Research Square

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Identification of CRF89_BF, a new member of an HIV-1 circulating BF intersubtype recombinant form family widely spread in South America

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Research Article

Keywords: HIV-1, CRF89_BF, circulating recombinant forms, clusters, Bolivia, Peru, South America

Posted Date: February 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-215082/v1

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Abstract

Circulating recombinant forms (CRFs) represent a substantial proportion of infections in the HIV-1 pandemic. Among 103 CRFs described in the literature, 16 are BF intersubtype recombinants, most of South American origin, of which CRF12_BF is the most widely spread. A BF recombinant cluster identified in Bolivia was suggested to represent a new CRF_BF. Here, we find that this cluster belongs to a larger cluster incorporating 38 viruses collected in 7 countries from 3 continents, 21 of them in Spain, most from Bolivian or Peruvian individuals, and 12 in South America (Bolivia, Argentina, and Peru). It comprises three major subclusters, two associated with Bolivian individuals and one associated with Peruvian individuals. Near full-length genome sequence analyses of five viruses collected in Spain, Bolivia, and Peru revealed coincident BF mosaic structures, with 13 breakpoints, 5 and 6 of which coincided with CRF12_BF and CRF17_BF, respectively, and grouping in a clade closely related to these CRFs and more distantly to CRF38_BF and CRF44_BF, all circulating in South America. These results allow us to identify a new HIV-1 CRF, designated CRF89_BF. Through phylodynamic analyses, CRF89_BF emergence was estimated in Bolivia around 1984. CRF89_BF is the fifth CRF member of the HIV-1 recombinant family related to CRF12_BF

Introduction

One of the distinguishing features of HIV-1 evolution is its high recombination rate, which can be similar to or even greater than the mutation rate [1,2]. HIV-1 features promoting recombination include large viral population sizes, rapid viral turnover [3], frequent multiply infected cells in lymphoid organs [4], and high genetic diversity, which facilitates superinfection with genetically divergent variants, since susceptibility to interclade immune responses correlates with genetic distance separating the variants eliciting them [5]. Recombination in HIV-1 can increase viral diversity [2,6], augment replicative fitness [7,8], promote evasion from immune responses [6,9,10], and facilitate propagation of drug resistance mutations [11].

Recombination has contributed extensively to the generation of genetic diversity in the HIV-1 pandemic [12,13]. Recombinant forms are generated in individuals infected with two or more HIV-1 clades. Those found in a single individual or a single epidemiologically linked cluster are designated unique recombinant forms (URFs), and those found in three or more epidemiologically unlinked individuals are designated circulating recombinant forms (CRFs) [14], of which 103 have been described in the literature. The proportion of CRFs has increased over time in the HIV-1 pandemic, representing approximately 17% of infections in 2010-15 [13]. Among the identified CRFs, the most numerous are those derived from parental strains of subtype B and subsubtype F1, of which 16 have been reported, most of which originated in South America. The first reported CRF_BF was CRF12_BF [15-17], which circulates widely in Argentina and Uruguay [15-22] and in lesser proportions in other countries from South America [23-25]. Subsequently, three other CRFs related to CRF12_BF, CRF17_BF [26], CRF38_BF [22], and CRF44_BF [27], were identified in different South American countries, mainly in the South Cone. Numerous URFs closely related to CRF12_BF, as shown by coincident breakpoints and grouping in phylogenetic trees, have also been identified in some of these countries [16,17,19,28]. It has been proposed that all these recombinants constitute a “family” [29,30] of viruses that derive from a common recombinant ancestor probably generated in Brazil from locally circulating B and F strains; subsequently, this ancestor would have gone through successive rounds of recombination with subtype B viruses, generating a great diversity of recombinant forms, some of which propagated epidemically, becoming CRFs [28]. Here, we identify a fifth CRF member of the CRF12_BF-related family.

Materials And Methods

Samples from HIV-1-infected individuals were collected in 14 Spanish regions for a molecular epidemiological study. An ~1.4 kb pol fragment in protease-reverse transcriptase (Pr-RT) was amplified by RT-PCR/nested PCR from plasma RNA as described previously [31] and sequenced with the Sanger method using a capillary automated sequencer. Near full-length genome (NFLG) sequences were obtained for selected samples by amplification in four overlapping segments from plasma RNA and sequenced by the Sanger method, as described [28,32]. Newly derived sequences are deposited in GenBank under accessions KX818199, KX818200, and MW344906-MW344922 (Table 1).

Sequences were aligned with MAFFT v7 [33]. Maximum likelihood phylogenetic trees were constructed with FastTree2 [34], using the general time reversible evolutionary model, with CAT approximation for among-site rate heterogeneity, and assessment of node support

with Shimodaira-Hasegawa (SH)-like)local support values, or with IQ-Tree [35] using the best-fit substitution model determined by the program, with assessment of node support with the ultrafast bootstrap approximation approach. Trees were visualized with MEGA v7.0 [36]. Mosaic structures were analyzed by bootscanning [37] with SimPlot v1.3.5 [38], with tree construction using the neighbor-joining method and a window width of 250 nucleotides; short potentially recombinant segments identified with SimPlot were further phylogenetically analyzed with IQ-Tree.

The time and most probable country location of the most recent common ancestor (MRCA) of the identified cluster and subclusters were estimated using Pr-RT sequences with the Bayesian Markov chain Monte Carlo (MCMC) coalescent method implemented in BEAST v1.8.4 [39]. For this analysis, the positions in the alignment corresponding to codons containing antiretroviral drug resistance mutations in any of the sequences, as determined with Stanford University's database HIVdb program [40], were removed. Prior to the BEAST analysis, the existence of temporal signal in the dataset was analyzed with Tempest [41]. Since, according to this analysis, there was insufficient temporal signal, we used as a prior parameter a normally distributed substitution rate ($1.33 \times 10^{-3} \pm 2.57 \times 10^{-4}$ subst/site/year) estimated from 65 CRF12_BF sequences, which exhibited an adequate temporal signal (r^2=0.389 in TempEst analysis) (Supplementary Fig. S1). BEAST analysis was performed using the SRD06 codon-based evolutionary model [42], an uncorrelated lognormal relaxed clock model and the Bayesian Skyline Plot population growth model [43]. The MCMC was run for 20 million generations, ensuring that the effective sample size values of all parameters were >200, indicating proper mixing. The posterior distribution of trees was summarized in a maximum clade credibility (MCC) tree with TreeAnnotator v1.8.4 after removal of a 10% burn-in. MCC trees were visualized with FigTree v1.4.2 (Rambaut, http://tree.bio.ed.ac.uk/software/figtree/). Parameter uncertainty was summarized in 95% highest posterior density (HPD) intervals.

Results

In an HIV-1 molecular epidemiological study in Spain, we identified a phylogenetic cluster of 18 Pr-RT sequences from samples collected in 5 regions nested within the CRF12_BF clade. In bootscan analyses, sequences from this cluster (henceforth, BF cluster) exhibited 5'-B/F/B/F-3' recombinant structures that were very similar to each other (Supplementary Fig. S2a-f). Their structures also showed some similarity with that of CRF12_BF, from which they differed in a longer subtype B segment in the Pr-RT junction (Supplementary Fig. S2g-i). To determine whether additional sequences in databases clustered with viruses of the BF cluster, all BF recombinant Pr-RT sequences ≥900 nt long deposited in the Los Alamos HIV-1 sequence database [44] and an additional BF recombinant sequence described in [45], not available in the Los Alamos database but deposited in GenBank [46] (accession MF109665), were downloaded and phylogenetically analyzed with FastTree2. We found that 20 additional database sequences grouped in the BF cluster, which was also well supported in an ML tree constructed with IQ-Tree (Fig. 1). Of the 38 viruses belonging to the BF cluster, 21 had been collected in Spain, 5 in Bolivia, 4 in Argentina, 3 in Peru, 2 in the United Kingdom, 2 in Japan, and 1 in Sweden. Epidemiological data from samples collected in Spain, available for all samples processed by us (Table 1) and from one database sequence, indicated that individuals in the BF cluster residing in Spain were predominantly male (but 6 of 18 were female), of South American origin (from Bolivia orPeru,but 4 were native Spanish), and infected via heterosexual contact (n=but 4 of 17 were MSM). Transmission route information was also available from one sample collected in Peru (DEURF13PE006), which was from an MSM.

In the BF cluster, there were three well-supported subclusters. One comprised 14 sequences, all collected in Western Europe, mostly in Spain but also 2 in the UK and 1 in Sweden; for 10 of the viruses collected in Spain, the country of origin of the patient was known, which was Bolivia in 8 and Spain in 2. A second subcluster comprised 5 viruses collected in Bolivia, all in La Paz. A third subcluster comprised 11 sequences from samples collected in Spain (n=6), Peru (n=3) and Japan (n=2), with all samples from Spain being from Peruvian individuals. These sublcusters were designated Euro-Bolivian, Bolivian, and Peruvian, respectively (Fig. 1). Interestingly, 3 viruses (M1131, MS0254, and DEURF13PE006) of 4 grouping in a sub-subcluster within the Peruvian subcluster were from MSM.

We obtained NFLG sequences from 3 viruses of the BF cluster from samples collected in the city of Bilbao from individuals without known epidemiological links, two (P2633 and P3177) from Bolivian individuals and one (P4464) from a Spanish individual. In a

phylogenetic tree, which included 2 other database NFLG sequences of viruses of the BF cluster collected in Bolivia (BOL0137) [17] and Peru (DEURF13PE006), the viruses of the BF cluster grouped in a clade closely related to CRF12_BF and CRF17_BF and more distantly to CRF38_BF and CRF44_BF (Fig. 2). The mosaic structures of the 5 NFLG sequences were analyzed through bootscan analyses, which showed highly similar structures (Fig. 3). Two ~7 kb-long sequences from viruses collected in the UK [45] branching in the BF cluster also showed mosaic structures highly similar to those of the NFLG (Supplementary Fig. S3). Two short segments in *gag* and *env* (signaled with arrows in Fig. 3) were apparently of subtype B, differing from CRF12_BF, which is of subtype F in those segments. To further examine the subtype assignment of those segments, they were analyzed with ML trees constructed with IQ-Tree, which confirmed the grouping of all viruses of the BF cluster with subtype B references, in contrast to CRF12_BF viruses, which grouped with F1 references (Fig. 4). The *env* subtype B segment was also found in CRF17_BF and CRF38_BF viruses (Fig. 4b). A short segment in *nef* (signaled with an arrow in Fig. 3), in which all viruses of the BF cluster, except P4464 and DEURF13PE006, appeared to be of subtype B in bootscan analyses, was also analyzed with an ML tree to determine whether this apparent discrepancy was real. The results showed that all viruses of the BF cluster, as well as the CRF12_BF and CRF17_BF viruses, were of subtype B in this segment (Fig. 4c).

Thus, these analyses show that viruses of the BF cluster have a coincident mosaic structure, which shows some similarity to those of CRF12_BF and CRF17_BF, but differ from both in the presence of a short subtype B segment in *gag,* absent from CRF12_BF and CRF17_BF, and from CRF12_BF in the presence of a short subtype B fragment in *env*, which is absent from CRF12_BF. Additionally, they also differ in breakpoint positions in $p17^{gag}$, RT and *vpu* (Fig. 5), where B-F1 transitions occur between nt positions 921-928, 2678-2765, and 6166-6179 in viruses of the BF cluster vs. 940-961, 2609-2652, and 6193-6235 in CRF12_BF, with transitions in CRF17_BF coinciding with those of CRF12_BF in $p17^{gag}$ and RT and being substantially displaced in the 3' direction in *vpu*.

These results, therefore, showing that NFLG sequences of viruses of a BF recombinant cluster group in a clade separate from other CRFs and exhibit a coincident and distinctive mosaic structure, indicate that they represent a new HIV-1 CRF, which was designated CRF89_BF.

The mosaic structure of CRF89_BF inferred from bootscan analyses and ML phylogenetic trees of partial segments (Fig. 6) indicates that it is predominantly of subtype F, with 13 breakpoints delimiting 7 subtype B and 7 subtype F segments. Its close relationship with CRF12_BF and CRF17_BF and more distant relationship with CRF38_BF and CRF44_BF are supported by phylogenetic clustering (Fig. 2) and coincidences in 5, 6, 3, and 2 breakpoints, respectively (Fig. 6).

We estimated the time and country of the MRCA of CRF89_BF using Pr-RT sequences and a Bayesian coalescent method. Since the CRF89_BF alignment lacked a sufficient temporal signal, we used as a prior parameter a substitution rate estimated from 65 CRF12_BF sequences (, which exhibited an adequate temporal signal (Supplementary Fig. S1). For the BEAST analysis, the country of origin of the individual, when known, was used. This was done because we found no definitive evidence of the epidemic spread of CRF89_BF in Spain (as reflected in clustering among Spanish individuals, which was seen only in two individuals – CU0019 and CU0020 – residing in the same city), and therefore, we assumed that Bolivian and Peruvian immigrants (who represented all foreign-born individuals in the data set) had probably acquired HIV-1 in their countries of origin. For individuals whose country of origin was unknown, country of sample collection was used as a location trait. According to this analysis (Fig. 7), the mean estimated time of the MRCA (tMRCA) of CRF89_BF was 1984 (95% HPD, 1975-1992), and its most probable location was Bolivia (PP=0.839), with the second most probable location being Argentina (PP= 0.106). Estimated times and locations of MRCAs of clusters were, for the Euro-Bolivian cluster 1990 (1983-1997) and Bolivia (PP=0.991); for the Bolivian cluster, 1991 (1985-1996) and Bolivia (PP=0.997); and for the Peruvian cluster, 1992 (1984-1998) and Peru (PP=0.961). Since we could not rule out the possibility that some subclusters of recent origin comprising samples collected in Spain reflected transmissions within the country, we performed an additional BEAST analysis in which the most recently diagnosed infections of two subclusters, comprising samples M1063, M1079 and NA0239, MS0360, respectively, of the Peruvian cluster and 4 infections from the city of Bilbao, P2345, P2346, P3174, and P3177, grouping in a sublcuster in the Euro-Bolivian cluster were assumed to have been acquired in Spain. This analysis also supported an origin of CRF89_BF, although with a lower PP (0.74) and an origin of Euro-Bolivian and Peruvian clusters in Bolivia (PP=0.88) and Peru (PP=0.95), respectively (Supplementary Fig. S4)

Among Pr-RT sequences deposited at the HIV Sequence Database from samples collected in Bolivia in 2005 (all from La Paz) [24], 4 (13.3%) of 30 were of CRF89_BF, which represented 4 (44.4%) of 9 BF recombinant viruses collected that year. By contrast, none of the 21 samples collected in Bolivia in 1996 (from La Paz, Cochabamba, and Santa Cruz) was of CRF89_BF. Among HIV-1-infected Bolivian individuals residing in Spain studied by us sequenced in Pr-RT, 7 (17.9%) of 39 were infected with CRF89_BF viruses, which represented 70% (7 of 10) infections with BF recombinant viruses.

Discussion

The HIV-1 epidemic in Argentina and the neighboring countries of Uruguay, Chile, Paraguay, and Bolivia is characterized by the cocirculation of B subtype and BF1 recombinant viruses [15-25]. Most of the BF1 recombinant forms in these countries appear to derive from a common recombinant ancestor, as inferred from coincident breakpoints and clustering in phylogenetic trees [16,17,22,26-28]. As the subtype F fragments of these recombinants cluster with viruses of the F subtype strain circulating in Brazil and there is no evidence of the circulation of this strain in other South American countries, it has been proposed that the common ancestor of these recombinants might have originated in Brazil, with subsequent recombination events giving rise to a great diversity of recombinant forms [16,28], some of which became circulating, of which CRF12_BF, CRF17_BF, CRF38_BF, and CRF44_BF had been identified previously [16,17,22,26,27]. Due to their common ancestry and similarity in recombination structures, all these viruses have been proposed to constitute a CRF "family" [29,30] (similarly, other CRF families could the CRF_BGs from Cuba, numbers 20, 22, and 23 [47], CRF_BGs from Spain and Portugal, numbers 14 and 73 [48], and CRF_01Bs from Malaysia, numbers 33, 53, 58, and 74 [49,50]). The first to be identified in the CRF_BF family from the Southern Cone of South America was CRF12_BF, which is widely circulating in Argentina and Uruguay [15-17] and in lower proportions in Chile [25], Paraguay [23], and Bolivia [24]. The second was CRF17_BF, representing a small proportion of infections in Argentina, Paraguay, and Bolivia [26]. Two other members of the family, CRF38_BF and CRF44_BF, were identified in Uruguay [22] and Chile [27], respectively. In a molecular epidemiological study in Bolivia, with samples collected in 1996 and 2005, a cluster of 4 BF recombinant viruses branching apart and differing in mosaic structure from CRF12_BF was identified among samples collected in the capital city of La Paz in 2005. The authors proposed that it could represent a new CRF of the CRF12_BF family [24]. Here, we show that this cluster (comprising the 4 viruses collected in 2005 and a fifth virus collected in 1999 [17] (Fig. 1)) forms part of a larger cluster, comprising 38 viruses collected in two other South American countries (Peru and Argentina), three European countries (Spain, United Kingdom, and Sweden) and Japan, with samples collected in Spain representing a majority, although most of them are from Bolivian or Peruvian individuals (Fig. 1). We show through the analysis of 5 NFLG sequences, three of which were newly derived from samples collected in Spain and two from databases from samples collected in Bolivia and Peru that the identified cluster represents a new CRF derived from subtypes B and F1, designated CRF89_BF (Figs. 2 and 3). This CRF is closely related to CRF12_BF and CRF17_BF, as deduced from multiple breakpoint coincidences and close phylogenetic clustering, and more distantly to CRF38_BF and CRF44_BF. CRF89_BF has a complex mosaic structure with 13 breakpoints, delimiting 7 subtype F and 7 subtype B fragments. One of the subtype B segments, in *gag*, is absent from CRF12_BF and related CRFs, and another segment in *env* is absent from CRF12_BF but found in CRF17_BF and CRF38_BF. Breakpoint coincidence with different CRF_BFs from the Southern Cone suggests a complex scenario of BF recombinant generation in this area through successive rounds of recombination with subtype B viruses, as previously proposed [28]. However, it seems unlikely that CRF89_BF derives from CRF12_BF or CRF17_BF, since in the NFLG phylogenetic tree, the CRF89_BF clade is not nested within CRF12_BF or CRF17_BF radiations but forms a separate clade (Fig. 2), and it exhibits several differences in breakpoint locations from both CRFs (Fig. 5).

CRF89_BF comprises three major clusters. One comprises exclusively samples collected in Western Europe (Spain, UK, and Sweden); however, out of 10 individuals with data on country of origin (all residing in Spain), 8 were Bolivian, and only 2 were Spaniards, whose viruses branch interspersed among those from Bolivian individuals. Therefore, it seems reasonable to assume that this cluster originated and spread initially in Bolivia, and its finding in Western Europe reflects the importation of infections acquired in Bolivia rather than local circulation of CRF89_BF. Otherwise, clustering of CRF89_BF strains among native European individuals would be expected but was not seen. Failure to identify viruses collected in Bolivia within the Euro-Bolivian cluster may be due to the low number of HIV-1 sequences from Bolivia available in public databases. A second CRF89_BF cluster comprises all five samples collected in Bolivia, all from La Paz. The third cluster comprises 3 sequences from Peru, 6 from Peruvians residing in Spain, and two from Japan, the last ones closely related to a Peruvian virus. Similar to the case of the Euro-Bolivian cluster, we assume that this cluster represents a variant originating and circulating in Peru, and its presence in Spain and Japan probably reflects the importation of infections acquired

in Peru rather than the local circulation of CRF89_BF. It is interesting to point out that although a small proportion of HIV-1 BF recombinant viruses have been identified in Peru (approximately 2% [18,51]), no evidence has been published of their circulation among the local Peruvian population. Therefore, the results presented here would be the first evidence indicating that an HIV-1 BF1 recombinant form, in this case CRF89_BF, is most likely circulating in Peru. It is also interesting to note that although heterosexual transmission is predominant among CRF89_BF infections, all 3 infections with information on transmission route in a subcluster of 4 individuals within the Peruvian cluster were in MSM. This reflects the circulation of CRF89_BF among Peruvian MSM and the linkage between HIV-1 heterosexual and MSM transmission networks. A similar linkage was observed in a CRF02_AG cluster in Spain, although in this case, the spread was from an MSM to a heterosexual network [52].

According to phylodynamic estimations, CRF89_BF probably emerged in Bolivia around the mid-1980s, with its major clusters emerging around the early 1990s, 2 of them in Bolivia and 1 in Peru (Fig. 7). These estimations were performed assuming that CRF89_BF infections in Bolivian and Peruvian individuals residing in Spain acquired their infections in their country of origin, which seems a reasonable assumption, as discussed above. However, since we could not rule out that subclusters of more recent origin comprising viruses sampled in Spain reflected local transmissions, a second analysis assuming HIV-1 acquisition in Spain of the most recently diagnosed infections of subclusters comprising Bolivian or Peruvian individuals was performed, yielding similar results (Supplementary Fig. S4) . The MRCA of CRF89_BF, according to our estimations, would be approximately 10 years more recent than that of CRF12_BF (Supplementary Fig. S5). However, we cannot rule out an earlier emergence of CRF89_BF, since estimations could change with more representative HIV-1 sampling in Bolivia.

In Bolivia, CRF89_BF was detected in only 5 samples from La Paz, 4 collected in 2005 and 1 in 1997. In 2005, CRF89_BF represented 13.3% HIV-1 samples collected in La Paz sequenced in Pr-RT. However, given the low proportion of Bolivian HIV-1 strains sequenced and the fact that no sequences from samples collected after 2005 are available in public databases, the current prevalence of CRF89_BF in Bolivia and its geographical spread in the country cannot be accurately estimated. Considering that in one of the major CRF89_BF clusters, 8 of 10 viruses, all of which were collected in Europe, were from Bolivian individuals and that 18% of HIV-1-infected Bolivian individuals residing in Spain studied by us harbored CRF89_BF viruses, we hypothesize that CRF89_BF could be widely circulating in some areas of Bolivia.

The identification of CRF89_BF infections in Spain and other European countries, mainly in South American immigrants, reflects the increasing relation between the South American and European HIV-1 epidemics, which is also reflected in the expansion in Western Europe of clusters of South American strains of subtypes C [53-56] and F1 [31,57-59], of CRF12_BF [60] and of CRF17_BF [54], and in the identification in Western Europe of CRFs derived from parental strains of South American ancestry [61-63].

The identification of CRF89_BF and other CRFs in NFLG sequences is relevant for molecular epidemiological studies because it allows for the proper characterization of HIV-1 strains circulating in different geographic areas and population groups. In this regard, some CRF89_BF viruses were misclassified as CRF12_BF viruses in GenBank submissions (accessions MF403410, MF403416), and such misclassification may not be irrelevant, since, even though both CRFs exhibit similar mosaic structures, they are not identical and form separate clades. It should also be pointed out that even relatively minor genetic differences in viral genomes may result in important biological differences. Examples in HIV-1 are CXCR4 coreceptor usage in CRF14_BG, which is associated with only four amino acid residues in the Env V3 loop [64], all or most of which are absent in viruses of the closely related CRF73_BG [48], which has a very similar, but not identical, mosaic structure, and differences in pathogenic potential or therapeutic response associated with clusters within HIV-1 genetic forms [65,66]. The identification of CRF89_BF may also be relevant for the development and testing of vaccines intended for use in areas where this CRF circulates, considering the correlation of susceptibility to protective immune responses with HIV-1 clades and with intraclade genetic diversity [5].

Declarations

Acknowledgments

We thank Daniel Zulaika, from Osakidetza-Servicio Vasco de Salud, for his support of this study and the personnel at the Genomic Unit, Instituto de Salud Carlos III, for technical assistance in sequencing.

Author contributions

MT, ED, and LPA conceived the study and supervised the work. MT, ED, HG, and AFG wrote the main manuscript text. MT, ED, AFG, and IFM performed phylogenetic and phylodynamic analyses. AFG, IFM, SB, VM, EGB, and MS performed experimental work. HG and ED performed data curation. SH, JM, MZS, JdelR, CR, LE, EB, EC, IRA, MLGA, CMS, CGG, JJGI, GC, and AMS recruited patients and obtained epidemiological data. All authors read and approved the manuscript.

Funding

This work was funded through Acción Estratégica en Salud Intramural (AESI), Instituto de Salud Carlos III, projects “Estudios sobre vigilancia epidemiológica molecular del VIH-1 en España”, PI16CIII/00033, and “Epidemiología molecular del VIH en España y su utilidad para investigaciones biológicas y en vacunas”, PI19CIII/00042; Red de Investigación en SIDA (RIS), Instituto de Salud Carlos III, Subdirección General de Evaluación y Fondo Europeo de Desarrollo Regional (FEDER), Plan Nacional I+D+I, project RD16ISCIII/0002/0004; and scientific agreement with Osakidetza-Servicio Vasco de Salud, Government of Basque Country, MVI 1001/16.

Competing interests

The authors declare no competing interests.

Data availability

Sequences are deposited in GenBank under accessions KX818199, KX818200, and MW344906-MW344922.

Ethics declaration

This study was approved by the Research Ethics Committee of Instituto de Salud Carlos III, Madrid, Spain. Informed consent was obtained from all participants. All methods were performed in accordance with the relevant guidelines and regulations.

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Tables

Table 1. Epidemiological data of patients residing in Spain studied by us and GenBank accessions of sequences.

Sample ID	City of sample collection	Region of sample collection*	Year of sample collection	Year of HIV diagnosis	Gender	Transmission route†	Country of origin	GenBank accession (Pr-RT)	GenBank accession (NFLG)
CU0019	Cuenca	Castilla-La Mancha	2016	2015	F	Heterosexual	Spain	MW344905	
CU0020	Cuenca	Castilla-La Mancha	2016	2009	M	Heterosexual	Spain	MW344906	
M0849	Madrid	Madrid	2016	2016	M	Heterosexual	Bolivia	MW344907	
M1063	Madrid	Madrid	2017	2017	F	Heterosexual	Peru	MW344908	
M1079	Madrid	Madrid	2017	2017	M	Heterosexual	Peru	MW344909	
M1131	Madrid	Madrid	2017	2017	M	MSM	Peru	MW344910	
MS0254	Madrid	Madrid	2018	2018	M	MSM	Peru	MW344911	
MS0360	Madrid	Madrid	2019	2019	M	MSM	Peru	MW344912	
NA0239	Pamplona	Navarra	2016	2016	M	Heterosexual	Peru	MW344913	
NA0379	Pamplona	Navarra	2018	2018	F	Heterosexual	Bolivia	MW344914	
P2345	Bilbao	Basque C.	2009	2009	F	Heterosexual	Bolivia	MW344915	
P2346	Bilbao	Basque C.	2009	2009	M	Sexual	Spain	MW344916	
P2633	Bilbao	Basque C.	2010	2010	F	Heterosexual	Bolivia	MW344917	KX818199
P3174	Bilbao	Basque C.	2012	2012	M	Heterosexual	Spain	MW344918	KX818200
P3177	Bilbao	Basque C.	2012	2012	F	Heterosexual	Bolivia	MW344919	
P4464	Bilbao	Basque C.	2016	2015	M	MSM	Spain		MW344920
P5090	Vitoria	Basque C.	2018	2018	M	Sexual	Bolivia	MW344921	
Z0275	Zaragoza	Aragon	2018	2018	M	n.a.	Bolivia	MW344922	

*Basque C.: Basque Country. †n.a. : not available.

Figures

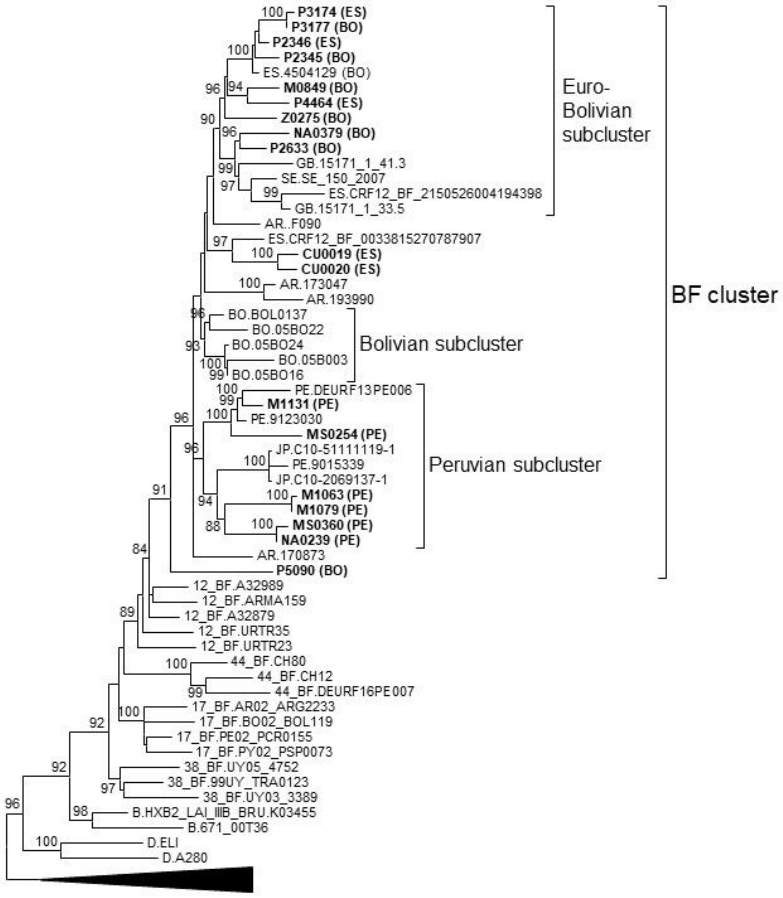

Figure 1

Maximum likelihood tree of Pr-RT sequences of BF cluster. Names of sequences obtained by us, all collected in Spain, are in bold type. Two-letter ISO code of country of origin of the individual, when known, is in parentheses after the virus name. In database sequences branching in the BF cluster, the country of sample collection is indicated before the virus name with the two-letter ISO country code. In reference sequences, subtype or CRF is indicated before the virus name. Branches corresponding to references of subtypes A, C, F, G, and H are compressed. Only bootstrap values ≥80% are shown.

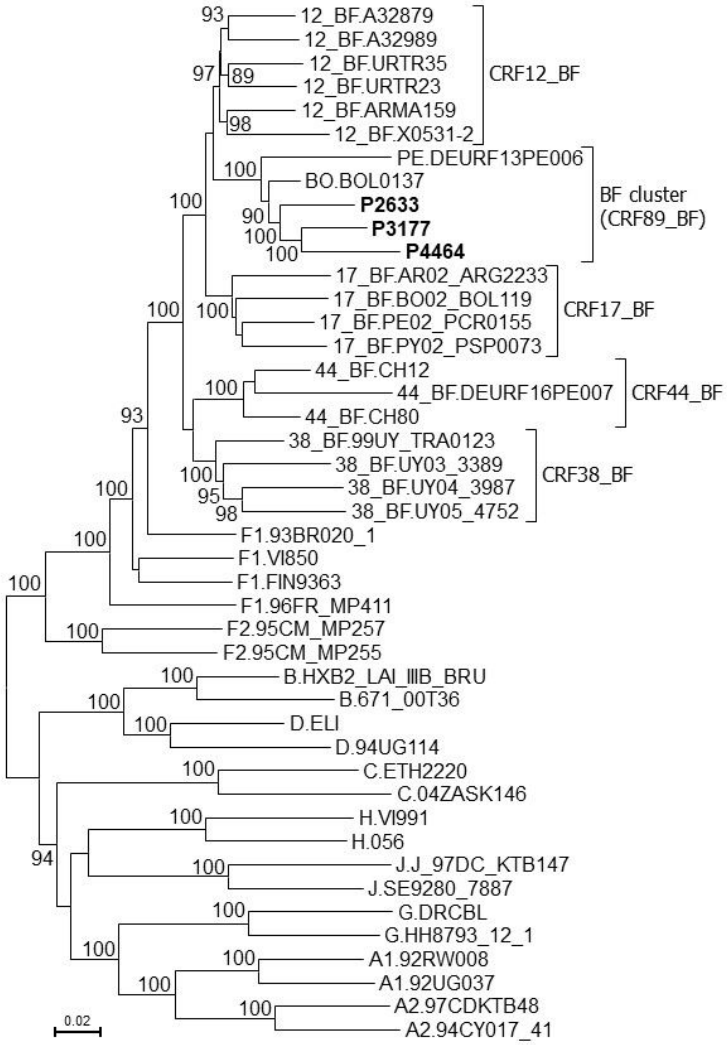

Figure 2

Maximum likelihood tree of NFLG sequences of the BF cluster and of references of CRF_BFs from the Southern Cone of South America and of subtypes. Names of sequences obtained by us are in bold type. In database sequences branching in the BF cluster, the country of sample collection is indicated before the virus name with the two-letter ISO country code. In reference sequences, the subtype or CRF is indicated before the virus name. Only bootstrap values ≥80% are shown.

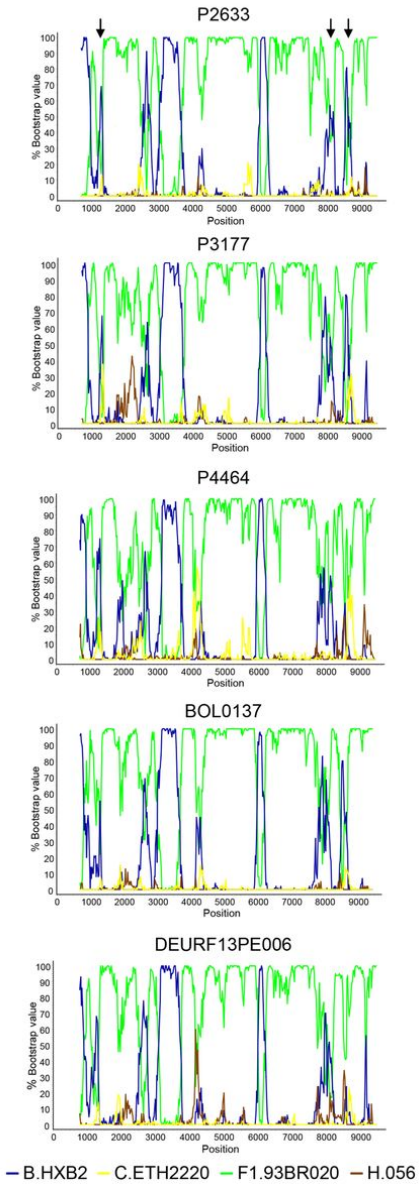

Figure 3

Bootscan analyses of 5 NFLG genomes of the BF cluster. The horizontal axis represents the position in the HXB2 genome of the midpoint of a 250 nt window moving in 20 nt increments, and the vertical axis represents bootstrap values supporting clustering with subtype reference sequences. Vertical arrows above P2633's bootscan plot indicate two short segments apparently of subtype B, whose subtype assignation was further analyzed through ML trees, as shown in Fig. 4.

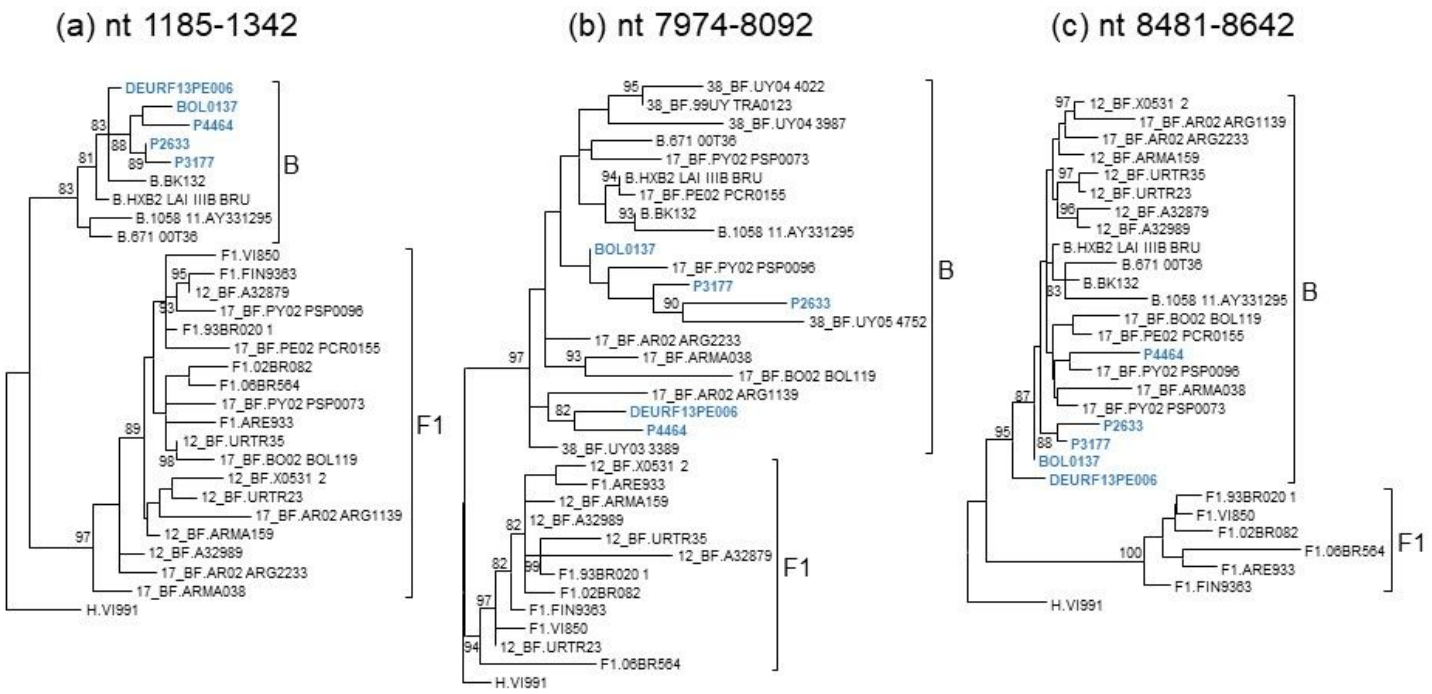

Figure 4

Maximum likelihood trees of genome segments of the BF cluster. Short segments for which bootscan analyses supported clustering with subtype B, signaled with arrows in Fig. 3, are analysed. HXB2 positions delimiting the analyzed segments are indicated on top of the trees. Sequence names of viruses of the BF cluster are in blue. Names of subtype and CRF references are preceded by the corresponding subtype or CRF. Only bootstrap values ≥80% are shown.

(a) p17^{gag}

Subtype/CRF	HXB2 position									
	858	882	889	921	928	940	961	964	970	972
B	A	A	A	C	A	T	A	C	C	G
F1	G	G	C	T	G	C	C	A	A	A
CRF12_BF	A	A	G	C	A	T	C	A	A	A
CRF17_BF	A	A	G	C	A	T	C	A	A	A
CRF89_BF	A	A	G	C	G	C	C	A	A	A

(b) RT

Subtype/CRF	HXB2 position											
	2537	2552	2585	2609	2652	2653	2660	2678	2765	2807	2816	2840
B	C	C	A	A	G	T	T	G	A	C	A	C
F1	T	A	G	G	A	C	A	A	G	T	G	T
CRF12_BF	C	C	A	A	A	C	A	A	G	T	G	T
CRF17_BF	C	C	A	A	A	C	A	A	G	T	G	T
CRF89_BF	C	C	A	A	G	T	C	G	G	T	G	T

(c) vpu

Subtype/CRF	HXB2 position												
	6153	6158	6160	6166	6179	6181	6188	6189	6193	6235	6238	6424	6425
B	G	A	A	A	G	C	A	T	T	A	A	A	T
F1	A	C	G	G	A	T	T	A	A	G	G	G	G
CRF12_BF	G	A	A	A	G	C	A	T	T	G	G	G	G
CRF89_BF	G	A	A	A	A	T	T	A	A	G	G	G	G

Figure 5

Differences in B/F1 breakpoint locations in CRF89_BF vs. CRF12_BF and CRF17_BF in P17gag, RT, and vpu. Identities of CRF_BF consensuses with B and F1 subtype consensuses at HXB2 positions where both subtype consensuses differ are shown. The shaded areas indicate positions where identities of CRF89_BF with B/F1 consensuses differ from those of CRF12_BF and CRF17_BF.

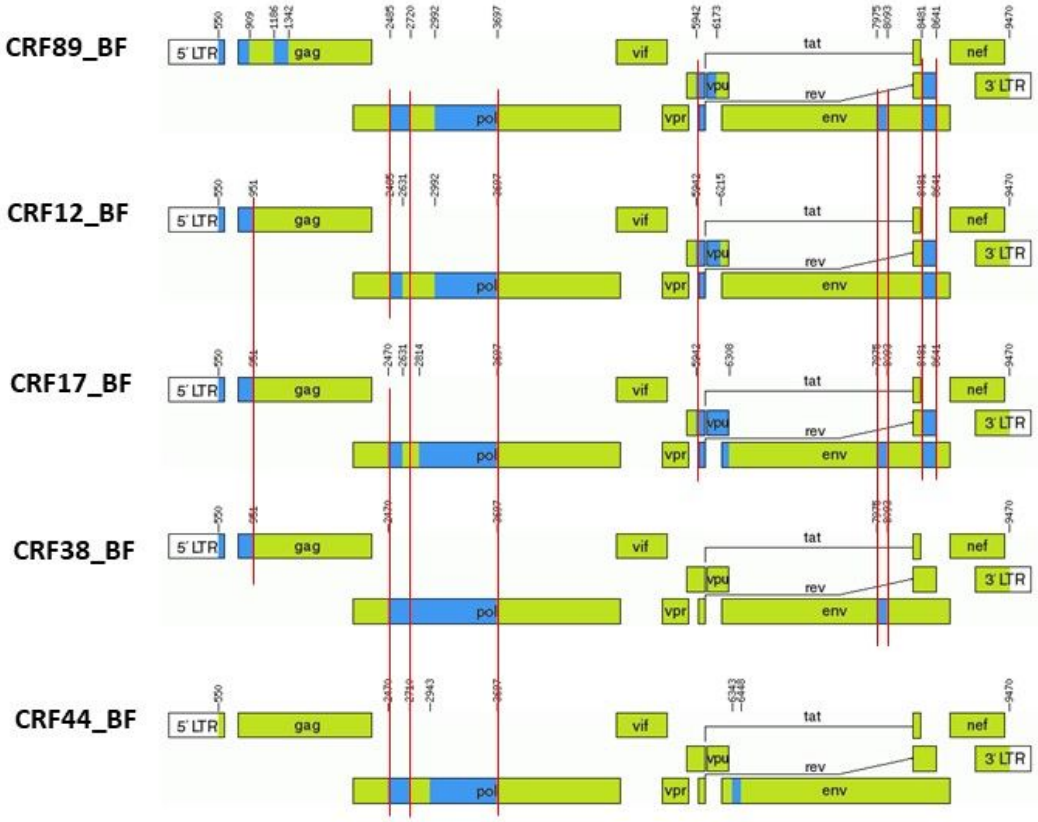

Figure 6

Mosaic structure of CRF89_BF compared to those of CRF12_BF, CRF17_BF, CRF38_BF, and CRF44_BF. Vertical red lines indicate coincident breakpoints. Positions correspond to the HXB2 genome.

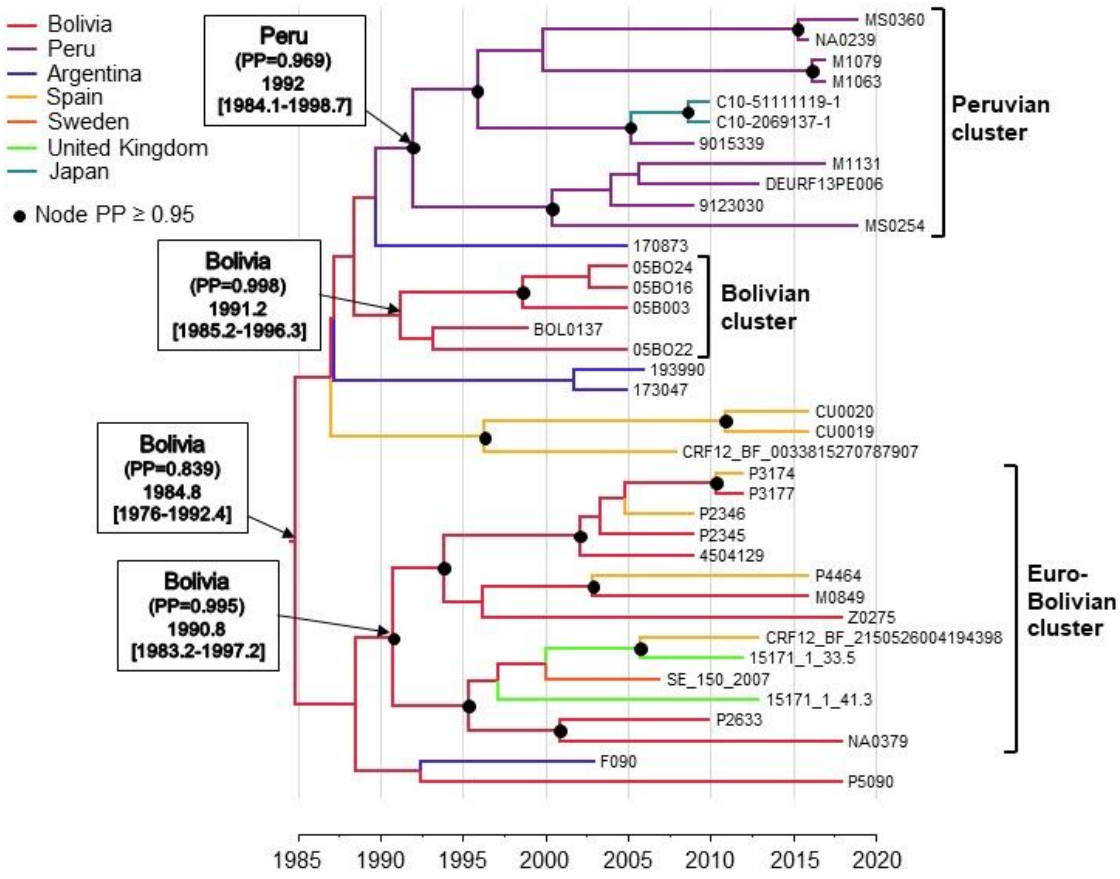

Figure 7

Maximum clade credibility tree of CRF89_BF Pr-RT sequences. Branch colors indicate, for terminal branches, country of sample collection or, if known, of origin of the individual, and for internal branches, the most probable location country of the subtending node, according to the legend on the left. Nodes supported by PP≥0.95 are indicated with filled circles. The most probable countries at the root of the tree and of the three major clusters are indicated, together with PP supporting the locations and tMRCA (mean values, with 95% HPD intervals in parentheses).

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