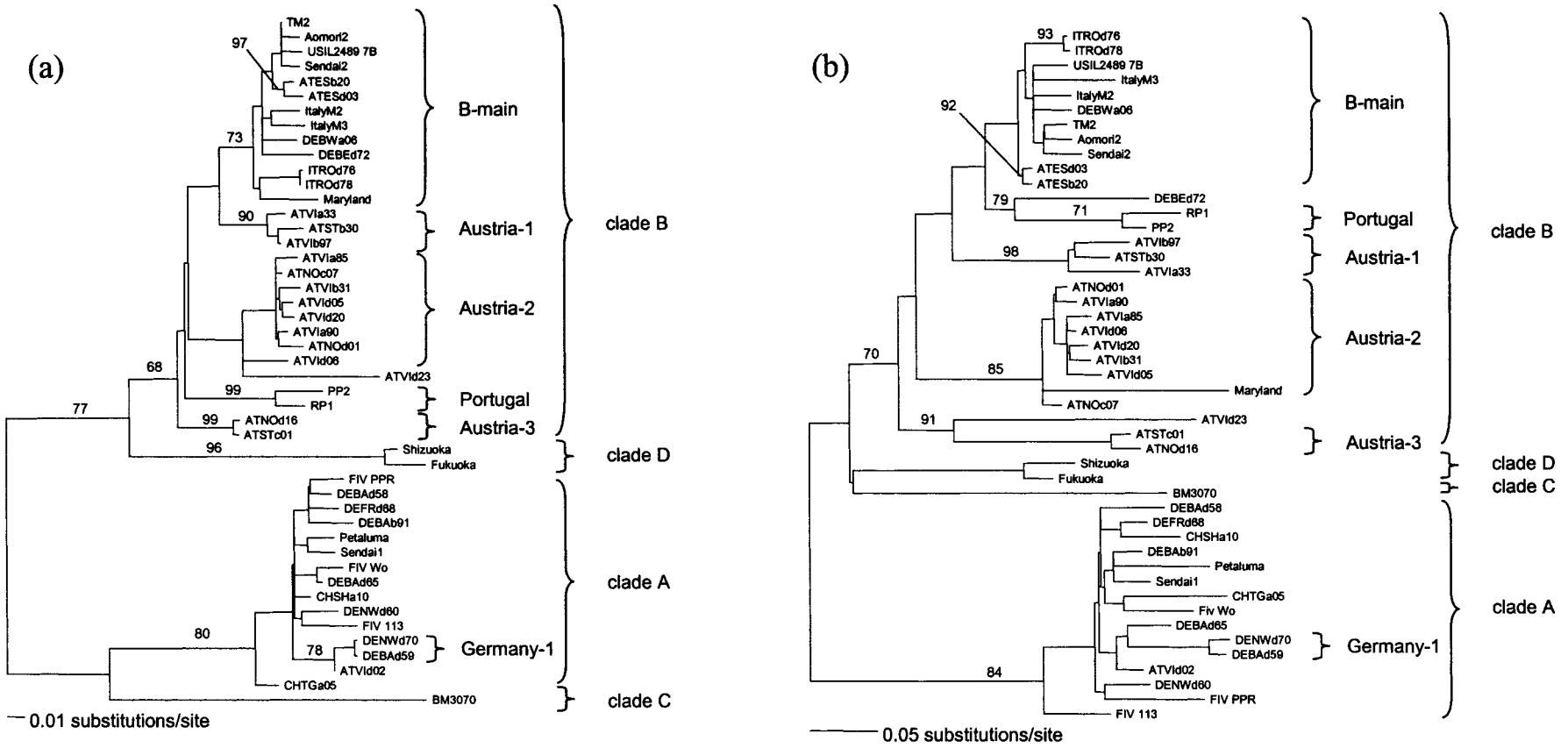

Fig. 1. Maximum-likelihood trees constructed by quartet puzzling. Numbers above the branches indicate the percentage of 1000 puzzling steps. Puzzle support values above 70 % are shown. Branch lengths are drawn to the scale at the bottom of each tree. The major subtypes A, B, C and D, as well as subclades of subtypes A and B, are indicated. Trees are midpoint-rooted. (a) Tree inferred from the 562 nt *gag* alignment. Reference sequences (GenBank): USIL2489_7B (U11820), ItalyM2 (Y13867), ItalyM3 (Y13866), Sendai2 (D37821), TM2 (M59418), Maryland (AF361320), Aomori2 (D37824), PP2 (AJ304959), RP1 (AJ304962), Shizuoka (D37818), Fukuoka (D37822), BM3070 (AF474246), Petaluma (M25381), FIV_Wo (L06135), FIV_PPR (M36968), Sendai1 (D37820) and FIV_113 (X68019). (b) Tree inferred from the 504 nt *env* alignment. Reference sequences were: ItalyM2 (X69501), ItalyM3 (X69502), Aomori2 (D37817), Sendai2 (D37814), Maryland (AF452126), PP2 (AJ304985), RP1 (AJ304988), Shizuoka (D37811), Fukuoka (D37815), FIV_Wo (L06135), FIV_113 (X60725) and Sendai1 (D37813). For accession numbers for Petaluma, FIV_PPR, USIL2489_7B, TM2 and BM3070 see (a).

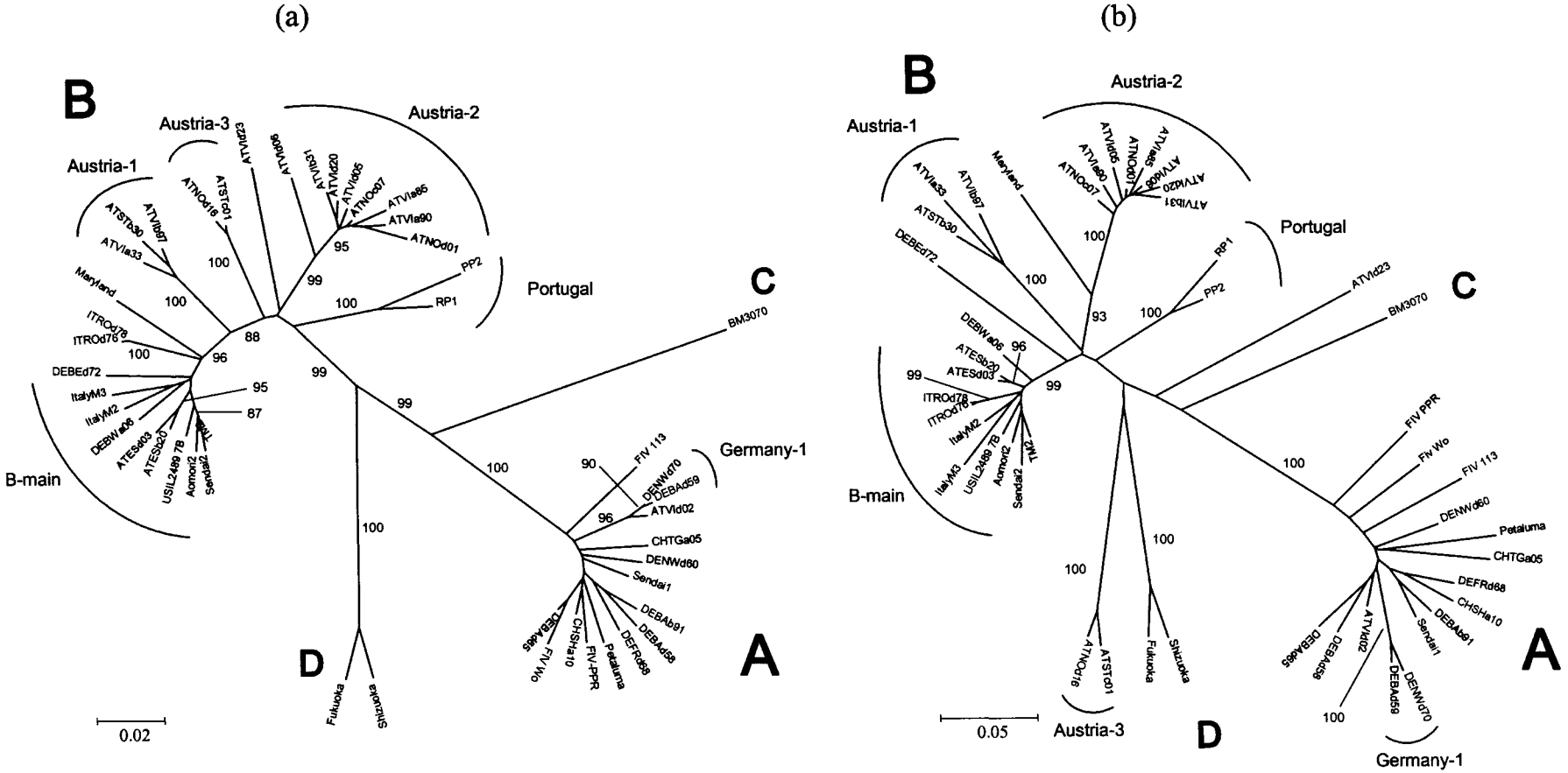

Fig. 2. Unrooted neighbour-joining trees. Numbers next to the branches indicate the percentage of 1000 bootstrap replicates. Bootstrap values above 80 % are shown. Branch lengths are drawn to the scale at the bottom of each tree. The major subtypes A, B, C and D, as well as subclades of subtypes A and B, are indicated. (a) Tree inferred from the 562 nt *gag* alignment. (b) Tree inferred from the 504 nt *env* alignment. See Fig. 1 for reference sequences.

and recombination frequency would necessitate the analysis of full-length genomes by analogy to the HIV-1 nomenclature proposal (Robertson *et al.*, 1999).

This work provides the first evidence that FIV of subtype B is predominant and highly divergent in Austria. Each of the described Austrian clusters within clade B consists of strains derived from different provinces of Austria (Table 1). Moreover, strains from the same provinces group into different subclades. Therefore these clusters represent real subclades rather than artefacts based on a common origin of the cats. Only a single subtype A strain was detected in Austria. In contrast, subtype A seems to occur more frequently in Germany. Our findings are in accordance with work by other groups, who found clade A and less frequently, clade B strains in Germany (Bachmann *et al.*, 1997) and a high prevalence of clade B FIV in Italy (Pistello *et al.*, 1997). However, more detailed screening studies would be necessary to precisely determine the prevalence of the occurring subtypes in Austria and Germany. The success of a vaccine might be largely dependent on the type and variety of circulating strains, which can differ substantially from region to region. In the light of the first commercial FIV-vaccine, it is even more important to consider the existing variety beforehand to prevent vaccination failure. Likewise, as new PCR-based diagnostic tools gain importance in the fields of diagnostics and research, knowledge about the genetic diversity and the compilation of new sequence data provide a valuable tool for the design of sensitive assays.

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