Contribution of transgender sex workers to the complexity of the HIV-1 epidemic in the metropolitan area of Milan

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Contribution of transgender sex workers to the complexity of the HIV-1 epidemic in the metropolitan area of Milan

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Key messages
- South American transgenders largely contribute to the heterogeneity of HIV-1 variants in Italy representing a source of viral diversity and intermixing
- In the transmission chains TSWs are frequently associated to HEs and MSM
- There is a need to reinforce prevention strategies addressing TSWs as well as their sex partners
Abstract

Objectives Transgender people are disproportionately affected by the HIV-1 epidemic. We evaluated the origin of HIV-1 variants carried by South American transgenders living in Milan through by combining accurate phylogenetic methods and epidemiological data.

Methods We collected 156 HIV-1 pol sequences obtained from transgender patients engaged in sex work (TSWs) followed between 1999 and 2015 at L. Sacco Hospital, Milan, Italy. Phylogenetic analyses were conducted by HIV-TRACE, MrBayes, MacClade and Beast programs. Reference sequences were retrieved from Los Alamos and local databases. Last negative testing or proxy data from clinical records of infected individuals were used to investigate the country of infection.

Results Among South American TSWs the most represented HIV-1 subtypes were B (70.5%), F1 (12.8%) and C (4.4%). Gene flow migrations of B subtype indicated significant fluxes from TSWs to Italians (21.3%) belonging to all risk groups (26.4% to HEs, 18.9% to MSM, 15.1% to IDUs). The largest proportion of bidirectional fluxes were observed between Italians and TSWs (24.6%). For F1 subtype bidirectional viral fluxes involved TSWs and Italians (7.1% and 14.3%) and a similar proportion of fluxes linked TSWs and Italian HEs or MSM (both 15.8%). Significant fluxes were detected from Italians to TSWs for subtype C involving both MSM (30%) and HEs (40%). Country of HIV-1 acquisition was identified for 72 subjects; overall, the largest proportion of patients with B subtype (73.5%) acquired HIV-1 infection in South America.

Conclusions Our results indicated that South American transgenders largely contribute to the heterogeneity of HIV-1 variants in our country. The high number of clusters based on all subtypes indicated numerous transmission chains in which TSWs were constantly intermixed with HEs and MSM. Our results strongly advocate interventions to facilitate prevention, diagnosis and HIV-1 care continuum among transgender people.
Introduction

HIV-1 infection remains a major public health concern with approximately 30,000 new infections reported each year for European countries in 2015. According to the European Centre for Disease Prevention and Control (ECDC) 32% of new diagnoses are detected in heterosexuals (HEs) (https://ecdc.europa.eu/en/publications-data/hiv-and-aids-annual-epidemiological-report-2016). Among the sexually active population, heterosexual males often acquire HIV-1 infection from female or male sex workers for whom the estimates of HIV-1 prevalence is higher compared to the general population.[1] Most of the available epidemiological studies regarding prostitution and HIV-1 infection are focused on female prostitution[2] even though commercialization of sex is common among men who have sex with men (MSM) and the male to male sexual transmission of HIV-1 was demonstrated to be more efficient.[3]

Migratory movements have been invoked for the spread of HIV-1 on a global scale, thus representing a vector of the virus.[4] Compared with native populations, migrants are at increased risk of many diseases attributable to individual behaviors, structural barriers and social inequalities that increase their vulnerabilities and limit the access to HIV-1 prevention, testing and care.[5]

Recent studies showed that the transgender population engaged in sex work (TSWs) has a disproportionate risk for HIV-1 acquisition, compared with female sex workers.[6][7] Worldwide, around 27.2% of transgenders are affected by HIV-1[7][8] and recent epidemiological research showed that they face unique structural, interpersonal, and individual vulnerabilities contributing to risk of HIV-1 acquisition (http://www.unaids.org/en/resources/campaigns/2014/2014gapreport/gapreport). These individuals, who in Italy are mostly from Latin America, often do not practice safe sex and seek medical care after the development of AIDS-related events, making difficult to establish the date and the place of their infection.

Although the B subtype is dominant in South America, F1 and C clades co-circulates at high levels, generating a growing number of Circulating Recombinant Forms (CRFs) and Unique Recombinant Forms (URFs), making the epidemic of this continent highly complex.[9][10] In Italy, among non-B subtypes, F1, A1 and C clades are prevalent, affecting 77.2% of HEs.[11] Nevertheless, MSM transmission networks have been reported recently as driver of the expansion of non-B Italian regional sub-epidemics.[12][13]

Despite recent data studied how transgender woman acquired HIV infection using phylogenetic approach to reconstruct transmission clusters,[14][15] to date no study has been undertaken to establish whether South American TSWs have been infected in their country of origin or acquired HIV-1 infection where they practice commercial sex. In this context, molecular data may trace the country of infection and make inferences on the possible source of individual strains when epidemiological data are lacking or incomplete. Because of the limited recruitment of TSWs in surveillance systems and the scarce data on HIV-1 genomic characterization among this population, this work aimed to study the contribution of
variants carried by TSWs to viral heterogeneity of the Italian HIV-1 epidemic and to investigate the origin of HIV-1 variants harbored by TSWs from South American countries.
Methods

Participants

We studied 156 HIV-1 pol sequences generated for routine drug resistance testing at diagnosis or at treatment failure obtained from TSWs residing in Milan during the 1999-2015 period. These patients referred themselves as transsexual women, transsexual men, crossdressing non-binary, or intersex people, involved in commercial sex. The clinical records were used to obtain demographics (country of birth, gender, age, date and country of 1st positive and last negative HIV-1 antibody testing and date of the arrival in Italy), clinical and laboratory data (clinical stage at presentation, CD4+ T-lymphocyte count and HIV-1 viral load).

Potential country of HIV-1 acquisition was inferred based on data obtained from clinical records. Stage of disease and CD4+T-cell count at HIV-1 diagnosis, together with reported dates of previous negative HIV-1 tests, were also used as proxy data for estimating the likely country of infection.

All participants gave informed consent for this study and to have their anonymized data stored on a local server. The study was conducted in accordance with the 1964 Declaration of Helsinki and the ethical standards of the Italian Ministry of Health.

Sequences subtyping

Sequences were trimmed to equivalent length (840 bp) and aligned with representative sequences available in the Los Alamos database (http://hiv-web.lanl.gov) using the CLUSTAL algorithm implemented in BioEdit v.7.2.6.1 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Subtype or CRF assignment was performed using MEGA7.[16]

Phylogenetic trees were generated with Maximum Likelihood method with bootstrapping on 1,000 replicates, Kimura 2-parameter method and a transition/trasversion ratio of 2.0. For sequences with unassigned subtype, similarity and bootscanning plots implemented in the SimPlot v.3.5.2(http://sray.med.som.jhmi.edu/SCRoftware/simplot/) were used to reconstruct recombination pattern.

Phylogenetic dataset

Separate datasets were constructed and studied for B, F1 and C subtypes (Supplementary Table 1).

Only sequences from South American TSWs were analyzed. References from Africa and South America were downloaded from Los Alamos database and additional Italian strains were obtained from patients residing in the metropolitan area of Milan, equally distributed among risk groups.

Phylodynamic and phylogeographic analysis

The Bayesian phylogenetic tree was reconstructed by means of both MrBayes[17] and Beast[18] using the general time reversible(GTR)+gamma distribution(G)+proportion of invariable sites (I) model. Different coalescent priors and different clock models were compared by calculating the Bayes Factor (BF). The BF analysis showed that the relaxed
lognormal molecular clock fitted the data better than the strict clock model (2lnBF=127.8). Bayesian Skyline coalescent
tree prior with a relaxed clock (uncorrelated Lognormal model) were selected for analyses. Uncertainty in the estimates
was indicated by 95% highest probability density (HPD) intervals. Only parameter estimates with Effective Sample
Size (ESS)>300 were accepted. The maximum clade credibility (MCC) tree was then selected from the posterior tree
distribution using TreeAnnotator v.1.8.4 and visualized with FigTree v.1.4.2(http://tree.bio.ed.ac.uk/software/figtree/).

Viral Gene Flow Analysis
The MacClade v.4 program was used on the global dataset and the subsets to test viral gene inflow/outflow among
different groups using a modified version of the Slatkin and Maddison test.[19]

Transmission clusters reconstruction
Molecular transmission networks were also evaluated in a largest dataset of genetic sequences using HIV-TRACE
(Transmission Cluster Engine).[20] The genetic distance threshold of 0.015 substitutions/site was selected as reported in
different studies for the investigation of epidemiological relatedness.[20]

More details are described in Supplementary methods.
Results

Participants

The majority of TSWs were from South America (96.8%), three individuals from Italy and two from Central America (El Salvador). Among South American TSWs (n=151), 97 subjects (64.2%) were Brazilian; 35 (23.2%), 8 (5.3%), 5 (3.3%), 4 (2.7%) individuals were from Peru, Paraguay, Argentina, Venezuela, and 1 (1.3%) from Bolivia and Chile, respectively. The median age was 46 years (range:29-65) The most frequent subtypes were B, F1 and C in 110 (70.5%), 20 (12.8%) and 7 (4.4%) patients, respectively. Among these groups no differences were observed in median age, median viral load values and median CD4 cell count. Other variants included 9 (5.8%) known CRFs (4 CRF12_BF, 3 CRF02_AG, 1 CRF01_AE, 1 CRF29_BF) and 9 URFs (8 URF BF, 1 URF BC).

Regarding the treatment status, 48.7% (n=76) of patients were naive for antiretroviral treatment at time of sequencing. The prevalence of any resistance among these subjects was 14.6% (n=11, 10 patients with B subtype); mutations conferring resistance to NRTI, NNRTI and PI, represented 81.8%, 36.4%, and 9%, respectively.

Phylogeographical analysis among countries and risk groups

B Subtype

In the B subtype dataset we identified 23 clusters, each including at least one TSW. Despite 53.2% of TSWs were included in clusters, a largest proportion were intermixed in the tree. Half of clusters (48%) involved only TSWs and Italians while four clusters (17.4%) encompassed only TSWs and South Americans (Supplementary Figure 1). No differences were observed among patient characteristics such as age, CD4 cell count and viral load in and out of clusters. Moreover, no differences were observed in the distribution of naïve and treated subjects or resistance.

Phylogeographic analysis showed a linkage between TSWs and Italians as well as between TSWs and South Americans (BF=1025.4)(Figure 1,part A).

The gene flow migrations calculated with MacClade program indicated bidirectional viral fluxes involving TSWs and Italians and TSWs and South Americans: 21.3% from TSWs to Italians, 24.6% from Italians to TSWs, 23% from TSWs to South Americans and 9.8% from South Americans to TSWs (Figure 1,part B).

The phylogenetic tree considering risk groups showed 26 clusters including two (n=19) to five sequences (n=1). Forty-three TSWs (45.7%) were included in clusters. Eight clusters included only TSWs and HEs (30.8%), six involved only TSWs and MSM (23%) and two included TSWs, HEs and MSM, or IDUs (7.7%)(Supplementary Figure 2).

Bayesian phylogeography showed a total of five rates with linkages between TSWs and all risk groups, HEs (BF=5621), MSM (BF=1873.3) and IDUs (BF=24.3) (Figure 1,part C). Fluxes involved all risk groups (26.4% from TSWs to HEs, 18.9% from TSWs to MSM and 15.1% from TSWs to IDUs)(Figure 1,part D).

F1 Subtype
The F1 tree showed 5 clusters, each including one TSW (27.8%). Two clusters, including six and five sequences, involved only Italians and TSWs; in these cases TSWs were external to clusters representing an outgroup. The largest cluster encompassed 11 strains from Italy and South America and one TSW, while the smallest clusters included only one South American or Italian and TSW. Thirteen TSWs are intermixed in the tree (Supplementary Figure 3). No differences were observed among patient in and out of clusters regarding age, CD4 cell count and viral load. Similarly, no differences were observed in the distribution of naïve and treated subjects or patients carrying resistance.

Rates indicated that TSWs were linked with both South Americans and Italians (BF=205 and 1025.5, respectively)(Figure 2,part A). Bidirectional viral fluxes were observed from TSWs to Italians (7.1%) and from Italians to TSWs (14.3%). The largest gene flow was observed from South Americans to TSWs (25%)(Figure 2,part B).

The phylogenetic tree of risk groups subset presented 5 clusters; one involved only MSM with one TSW while the all the others were mixed; in particular the largest cluster of 11 stains encompassed all risk groups and three TSWs. In three out of five clusters, TSWs were external to clusters as an outgroup (Supplementary Figure 4). F1 migration analyses identified three rates, one between TSWs and HEs (BF=5621)(Figure 2,part C). Gene flows involved all risk groups (15.8% from TSWs to HEs, 15.8% from TSWs to MSM and 5.3% from TSWs to IDUs)(Figure 2,part D).

**C Subtype**

In the phylogenetic tree of C subtype, all TSWs (n=6) were included in a large cluster of 47 sequences together with all South American references and six Italians. TSWs formed a significant subclade with Italians (Supplementary Figure 5).

Only one non-zero rate was present involving TSWs and Italians (BF=5621)(Figure 3,part A).

The gene flow indicated bidirectional fluxes from TSWs to Italians (5.9%) and from Italians to TSWs (23.5%); another flux was reported from South Americans to TSWs (5.9%)(Figure 3,part B).

The phylogenetic tree considering the risk groups highlighted 5 clusters, each containing one TSW. One TSW was intermixed in the tree. Two clusters contained only MSM and TSWs and only one HE and TSWs (Supplementary Figure 6). Migration analyses identified two rates all involving TSWs (TSWs and MSM, BF=1025; TSWs and HEs, BF=9.5)(Figure 3,part C). Bidirectional fluxes involved TSWs and MSM (both 20%) while the largest flux was from HEs to TSWs (40%)(Figure 3,part D).

**Country of HIV-1 infection acquisition based on epidemiological data**

Based on patient records, the country of HIV-1 acquisition was identified for 72 (46.1%) subjects (n=51 for South America, n=21 for Italy). The majority of TSWs carrying B subtype (36/49, 73.5%) acquired HIV-1 infection in South America. Six out of nine subjects (66.7%) with F1 clade resulted infected in Italy. For all subjects with C subtype with known data (n=4), South America resulted the country of HIV-1 acquisition.
Phylodynamic analysis

The phylodynamic analysis on TSWs carrying B subtype (Supplementary Figure 7) showed 6 transmission clusters each involving a limited number of strains (n=3-5, total n=24, 22.6%).

The root of the tree dated 60.3 years before 2015 (95%HPD 36–241) and clusters showed a mean TMRCA (Time of the Most Recent Common Ancestor) of 46 years (95%HPD 22–193).

The mean substitution rate for this dataset was 0.84x10^{-3} (95%HPD: 1.1x10^{-4}–1.56x10^{-3}).

Networks reconstruction

In the first dataset, 68 strains clustered in 24 clusters composed of 2-12 sequences (mean 2.8)(Figure 4,part A). Half of clusters involved TSWs (n=13, 10.5%) mainly associated with MSM (57.1%) and HEs (32.2%); only one cluster contained more than one TSW. MSM showed a highest probability to be included in clusters compared to other risk categories (34.6% for MSM vs. 19.8% for HEs, 9.8% IDUs and 10.5% TSWs; p<.001).

In the second dataset we identified 83 clusters including 2-37 sequences (mean 2.9)(Figure 4,part B). Twenty TSWs (15.9%) were involved in 19 clusters (22.9%); 12 clusters (63.1%) included only TSWs and Italians, three (15.8%) only TSWs and South Americans, three (15.8%) TSWs, South Americans and Italians; one cluster is composed by two TSWs.

Italians showed a highest probability (39.9%, p<.001) to be included in clusters compared to South Americans (9.2%), TSWs (15.9%) and Africans (24.5%).
Discussion

According to the ECDC report, 37% of all newly diagnosed cases of HIV-1 in the EU/EEA were in people born outside of the reporting country (https://ecdc.europa.eu/en/publications-data/thematic-report-hiv-and-migrants). These subjects most often received a late HIV-1 diagnosis in European Countries and in such cases, HIV-1 infection is mostly assumed to have occurred in their country of origin. Recently, growing evidence supports that migrants acquire HIV-1 infection postmigration. Determining when HIV-1 acquisition occurs (premigration or postmigration) is critical to design adequate HIV-1 prevention programs, testing strategies and treatment services. Alvarez-del Arco et al.[5] reported that 63% of migrants acquired HIV infection after migrating into Europe and the proportion of postmigration infection varies according to patient origin and mode of transmission with highest percentage of HIV-1 diagnosis for subjects from Latin America and Caribbean (71%), in particular in MSM (79%). Similarly, Paraskevis et al. found high percentage of postmigration in Greece frequently related to contacts among migrants.[21] Furthermore, Pantazis et al. indicated postmigration infection in half of studied subjects (55.3%).[22] However, the results of these studies were difficult to compare because of distinct characteristics of migrant population.

In this context, our evaluation focused on a peculiar population of South American transgender that have been very poorly investigated and underrepresented in HIV-1 epidemiological studies. Despite the growing literature on HIV-1 among transgender people since 2012, they are still frequently associated with MSM and large gaps in knowledge are still present. Most of studies included less than 100 participants[23][24] and were often limited to Latin America countries such as Peru, Brazil and Argentina, or Asia.

The high probability of HIV-1 acquisition in transgender people is related to the legal and economic marginalization that limit their employment options to sex work.[7] The findings from Logie at al. suggested that transgender women who are sex workers have a HIV-1 infection rates nine-fold higher compared to transgender women not involved in sex work.[25]

Recent estimates reported the presence of more than 40,000 transgender in Italy, the majority of these subjects being South Americans (about 60%) (http://www.ansa.it/web/notizie/rubriche/cronaca/2010/06/05/visualizza_new.html_1819711343.html).

Estimates related to the main Italian metropolitan areas indicated the presence of a fluctuating but consistent population of about 3,000 and 2,000 TSWs in Rome and Milan, respectively (http://www.liberazionesperanza.it/wp-content/uploads/2018/05/2-MAPPATURA-NAZIONALE-DELLA-PROSTITUZIONE-DI-STRADA-27-ottobre.pdf).

A recent study [14] evaluated transmission networks in transgender woman, however the major limitation of this work is that no data could infer about directionality of the transmission with the consequent impossibility to distinguish
between transmitters and recipients. With the purpose to clarify this point we adopted advanced phylogenetic methods that permit us to identify viral fluxes and the most probable country of HIV acquisition.

Sequence analysis of HIV-1 positive transgender women of South American origin, who practice sex work in the metropolitan area of Milan revealed adverse landscape of HIV-1 variants, with non-B subtypes carried by around 30% of patients.

Bidirectional fluxes among TSWs, Italians, and South Americans and a high number of clusters indicated numerous transmission chains. Postmigration acquisition, inferred on half of the patients based on proxy data, was higher in patients with subtype B and C compared with those with subtype F1. Indeed, half of subtype B clusters included only TSWs and Italians and C sequences formed a subclade with Italian strains. Differently, where clusters of F1 subtype involved only TSWs and Italians, TSWs were located external to clusters as an outgroup, suggesting that they represent the possible source of that transmission chain.

The comparison between epidemiological and molecular data gave partially overlapping results indicating that around 40% and 50% of patients carrying B subtype acquired HIV-1 infection in Italy, respectively. However, the country of HIV-1 acquisition derived from clinical data was available for around half of analyzed patients making these results partially representative. Differently from the observation by Ragonnet-Cronin et al.[14] where transgender women frequently shared the same cluster and consequently presented a direct linkage or shared partners, we did not have evidence of contacts among TSWs as indicated by a limited proportion of clusters containing more than one TSW indicating a largest number of clients/partners.

By a phylodynamic approach, we observed that the time of penetration of HIV-1 B variant carried by TSWs took place as early as that established for B subtype in Italy.[26]

Despite a consistent proportion of postmigration HIV-1 acquisition in the study population, our study indicated that TSWs largely contribute to the spread of South American HIV-1 variants in Italy representing a source of viral diversity and intermixing among HIV-1 variants of different origins.

Our results regarding C and F1 variants are in agreement with previous Italian data indicating a high relation between the South American strains and those circulating in Italians and further showed that B subtype was imported from Latin American countries.[27][28] Uncertainty cannot be completely avoided, as these individuals frequently travel abroad and we cannot rule out transmission events in other countries during their permanence in Italy.

Our results suggested that fluxes from TSWs mainly involved Italian MSM and HEs confirming the need of considering TSWs as a group distinct from MSM or cisgender female sex workers to better understand the transmission dynamics among these groups. In this context, TSWs could have a bridging role in the spread of HIV-1 among MSM and HEs.
The major limitation of the present study is the relative lack of epidemiological information, which has weakened the correlation with phylogenetic results. Additionally, our dataset includes only patients from a metropolitan area of Milan that could not fully represent all TSWs living with HIV-1 in Italy.

In conclusion, this study represents the first effort to use molecular methods to investigate the role of TSWs in the HIV-1 epidemic, confirming that computational approaches like phylogeographic analysis can be used not only to trace the most probable country of infection but also to infer additional features of the Italian HIV-1 epidemic. Our findings can be used to reinforce prevention strategies addressing TSWs as well as their sex partners, including targeted testing for HIV-1 and other sexually transmitted infections and early initiation of antiretroviral treatment.
Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

AL, AB and FRS conceived and designed the study. GB, MF, VM, CA, AR collected and analyzed the epidemiological and viral data of patients. GZ and MC participated to phylogenetic analyses. AL, AB, FRS, MG and CB wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Figures Legend

Figure 1. B subtype dataset: Panels A and B show significant non-zero migration rates of supported by a BF>3 as indicated by colour lines. The migrations were calculated using SPREAD3 program.

Panels C and D report the bubblegrams indicating the frequency of the gene flows (migrations) to/from different locations and risk categories; the area of each circle is proportional to the percentage of observed migrations in the ML genealogy. The migrations were inferred using a modified version of the Slatkin and Maddison algorithm. IT: Italy, SA: South America, TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

Figure 2. F1 subtype dataset: Panels A and B show significant non-zero migration rates of supported by a BF>3 as indicated by colour lines. The migrations were calculated using SPREAD3 program. Panels C and D report the bubblegrams indicating the frequency of the gene flows (migrations) to/from different locations and risk categories; the area of each circle is proportional to the percentage of observed migrations in the ML genealogy. The migrations were inferred using a modified version of the Slatkin and Maddison algorithm. IT: Italy, SA: South America, TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

Figure 3. C subtype dataset: Panels A and B show significant non-zero migration rates of supported by a BF>3 as indicated by colour lines. The migrations were calculated using SPREAD3 program. Panels C and D report the bubblegrams indicating the frequency of the gene flows (migrations) to/from different locations and risk categories; the area of each circle is proportional to the percentage of observed migrations in the ML genealogy. The migrations were inferred using a modified version of the Slatkin and Maddison algorithm. IT: Italy, SA: South America, AF: Africa, TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

Figure 4. Molecular transmission clusters identified using HIV-TRACE. Part A: colour indicates risk categories, Part B: colour indicates locations. Edges represent genetic distance of 0.015 substitutions per site. IT: Italy, SA: South America, AF: Africa, TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.
Supplementary information

Supplementary

Supplementary Figure 1. Bayesian phylogeographical tree of HIV-1 B pol sequences with branches coloured on the basis of the most probable location of the descendent nodes. The correspondences between the locations and colours are shown in the panel (left). IT: Italy, SA: South America, TSWs: transgender sex workers. Dots indicate significant posterior probabilities (pp>0.8).

Supplementary Figure 2. Bayesian phylogeographical tree of HIV-1 B pol sequences with branches coloured on the basis of the most probable risk category of the descendent nodes. The correspondences between the risk categories and colours are shown in the panel (left). Dots indicate significant posterior probabilities (pp>0.8). TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

Supplementary Figure 3. Bayesian phylogeographical tree of HIV-1 F1 pol sequences with branches coloured on the basis of the most probable location of the descendent nodes. The correspondences between the locations and colours are shown in the panel (left). Dots indicate significant posterior probabilities (pp>0.8). IT: Italy, SA: South America, TSWs: transgender sex workers.

Supplementary Figure 4. Bayesian phylogeographical tree of HIV-1 F1 pol sequences with branches coloured on the basis of the most probable risk category of the descendent nodes. The correspondences between the risk categories and colours are shown in the panel (left). Dots indicate significant posterior probabilities (pp>0.8). TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

Supplementary Figure 5. Bayesian phylogeographical tree of HIV-1 C pol sequences with branches coloured on the basis of the most probable location of the descendent nodes. The correspondences between the locations and colours are shown in the panel (left). Dots indicate significant posterior probabilities (pp>0.8). IT: Italy, SA: South America, AF: Africa, TSWs: transgender sex workers.

Supplementary Figure 6. Bayesian phylogeographical tree of HIV-1 C pol sequences with branches coloured on the basis of the most probable risk category of the descendent nodes. The correspondences between the risk categories and colours are shown in the panel (left). Dots indicate significant posterior probabilities (pp>0.8). TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

Supplementary Figure 7. Dated tree of TSWs harboring B subtype showing clustering sequences (in gray). Asterisks indicate significant posterior probabilities (>0.8). Time-line scale is displayed underneath the tree.
References


Figure 1. B subtype dataset: Panels A and B show significant non-zero migration rates of supported by a BF>3 as indicated by colour lines. The migrations were calculated using SPREAD3 program. Panels C and D report the bubblegrams indicating the frequency of the gene flows (migrations) to/from different locations and risk categories; the area of each circle is proportional to the percentage of observed migrations in the ML genealogy. The migrations were inferred using a modified version of the Slatkin and Maddison algorithm. IT: Italy, SA: South America, TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.
Figure 2. F1 subtype dataset: Panels A and B show significant non-zero migration rates of supported by a BF>3 as indicated by colour lines. The migrations were calculated using SPREAD3 program. Panels C and D report the bubblegrams indicating the frequency of the gene flows (migrations) to/from different locations and risk categories; the area of each circle is proportional to the percentage of observed migrations in the ML genealogy. The migrations were inferred using a modified version of the Slatkin and Maddison algorithm.

IT: Italy, SA: South America, TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

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Figure 3. C subtype dataset: Panels A and B show significant non-zero migration rates of supported by a BF>3 as indicated by colour lines. The migrations were calculated using SPREAD3 program. Panels C and D report the bubblegrams indicating the frequency of the gene flows (migrations) to/from different locations and risk categories; the area of each circle is proportional to the percentage of observed migrations in the ML genealogy. The migrations were inferred using a modified version of the Slatkin and Maddison algorithm.

IT: Italy, SA: South America, AF: Africa, TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

81x60mm (300 x 300 DPI)
Figure 4. Molecular transmission clusters identified using HIV-TRACE. Part A: colour indicates risk categories, Part B: colour indicates locations. Edges represent genetic distance of 0.015 substitutions per site. IT: Italy, SA: South America, AF: Africa, TSWs: transgender sex workers, MSM: men who have sex with men; HEs: Heterosexuals, IDUs: injecting drug users.

96x60mm (300 x 300 DPI)
Methods

Subtyping of protease and RT sequences

Two to five isolates representative of each of the nine pure subtypes (A–D, F–H, J, and K) and 98 known CRFs were chosen as references.

Phylogenetic dataset

Reference sequences were selected according to the following inclusion criteria: i) sequences had already been published in peer-reviewed journals; ii) the subtype assignment of each sequences classified as non-recombinant was certain; iii) the city/state of origin and the year of sampling were known and clearly established in the original publication.

The phylogenetic signal was evaluated on all datasets by performing likelihood mapping analyses with the program TREEPUZZLE.[1]

Phylodynamic and phylogeographic analysis

All sites of major antiretroviral drug resistance mutations in the protease and reverse transcriptase regions, identified through the last updated Surveillance Drug Resistance Mutations (http://hivdb.stanford.edu/cgi-bin/AgMutPrev.cgi) and International AIDS Society (IAS) (http://www.iasusa.org/guidelines/index.html) lists for naïve and treated patients respectively were removed to avoid bias due to convergence related to drug resistance.

The best model for phylodynamic and phylogeographic analyses was selected using the program Modeltest v.3.7.[2] A Markov Chain Monte Carlo (MCMC) search in MrBayes was made for 10x10^6 generations using tree sampling every 100th generation and a burn-in fraction of 50%. Statistical support for specific clades was obtained by calculating the posterior probability (pp) of each monophyletic clade, and a posterior consensus tree was generated after a 50% burn-in. Clades with a pp >0.8 were considered epidemiologically related clusters.

Dated trees, evolutionary rates and population growth were co-estimated by using Beast v.1.8.4 (http://beast.bio.ed.ac.uk) using the previous selected model. Different coalescent priors (constant population size, exponential growth, logistic growth, and Bayesian skyline plot) were tested using both strict and relaxed molecular clock models. The difference (in log space) of marginal likelihood between any two models is log BF (Bayes Factor). The best fitting models were selected using a BF with marginal likelihoods implemented in BEAST. In accordance with Kass and Raftery,[3] the strength of the evidence against H0 was evaluated as follows: 2lnBF<2 no evidence; 2-6 weak evidence; 6-10 strong evidence, and >10 very strong evidence. A negative 2lnBF indicates evidence in favor of H0. Only values of >6 were considered significant. BF calculations were performed with Tracer v.1.6 (http://beast.bio.ed.ac.uk/Tracer). Chains were run for a minimum of 300 million generations with sampling every 30,000 generations. Convergence was assessed based on the ESS (effective-sample size) value.

Viral Gene Flow Analysis

The gene flow analysis was made by classifying the sequences in different groups based on geographical origin of isolates or risk group. A one-character data matrix was obtained from the original data set by assigning a one-letter code to each taxon in the tree to indicate its group of origin. The putative origin of each ancestral sequence (i.e. internal node) in the tree was then inferred by finding the most parsimonious reconstruction of the ancestral character. The final tree length (i.e. the number of observed migrations in the genealogy) was compared with the tree-length distribution of 10,000 trees, after random joining and splitting. Observed genealogies that are significantly shorter than random trees
indicate the presence of subdivided populations with restricted gene flow. Specific migrations among different areas (character states) were traced using the state changes and stasis tool.

**Transmission clusters reconstruction**

We used two aligned dataset including 364 and 1,158 sequences comprising B, C and F1 subtype strains where codons associated with drug resistance were removed as previously described. The first dataset was used to study transmission clusters among risk categories using all references already selected for phylogenetic analysis (TSWs=124, HEs=111, MSM=78, IDUs=51). The second dataset included a largest number of reference sequences from Italy (IT=351), South America (SA=542), Africa (AF=139) and TSWs (n=126) to study transmission clusters among countries.

**References**


Supplementary Table 1. Reference sequences used for phylodynamic and phylogeographic analyses.

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Supplementary Figure 1

81x60mm (300 x 300 DPI)
Supplementary Figure 2

81x60mm (300 x 300 DPI)
Supplementary Figure 3

81x60mm (300 x 300 DPI)
Supplementary Figure 4

81x60mm (300 x 300 DPI)
Supplementary Figure 6

81x60mm (300 x 300 DPI)