MAJOR ARTICLE

Increasing Prevalence of HIV-1 Subtype A in Greece: Estimating Epidemic History and Origin

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(See the editorial commentary by Thomson and Nájera, on pages 1120–4.)

Background. In North America and Europe, human immunodeficiency virus (HIV)–1 infection has typically been dominated by subtype B transmission. More recently, however, non-B subtypes have been increasingly reported in Europe.

Methods. We analyzed 1158 HIV-1–infected individuals in Greece by DNA sequencing and phylogenetic analyses of protease and partial reverse-transcriptase regions.

Results. We found that the prevalence of non-B subtypes has increased over time and that this significant trend can be mainly attributed to subtype A, which eventually surpassed subtype B in prevalence in 2004 (42% and 33%, respectively). Multivariate analysis revealed that the year of HIV diagnosis was independently associated with subtype A infection (odds ratio for being infected with subtype A for a 10-year increase in the time period of diagnosis, 2.09 [95% confidence interval, 1.36–3.24]; $P < .001$). Phylogenetic analysis revealed that the subtype A epidemic in Greece is the result of a single founder event. The date of the most recent common ancestor of the subtype A in Greece was estimated to be 1977.9 (95% highest posterior density interval, 1973.7–1981.9).

Conclusions. Subtype A circulates among the long-term residents of Greece. This is in contrast to the situation in most European countries, in which infection with non-B genetic forms is associated either with being an immigrant or heterosexual or with intravenous drug use.

HIV-1 originated in Africa via cross-species transmission from chimpanzees and gorillas infected with simian immunodeficiency virus (SIVcpz and SIVgor) [1–5]. The majority of HIV-1 infections worldwide have been caused by group M viruses, in contrast to group O infections (which are localized to west-central Africa) and group N infections (which have been documented only among a few individuals in Cameroon) [6, 7]. Group M genetic diversity has been classified into subtypes, sub-subtypes, and circulating recombinant forms (CRFs) (http://hiv-web.lanl.gov). CRFs are mosaic viruses that have been detected in 2 or more epidemiologically unlinked individuals [8].

The prevalences of the different HIV-1 subtypes and CRFs differ according to geographic location, and the global distribution of HIV-1 variants provides information about the origin and the dissemination of the virus. The epidemic initially spread to Western countries in the form of subtype B, originally from Africa.

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The introduction of the HIV-1 epidemic into Europe is thought to have occurred through sexual contact with men in or from the United States [9] or through heterosexual contacts with individuals from Central Africa [10]. During the early stages of the AIDS epidemic (the early 1980s), the prevalence of HIV-1 infection was higher among men who have sex with men (MSM) than among heterosexual persons. For this reason, subtype B—which was identified at a high prevalence among MSM in the United States—was the predominant clade in Europe before the 1990s. The prevalence of non-B subtypes in Europe has been increasingly recognized over the last few years. Non-B infections in Europe are mainly associated with immigrants or heterosexual persons epidemiologically linked with sub-Saharan Africa [11–22]. In a previous study, non-B subtypes were more frequently detected among immigrants or heterosexual persons in Greece [23], whereas non-B subtypes have been increasingly identified among individuals with newly diagnosed HIV-1 infection during the past few years [24].

The objective of the present study was to estimate longitudinally the prevalence of HIV-1 subtypes in Greece, using virus sampled from patients since the beginning of the epidemic in Greece; to identify any risk factors associated with the increasing prevalence of subtype A; and to phylogenetically investigate the origin of subtype A infections.

MATERIALS AND METHODS

Study population. HIV-1 protease (PR) and partial reverse-transcriptase (RT) sequences were generated from plasma samples submitted for routine drug resistance testing by use of the HIV-1 TRUGENE Genotyping Kit (Bayer HealthCare) and the ViroSeq HIV-1 Genotyping System (Celera Diagnostics). The samples were collected anonymously from treated individuals who had experienced treatment failure (HIV RNA level $>1 \times 10^3$ copies/mL) as well as from treatment-naive patients. Specifically, 101 treatment-naive individuals were studied for the prevalence of resistance-associated mutations, as reported elsewhere [24].

The demographic, immunologic, and virologic parameters were stored in a database. HIV-1 sequences were generated from a total of 1158 individuals sampled from 1998 through 2005. For each individual, the earliest available sample was included in the analysis. The dates of HIV diagnosis were provided by the Hellenic Centre for Diseases Control and Prevention, after anonymous matching approved by the Hellenic Data Protection Authority. The population characteristics are summarized in table 1.

Study design. PR and partial RT sequences of 624 nt in length were characterized from 704 treated and 200 treatment-naive individuals who were tested for genotypic resistance to antiretroviral drugs from 1998 through 2005. Moreover, we analyzed 254 samples collected prospectively from treatment-naive patients with newly diagnosed HIV-1 infection in Greece since 2002, as part of the SPREAD study (http://www.spread-europe.org). In total, PR and partial RT sequences were generated from 1158 HIV-1–seropositive individuals, who comprised 15% of the total HIV-1–infected population (7718 subjects) in Greece, as reported up to December 2005 (http://www.keel.org.gr). The GenBank accession numbers of all of the sequences used in this study are EF447435–EF449504.

Table 1. Characteristics of the study population.

Characteristic	Value (n = 1158)
Sex	
Male	958 (82.7)
Female	200 (17.3)
Treatment	
Yes	704 (60.8)
No	454 (39.2)
Patient ethnicity	
Hellenic	644 (55.6)
Sub-Saharan African	35 (3.0)
African other than sub-Saharan	7 (0.6)
European other than Hellenic	21 (1.8)
Asian	6 (0.5)
Australian	1 (0.1)
American	2 (0.2)
Undetermined	442 (38.2)
Transmission risk group	
MSM	631 (54.5)
Heterosexual persons	245 (21.2)
IDUs	43 (3.7)
Vertically infected children	16 (1.4)
Persons with hemophilia	26 (2.2)
Undetermined	197 (17)
Known date of HIV diagnosis	
No	360 (31.1)
Yes	798 (68.9)
Age, mean ± SD, years	39.5 ± 12

NOTE. Data are no. (%) of patients, unless otherwise indicated. IDUs, injection drug users; MSM, men who have sex with men.

HIV-1 subtype classification. All PR and partial RT sequences were aligned against HIV-1 reference sequences, including all previously described subtypes and CRFs available at http://hiv-web.lanl.gov, by use of CLUSTAL W (version 1.74) [25]. Phylogenetic analysis of HIV-1 subtypes and recombinants was performed using several methods: (1) the neighbor-joining (NJ) method with a F84 model of nucleotide substitution, as implemented in PAUP* (version 4.0β) [26]; (2) bootscanning analysis, as implemented in SimPlot (version 3.2β; http://sray.med.som.jhmi.edu/SCRoftware); and (3) maximum-likelihood phylogeny estimation with a Tamura-Nei substitution model and a γ distribution model of among-site rate heterogeneity, as implemented in TREE-PUZZLE (version 5.2) [27].

The HIV-1 subtype classification procedure is described in detail in the Appendix.

Phylogenetic analyses. Exploratory analyses of subtype A sequences were performed using the NJ method under the HKY+Γ model; branch lengths were calculated using maximum likelihood, as implemented in PAUP*. Phylogenetic trees were also estimated using a Bayesian method under the HKY+Γ model, as implemented in MrBayes (version 3.0B4) [28]. For the Bayesian analysis, 4 Metropolis-coupled Markov chain Monte Carlo (MCMC) calculations were run for 2×10^6 generations, with a burn in of 2×10^5 generations. Similarity searches were performed using the nucleotide-nucleotide basic local alignment search tool (BLAST) available at http://www.ncbi.nlm.nih.gov/BLAST.

Estimation of evolutionary rate and epidemic history of subtype A in Greece. Preliminary analysis of the HIV-1 subtype A data from Greece indicated that the time period (6 years) over which the Greek sequences were sampled was not sufficiently long to accurately estimate the rate of evolution. To provide a time scale for our phylogenetic analysis, we therefore estimated the substitution rate using an independent data set of subtype A sequences sampled from 1986 through 2000. The sequences used for the estimation of the substitution rate were A1.KE.86.ML170, A1.KE.86.ML013, 01_AE.TH.90.CM23, A2C.ZM.90.ZAM184, A1.UG.92.UG029, A1D.UG.92.UG035, 01_AE.TH.93.93TH902, 01_AE.TH.93.93TH253, A1.SE.94.SE7535, A1C.KE.95.ML170_1995, A1.SE.95.UGSE8131, 01_AE.CN.96.96CNKM003, A2.CD.97.97CDKTB48, 01A1.CM.97.CM53122, 01A1.MM.99.mCSW105, A1.UA.00.98UA0116, and A1.KE.00.CQ891776. Importantly, the independent data set exactly matched the genome region (PR and partial RT) obtained for the Greek sequences. This 2-step approach has proved useful in previous analyses of HIV-1 [29]. Coestimation of population dynamics, substitution model parameters, and tree topology was performed using Bayesian inference, as implemented in BEAST (version 1.3) [30, 31]. In this Bayesian analysis, the independent estimate of the substitution rate was implemented as a normally distributed prior (mean number of substitutions per site per year, 0.0018; variance, 0.1×10^{-3}). Three separate MCMC runs were calculated, each for 15×10^6 generations with a burn in of 1.5×10^6 generations. The nucleotide substitution model used was HKY+Γ, and the Bayesian skyline plot model was used to estimate epidemic history. Plots of the effective number of infections through time were generated using the program Tracer (http://evolve.zoo.ox.ac.uk/software.html?id=tracer).

Statistical analysis. The temporal trend in the prevalence of newly diagnosed subtype A or B infections was initially assessed graphically. Subsequently, a nonparametric Wilcoxon-type test for trend through time was used to examine whether the prevalence of subtype A or B changed over time among the subjects with newly diagnosed HIV-1 infection [32].

Potential associations between the prevalence of subtype A and variables such as patient age, sex, year of HIV diagnosis, ethnicity, and transmission risk group were examined using a χ^2 test or a *t* test, as appropriate. A multiple logistic regression model was applied to identify factors independently associated with the diagnosis of subtype A infection.

RESULTS

Prevalence of HIV-1 subtypes. According to the phylogenetic and bootscanning analyses, subtype B was the most common subtype (65.4%), whereas the prevalences of non-B subtypes and recombinants were 27.5% and 6.6%, respectively (table 2). Among the non-B subtypes, subtype A was the most prevalent (20.6%), followed by subtypes C (4.4%), G (0.9%), F (0.8%), D (0.7%), and H (0.1%); the prevalences of CRFs, unique recombinants, and unclassified sequences (which were not recombinants and showed no similarity with any of the previously characterized subtypes and CRFs) were 3.5%, 3.2%, and 0.5%, respectively (table 2). Among the recombinants, CRF02_AG was the most frequently detected CRF (1.7%), whereas 8 additional CRFs were detected (table 2). The prevalences of dif-

Table 2. Prevalence of HIV-1 subtypes, circulating recombinant forms (CRFs), unique recombinants, and unclassified sequences.

HIV-1 classification	No. (%)
Pure subtype	
B	757 (65.4)
A	239 (20.6)
C	51 (4.4)
G	10 (0.9)
F	9 (0.8)
D	8 (0.7)
H	1 (0.1)
Subtotal	1075 (92.8)
CRFs	
CRF02	20 (1.7)
CRF01	3 (0.3)
CRF03	1 (0.1)
CRF04	8 (0.7)
CRF06	3 (0.3)
CRF10	1 (0.1)
CRF11	1 (0.1)
CRF12	1 (0.1)
CRF14	2 (0.2)
Subtotal	40 (3.5)
Unique recombinants[a]	37 (3.2)
Unclassified sequences	6 (0.5)
Total	1158 (100)

[a] CRF02/B, CRF02/U, A/B, A/C, A/G, B/A, B/F, CRF7/CRF15, F/B, G/U, 99GR303/U, and 99GR303/U/K.

ferent subtypes and recombinants among different transmission risk groups are shown in table 3. The distribution of the different subtypes and recombinants varied significantly among the different risk groups ($P < .001$). Recombinants were detected more frequently among injection drug users (IDUs) than among other patients (20.9% vs. 6.1%; $P < .001$).

Temporal trends in the prevalences of different subtypes. The proportion of infections with subtype A, subtype B, or other strains diagnosed each year was investigated for 798 patients with known dates of HIV diagnosis (spanning the period from 1984 through 2004) to evaluate temporal trends in subtype distribution. The prevalence of subtype B declined from 94% in 1984 to 33% in 2004 ($P < .001$ for trend). At the beginning of the epidemic (around 1983–1985), the prevalence of subtype B was high (>90%), and although a decline was first observed in the late 1980s, the prevalence remained at a high level (between 80% and 90%) (figure 1*A* and 1*B*). In the 1990s, the decline in the prevalence of subtype B continued, ending up lower than the non-B prevalence in 2003 and 2004 (figure 1*A* and 1*B*).

Further investigation of the increasing prevalence of diagnoses of non-B infections through time revealed a marked increase in the prevalence of subtype A—its prevalence increased significantly from 6% in 1984 to 42% in 2004 ($P < .001$ for trend; figure 1*A* and 1*B*). We note that, although the prevalence of subtype A was lower than that of subtype B at the beginning of the 1980s, it increased continuously and, in 2004, overtook the prevalence of subtype B (figure 1*A* and 1*B*).

Associations between subtype A and patient characteristics. A univariate analysis was performed on 798 patients to assess risk factors (year of HIV diagnosis, age, sex, transmission risk group, and ethnicity) associated with subtype A infection. The prevalence of subtype A was significantly associated with the year of HIV diagnosis ($P < .001$) and transmission risk group ($P = .008$). In particular, infection with subtype A was higher among individuals whose condition was diagnosed in more recent years. Subtype A was also more frequent among MSM (21.5%), heterosexual persons (22.8%), those who belonged to the undetermined risk group (25.8%), and vertically infected children (18.2%), compared with other risk groups (persons with hemophilia, 0%; IDUs, 9.8%). (Note that the prevalences of subtype A infections among the different risk groups given here was estimated using the 798 patients with available dates of HIV diagnosis and for this reason are slightly different from those reported in table 3.)

To identify any risk factors independently associated with subtype A, we performed an analysis using a multiple logistic regression model. The studied parameters were sex, age, risk group (MSM, heterosexual persons, IDUs, vertically infected children, and undetermined), year of HIV diagnosis, and ethnicity (Hellenic vs. other and sub-Saharan vs. other). The analysis revealed that the year of HIV diagnosis was independently associated with subtype A infection (odds ratio for being infected with subtype A for a 10-year increase in the time period of diagnosis, 2.09 [95% confidence interval, 1.36–3.24]; $P < .001$) (table 4).

Phylogenetic analyses of subtype A. To investigate the origin of subtype A in Greece, we performed phylogenetic analyses using HIV-1 sequences classified as subtypes A1, A2, and A3 from different geographic regions. As an exploratory analysis, we analyzed 287 sequences from different areas (eastern Africa [Rwanda, Uganda, Kenya, and Tanzania], western and west-central Africa [Ivory Coast, Cameroon, Equatorial Guinea, Gabon, and the Democratic Republic of Congo], Germany, Italy, Sweden, Ukraine, Uzbekistan, Cyprus, the United States, and Greenland), including 110 sequences from Greece. NJ analysis revealed that almost all subtype A sequences from Greece (100 [91%]) fell within a monophyletic cluster.

A consensus sequence of the Greek subtype A strains was compared against all HIV-1 sequences in the National Center for Biotechnology Information database using a BLAST similarity search. BLAST search results revealed a high similarity to the following sequences: AY322190 (Kenya), AY322185 (Kenya), AF457065 (Kenya), AF457063 (Kenya), AY435228 (Uganda), AJ413012 (Rwanda), AY677554 (United States), AJ419454 (Denmark), and a set of 22 sequences from Albania

Table 3. Prevalence of HIV-1 subtype B, subtype A, other subtypes, recombinants, and unclassified sequences among different transmission risk groups.

Risk group	B	A	Other subtypes	Recombinants	Unclassified sequences	Total
MSM	449 (71.2)	135 (21.4)	13 (2.1)	31 (4.9)	3 (0.5)	631
Heterosexual persons	122 (49.8)	57 (23.3)	43 (17.6)	22 (9.0)	1 (0.4)	245
IDUs	26 (60.5)	7 (16.3)	1 (2.3)	9 (20.9)	0 (0)	43
Persons with hemophilia	26 (100)	0 (0)	0 (0)	0 (0)	0 (0)	26
Vertically infected children	7 (43.8)	5 (31.3)	3 (18.8)	1 (6.3)	0 (0)	16
Undetermined	127 (64.5)	35 (17.8)	19 (9.6)	14 (7.1)	2 (1.0)	197
Total	757	239	79	77	6	1158

NOTE. Data are no. (%) of patients. IDUs, injection drug users; MSM, men who have sex with men.

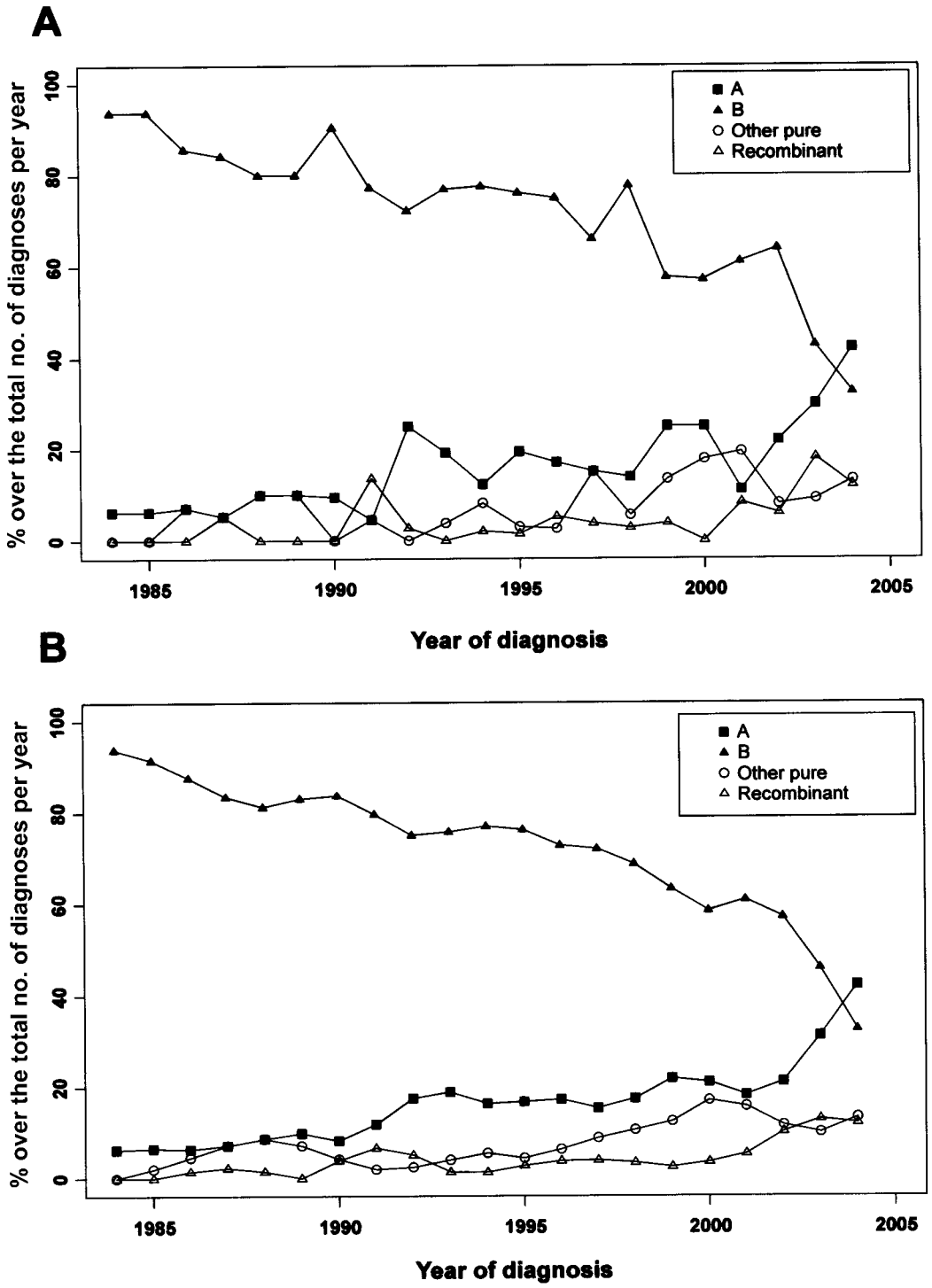

Figure 1. Prevalence of HIV-1 subtype A, subtype B, other pure subtypes, and recombinants according to the year of HIV diagnosis. *A,* unsmoothed; *B,* smoothed. The observed points were smoothed using locally weighted regression with a bandwidth of 0.30 (i.e., for each point in the data, centered subsets of 30% of the observations were used to calculate the corresponding smoothed value).

[33]. A further phylogenetic analysis that used the NJ method and that included these strains showed that the Greek monophyletic cluster does not fall within any of the subtype A subclusters (A2 and A3) (figure 2*A*) and, moreover, that almost all the sequences from Albania formed a distinct subclade within the Greek clade (figure 2*A* and 2*B*). In other words, there was a cluster of sequences sampled almost exclusively from Greece and Albania (denoted I in figure 2*B*; 3 additional

Table 4. Results of the multivariate analysis of the parameters associated with subtype A.

Risk factor	OR (95% CI)	*P*
Male sex/female sex	1.10 (0.58–2.08)	.771
Age, per year	1.01 (0.99–1.03)	.227
Transmission group		
Heterosexual persons/MSM	1.60 (0.93–2.77)	.090
IDUs/MSM	0.44 (0.13–1.51)	.192
Vertically infected children/MSM	1.59 (0.29–8.60)	.589
Undetermined/MSM	1.67 (0.60–4.66)	.322
Year of HIV diagnosis, per year	1.08 (1.03–1.12)	<.001
Ethnicity		
Hellenic/other	1.15 (0.45–2.97)	.769
Sub-Saharan/other	0.41 (0.10–1.66)	.209

NOTE. CI, confidence interval; IDUs, injection drug users; MSM, men who have sex with men; OR, odds ratio.

sequences—1 from Kenya, 1 from Greenland, and 1 from the United States—fell within the monophyletic cluster of the Greek/Albanian monophyletic cluster) and another cluster containing sequences from Greece and eastern Africa (Kenya, Uganda, and Tanzania) (figure 2*B*). To further confirm this finding, we repeated the analysis using only the third codon positions. This confirmed the monophyletic clustering of subtype A sequences sampled from Greece (data not shown).

Sequences from patients who received a diagnosis of HIV-1 infection during the early phase of the epidemic in Greece (before 1990) fell within clade I, thus confirming the notion that the subtype A epidemic in Greece was the result of a single founder event. Sequences from Albania falling within clade I showed, on average, a much lower among-strain evolutionary distance (0.044) than the Greek sequences (0.1), suggesting that the Albanian subtype A cluster began later as a single founder event, most probably originating from Greece.

Bayesian methods were used to further confirm the monophyletic nature of the subtype A sequences from Greece. The final analysis was performed with the inclusion of 1 or 2 sequences from each of the different clusters identified in the NJ tree and 18 sequences from Greece (the total number of sequences analyzed using Bayesian methods was 48). The Greek sequences again appeared as monophyletic in this analysis with high posterior probability support (0.94; data not shown), further supporting our previous results.

Estimation of the evolutionary rate and epidemic history of subtype A in Greece. All MCMC independent runs converged to almost identical values for all parameters (data not shown). The mean substitution rate (combined runs) for the PR and partial RT regions analyzed was 1.73×10^{-3} (95% highest posterior density [HPD] interval, 1.54×10^{-3} to 1.92×10^{-3}) substitutions per site per year. The date of the most recent common ancestor (MRCA) of the tree was estimated to be 1977.9 (95% HPD interval, 1973.7–1981.9). This is in accordance with the epidemiological data indicating that subtype A was introduced into Greece a long time ago and is found among patients who received a diagnosis in the early 1980s. The Bayesian skyline plot (figure 3) suggests that the effective number of infections grew exponentially between approximately 1990 and 1999. The effective number of infections corresponds to the number of infected individuals transmitting the infection to other subjects rather than the total number of infected individuals. After the period of exponential epidemic growth, the effective number of infections stabilized around the late 1990s.

DISCUSSION

Analysis of PR and partial RT sequences from 1158 patients representing approximately one-sixth of all HIV-1 diagnoses since the beginning of the epidemic in Greece revealed high genetic diversity of HIV-1 in Greece. The prevalence of the non-B subtypes has increased over time, and this rise can be attributed mainly to an increasing frequency of subtype A infections. Subtype A became the most common subtype in 2004. Phylogenetic analysis showed that almost all of the Greek subtype A sequences fell into a single monophyletic cluster, indicating that the Greek subtype A epidemic was the result of a single introduced infection, probably from Africa, which subsequently spread among the long-term residents of Greece. We should note that our present findings are based on partial genomic sequences (of PR and RT) and that further analysis will be done in the future to characterize the full-length sequence of the subtype A variants in Greece. Moreover, in accordance with the epidemiological data, the MRCA of the subtype A in Greece was estimated to be in the late 1970s, thus suggesting that it was introduced during the early stage of the epidemic in Greece.

The characteristics of the HIV-1 epidemic in Greece are somewhat unusual in comparison to those in most European countries. Specifically, non-B subtypes have been introduced into Europe because of immigration from and international travel to sub-Saharan Africa, often reflecting the historical and socioeconomic links of European countries and their former colonies. The prevalence of non-B subtypes has shown a remarkable increase in several European countries in recent years (e.g., France, the United Kingdom, Italy, Portugal, Spain, Luxembourg, Belgium, and Sweden), and the majority of these infections have been acquired abroad via heterosexual contact [11–20, 22]. In this way, travelers who acquire the infection abroad serve as a conduit for the introduction of diverse HIV-1 subtypes into European countries. The propagation of non-B variants has also been reported outside this risk group, suggesting the onward transmission of non-B viruses in Western countries.

Non-B subtypes are relatively rare in most European coun-

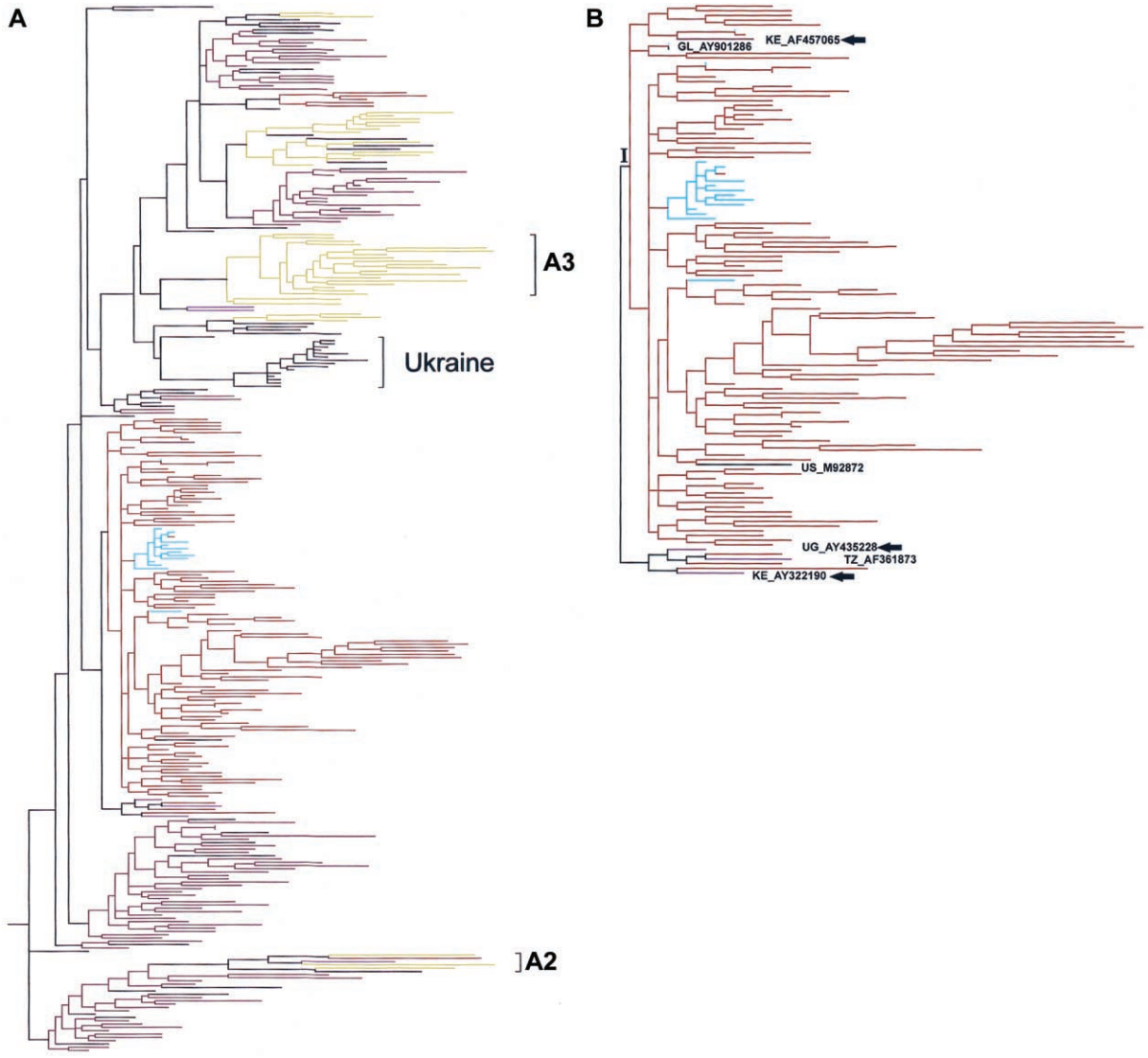

Figure 2. *A,* Phylogenetic analysis of HIV-1 subtype A sequences (classified as A1, A2, and A3) from different geographic regions (Africa, Europe, Asia, and the United States), including sequences from Greece and Albania. Taxa sampled from Greece, Albania, and eastern and west-central Africa are shown in red, cyan, purple, and yellow, respectively. Major subtype A subclades (A2, A3, and Ukraine strains) are indicated by brackets. *B,* Monophyletic cluster of the subtype A sequences sampled from Greece. Sequences identified by a BLAST similarity search as being the most similar to those from Greece are identified by arrows.

tries, with the exception of Portugal, Romania, and Russia as well as other European countries of the former Soviet Union [34–38]. Results from the CATCH (Combined Analysis of Resistance Transmission over Time of Chronically and Acutely Infected HIV Patients) study, based on 1633 sequences sampled from naive patients across Europe during 1996–2002, confirm this pattern [39]. In particular, subtype G is detected more frequently among patients with a recent diagnosis in Portugal, as a result of transmission among IDUs (R. Camacho [Laboratório de Virologia, Serviço de Imuno-hemoterapia, Hospital de Egas Moniz, Lisbon, Portugal], personal communication). Similarly, in Russia and other countries that were formerly part of the Soviet Union, subtype A is the predominant clade among IDUs. Since 1997, when the number of infections reported in the Russian Federation began to grow remarkably, 90% of the patients with known risk factors have been IDUs, and subtype A has been the most common strain [35]. Romania also provides an exception, with subtype F being the predominant clade since the beginning of the epidemic in that country.

Notably, the extensive genetic heterogeneity of the virus has direct implications on drug resistance evolution as well as on the potential difference in disease progression among individuals infected with different subtypes [40]. Specifically, subtype-specific resistance mutations have been reported [41–43] and,

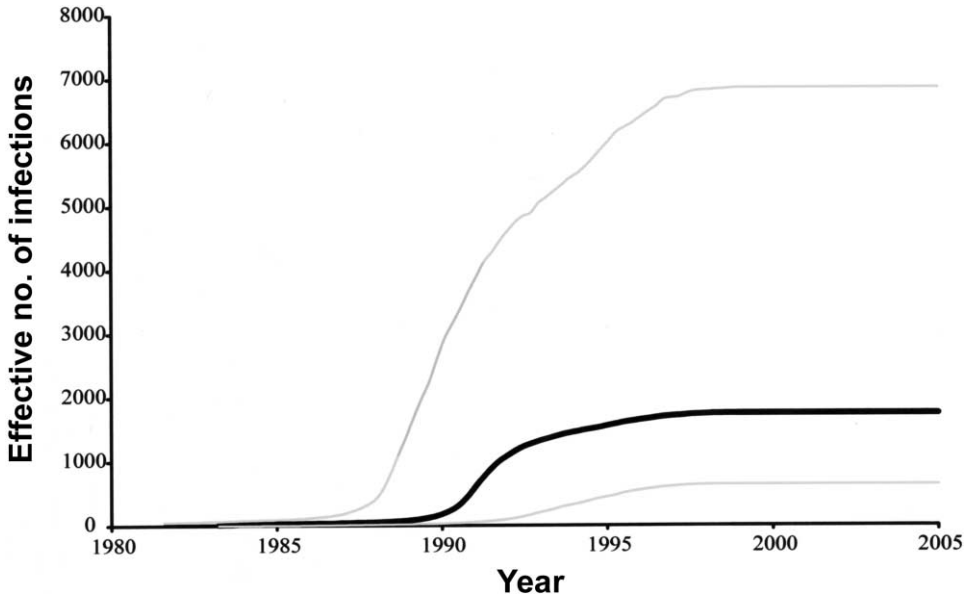

Figure 3. Epidemic history of HIV-1 subtype A in Greece. The black and gray lines indicate the median and the 95% highest posterior density interval of the estimate.

moreover, different subtypes have been recently shown to differ with respect to replicative fitness as well as the rate of CD4 cell count decline over time [40].

The epidemic started similarly in Greece relative to the rest of western Europe. In contrast to other European countries, however, subtype A has predominated over subtype B among long-term residents. These characteristics of the subtype A epidemic in Greece are unusual. Our findings concerning the increased prevalence of subtype A versus B could possibly be explained by an increased transmissibility of A compared with B or recent chance introductions among certain sexually promiscuous groups.

The present phylogenetic analyses showed that the subtype A cluster in Greece probably originated in eastern Africa (Tanzania, Kenya, and Uganda), where subtype A is the most prevalent clade (http://hiv-web.lanl.gov). Moreover, we demonstrated that the subtype A epidemic in Albania is also monophyletic and most probably originated in Greece. A possible explanation for the similarities in HIV-1 spread between Greece and Albania could be the large quantity of immigration and travel between Albania and Greece that started in the early 1990s. During the past 15 years, Greece has experienced vast waves of immigration from Balkan countries, eastern Europe, the Middle East, Asia, and Africa, and these immigrants comprise ~7% of the inhabitants of Greece (according to the General Secretariat of the National Statistical Service of Greece). The majority of immigrants originate from neighboring countries—and especially from Albania, from which travel is frequently seasonal.

The estimation of the demographic history of subtype A revealed that the epidemic growth occurred mainly during the 1990s. These findings are in accordance with the increasing prevalence of subtype A infections among individuals receiving a diagnosis after the 1990s. The stabilization of the epidemic around the late 1990s can be probably explained by the fact that highly active antiretroviral therapy was introduced into Greece in 1996; however, other hypotheses are also plausible.

In conclusion, a significant increase if subtype A infections through time has been identified in Greece, resulting in a clear predominance of subtype A over subtype B among individuals with a recent diagnosis of HIV-1 infection. The subtype A epidemic in Greece arose from a founder event, suggesting that it was introduced once and sustained by onward transmission within Greece. The increasing prevalence of non-B subtypes in Europe gives cause for concern regarding the potential differences among infections with respect to resistance profiles, response to antiretroviral treatment, and other biological properties of the virus.

MULTICENTRE STUDY ON HIV HETEROGENEITY GROUP MEMBERS

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APPENDIX

HIV-1 SUBTYPE CLASSIFICATION

Subtype analysis was performed by including 20–30 unknown sequences each time. The significance of the clustering of each sequence with the reference sequences was assessed by bootstrap analysis (1000 replicates); only clusters supported by bootstrap values or quartet puzzling support of >75% were considered to be significant. In the second step, we included as reference sequences all CRFs in addition to the pure subtypes, and phylogenetic analysis was repeated (this step is necessary for the identification of CRFs). CRFs were not included in the initial tree for the reason that they are recombinants and, therefore, in some cases decrease the bootstrap support for subtype clusters. For unclassified sequences (those with no significant bootstrap values compared with any of the previously known subtypes or CRFs), we performed additional analysis using SimPlot (3.2β version) (http://sray.med.som.jhmi.edu/SCRoftware). Specifically, exploratory analysis for the presence of any recombination events was performed by bootscanning plots using a sliding window of 400 nt moving in steps of 50 nt and maximum-likelihood estimated distances (F84 model), as implemented in SimPlot. The method for tree reconstruction was NJ. A putative recombination pattern was further confirmed by phylogenetic analysis in each individual fragment, with distinct subtype assignment. In the event that there was no evidence for recombination, the sequence was considered to be unclassified.

Phylogenetic analysis was performed with the inclusion of 2 representative sequences for each HIV-1 subtype and CRF, as follows: U455 and 92UG037 (subtype A1); SF2 and JRFL (subtype B); 92BR025 and C2220 (subtype C); ELI and NDK (subtype D); VI850 and FIN9363 (subtype F1); 92NG083 and HH8793 (subtype G); VI991 and 90CF056 (subtype H); SE7887 and SE7022 (subtype J); 97EQTB11C and 96CM-MP535C (subtype K); 90CF402 and 93TH253 (CRF01_AE); IbNg and 97CM_MP807 (CRF02_AG); KAL153 and RU98001 (CRF03_AB); 97PVCH and 94CY032 (CRF04_cpx); VI1310 and VI961 (CRF05_DF); BFP90 and 95ML84 (CRF06_CPX); 97CN001 and 98CN009 (CRF07_BC); 98CN006 and 98CNGX7 (CRF 08_BC); 96TZ_BF061 and 96TZ_BF071 (CRF10_CD); GR17 and MP818 (CRF11_cpx); A32879 and URTR17 (CRF12_BF); 96CM_4164 and 96CM_1849 (CRF13_cpx); and X397 and X421 (CRF14_BG).

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