Infection, Genetics and Evolution 19 (2013) 152–163

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Molecular epidemiology and genetic diversity of hepatitis B virus in Mar del Plata city, Argentina

Luciana Barbini *, Mercedes Elizalde, Carolina Torres, Rodolfo Campos

Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 4^to piso. (1113), Ciudad Autónoma de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 6 March 2013
Received in revised form 2 July 2013
Accepted 8 July 2013
Available online 17 July 2013

Keywords:
Diversity
Epidemiology
Hepatitis B virus
Mar del Plata
Recombination
Coalescence

ABSTRACT

The aim of this work was to describe the current molecular epidemiology and genetic diversity of HBV in Mar del Plata, an important Argentinean touristic city. The phylogenetic analysis of 29 HBV DNA positive serum samples showed that F1b was the predominant subgenotype (sgt, 62.1%), followed by sgt A2 (13.8%) and sgt F4, gt D and gt G (6.9% each). Among anti-HBc IgM positive samples, 75.0% were sgt F1b, followed by sgt F4 (12.5%), sgt A2 (6.25%) and sgt D (6.25%). Three recombinant full length genomes were found: two G/F1b (some of the first gt G detected in Argentina) and one F4/D2. The circulation of clinical important mutations in the city was described. Mutations at the HBsAg were detected in 34.5% of the analyzed samples, associated with laboratory diagnosis and antiviral treatment failures, immune escape and hepatocellular carcinoma. Most of the samples presented *wild type* BCP/PC sequences. Coalescence analysis for the most prevalent sgt F1b estimated that the diversification mainly occured during mid '90s and the tMRCA was estimated in 1987. Finally, the high presence of the autochthonous sgt F1b, associated with the anti-HBc IgM positive infection and its present-day diversification process, shows the strong impact of internal human migratory movements into the current population of Mar del Plata.

1. Introduction

Hepatitis B virus (HBV) is one of the major causative agents of liver disease including acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma. More than 400 million people worldwide are chronically infected (Lai et al., 2003). HBV belongs to the *Hepadnaviridae* family and contains a partially double-stranded circular DNA genome, approximately 3.2 kb length. It presents four partially overlapping open reading frames encoding: polymerase, surface, core and X proteins.

Based on an intergroup divergence greater or equal to 8%, HBV has been classified in at least eight genotypes (A–H) (Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002). These genotypes show a distinctive geographical distribution (Norder et al., 1994). The most cosmopolitan genotypes are A and D, predominant in Europe, Africa, and North America, and in Mediterranean and Near and Middle East countries, respectively. Genotypes B and C are found in East and South-East Asia, and genotype E in West Africa. Genotype G was isolated in different countries, as Germany, France, Japan and USA. Genotypes F and H are considered indigenous to the native population of the Americas, being genotype F restricted to Central and South America and genotype H to North and Central America (Arauz-Ruiz et al., 1997; Piñeiro y Leone et al., 2003; Di Lello et al., 2009). Genotype's distribution varies in different regions of Argentina. In Buenos Aires, genotypes A, D, and F are the most prevalent, whereas in the north region of the country, the most prevalent is genotype F (França et al., 2004). The different genotype distribution in Argentine regions indicates that the epidemiological landscape of HBV infection appears to be the result of the diverse human migratory movements that have given shape to the present population (Piñeiro y Leone et al., 2008).

An increasing number of HBV intergenotype recombinants have been identified. Hybrids between genotypes B and C were found in Asia (Sugauchi et al., 2003). Recombination between genotypes A and D were reported in Italy, South Africa and India (Morozov et al., 2000; Owiredu et al., 2001; Chauhan et al., 2008) and between genotypes C and D were identified in Tibet and China (Cui et al., 2002; Wang et al., 2002). Other A/E, A/G and C/G hybrids were also described elsewhere (Kurbanov et al., 2005; Suwannakarn et al., 2005; Osiowy et al., 2008).

HBV prevalence is low in Argentina (Paraná and Almeida, 2005). An epidemiologic report from the "National Program of Viral Hepatitis Control" informs an anti-HBc prevalence of 2.08% and a positivity of 0.15% for HBsAg, in a blood donor group in 2009. For the same group in Mar del Plata, prevalences of 1.19% and 0.09% were informed, respectively (González, 2009).

* Corresponding author. Address: Junín 956 4^to piso. (1113), Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 1149648264.
E-mail address: lbarbini@ffyb.uba.ar (L. Barbini).

1567-1348/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.meegid.2013.07.007

Table 1
Polymerase chain reactions information.

Genes	PCR round	Primer name	Sequence 5′–3′	Position (nt)
S	1st	HBV37	TTTTTCACCTCTGCCTAATCATC	1821–1843
S	1st	HBV40	AAAAAGTTGCATGGTGCTGG	1828–1809
S	2nd	HBV27	CTGCTGGTGGCTCCAGTTC	59–76
S	2nd	HBV26	AGAAAATTGGTAACAGMGGYA	814–794
X/BCP/PC	1st	HBV71	TGCCAAGTGTTTGCTGACGC	1174–1193
X/BCP/PC	1st	HBV50	GAACTGTTTCTCTTCCAAAAGTAAG	2247–2222
X/BCP/PC	2nd	HBV69	GCCGATCCATACTGCGGAACT	1260–1280
X/BCP/PC	2nd	HBV64	ACGGGAAGAAATCAGAAGG	1976–1958
Full length	1st	HBV37	TTTTTCACCTCTGCCTAATCATC	1821–1843
Full length	1st	HBV40	AAAAAGTTGCATGGTGCTGG	1828–1809
Fragment 1	2nd	HBV19	ATTTGTTCAGTGGTGCGTAGG	688–708
Fragment 1	2nd	HBV20	AAAGGTTCCACGCATGCGCT	1248–1229
Fragment 2	2nd	HBV21	CTGTGCCAAGTGTTTGCTGA	1171–1190
Fragment 2	2nd	HBV66	CAGACCAATTTATGCCTACA	1801–1782
Fragment 3	2nd	HBV55	TACATCAAAGACTGTGTATTTAAGG	1704–1728
Fragment 3	2nd	HBV54	GAGTTCTTCTTCTAGGGGACCTG	2381–2359
Fragment 4	2nd	HBV63	AGTGTGGATTCGCACTCCT	2269–2287
Fragment 4	2nd	HBV16	TGAGGCGCTACGTGTGGATTC	2807–2787
Fragment 5	2nd	HBV77	ACCAGACAYTATTTGCATA	2736–2754
Fragment 5	2nd	HBV12	ACACGAGCAGGGGTCCTAGG	197–178
Fragment 6	2nd	HBV27	CTGCTGGTGGCTCCAGTTC	59–76
Fragment 6	2nd	HBV26	AGAAAATTGGTAACAGMGGYA	814–794

The diversity of HBV genome has led to the description of mutations with clinical significance. Aminoacid substitutions in the "a" determinant of HBsAg (aa 124–147) can modify the antigenicity of the protein and may impair virion secretion, leading to immune escape, diagnostic failure and occult HBV infection (Carman, 1997; Coleman, 2006; Wu et al., 2010). HBV variants harboring mutations in the viral polymerase gene confer resistance to antiviral drugs and are gradually selected during long-term antiviral therapy with nucleoside analogs (Cao, 2009). Our group has previously shown that the prevalence of HBsAg variants is significant among the Argentine population (Piñeiro y Leone et al., 2008).

Despite Mar del Plata is one of the most important tourist Argentinean cities, with approximately 700,000 inhabitants and characterized by a strong internal migration during the last 50 years, until now there is no data about the local genotype distribution and the circulation of HBV variants. The aim of this study was to describe the current local molecular epidemiology and the genetic diversity of HBV in this city.

Table 2
Main characteristics of the studied samples.

Sample	HBsAg	Anti-HBs	Anti-HBc	Anti-HBc IgM	HBeAg	Anti-HBe	Genotype/Subgenotype	Serotype
HBV_MDQ1	+	–	+	+	ND	ND	F1b	adw4
HBV_MDQ2	+	–	+	+	ND	ND	F1b	adw4
HBV_MDQ3	+	–	+	+	ND	ND	F1b	adw4
HBV_MDQ4	+	+	+	+	ND	ND	F1b	adw4
HBV_MDQ5	+	ND	+	–	ND	ND	G	adw2
HBV_MDQ6	+	–	+	+	ND	ND	F1b	adw4
HBV_MDQ7	+	–	+	+	ND	ND	F1b	adw4
HBV_MDQ8	+	ND	+	+	+	–	F1b	adw4
HBV_MDQ9	+	–	+	+	+	–	A2	adw2
HBV_MDQ10	+	–	+	ND	–	+	D2	adw3
HBV_MDQ11	+	–	+	–	+	–	F1b	adw4
HBV_MDQ12	+	–	+	+	ND	ND	F1b	adw4
HBV_MDQ13	+	–	+	–	–	+	F4	adw4
HBV_MDQ14	+	–	+	–	+	–	F1b	adw4
HBV_MDQ15	+	–	+	+	ND	ND	D3	ayw2
HBV_MDQ16	+	–	+	–	+	–	A2	adw2
HBV_MDQ17	+	–	+	ND	ND	–	F1b	adw4
HBV_MDQ18	+	–	+	+	+	–	F1b	adw4
HBV_MDQ19	+	–	+	+	ND	ND	F4	adw4
HBV_MDQ20	+	–	+	ND	+	–	F1b	adw4
HBV_MDQ21	+	–	+	+	ND	ND	F1b	adw4
HBV_MDQ22	+	–	+	+	–	+	F1b	adw4
HBV_MDQ23	+	–	+	+	–	+	F1b	adw4
HBV_MDQ24	+	ND	+	ND	*ND*	ND	G	adw2
HBV_MDQ25	+	ND	+	ND	ND	ND	A2	adw2
HBV_MDQ26	+	ND	+	ND	ND	ND	A2	adw2
HBV_MDQ27	+	ND	+	ND	ND	ND	F1b	adw4
HBV_MDQ28	+	+	+	ND	ND	ND	F1b	adw4
HBV_MDQ29	+	–	+	+	–	+	nd	adw4

Serological markers, genotypes and serotypes of the twenty-nine samples. HBsAg: hepatitis B surface antigen; anti-HBs: anti HBsAg antibodies; anti-HBc: total anti core antibodies; anti-HBc IgM: anti core IgM antibodies; HBeAg: hepatitis B e antigen; anti-HBe: anti HBeAg antibodies. ND: not determined.

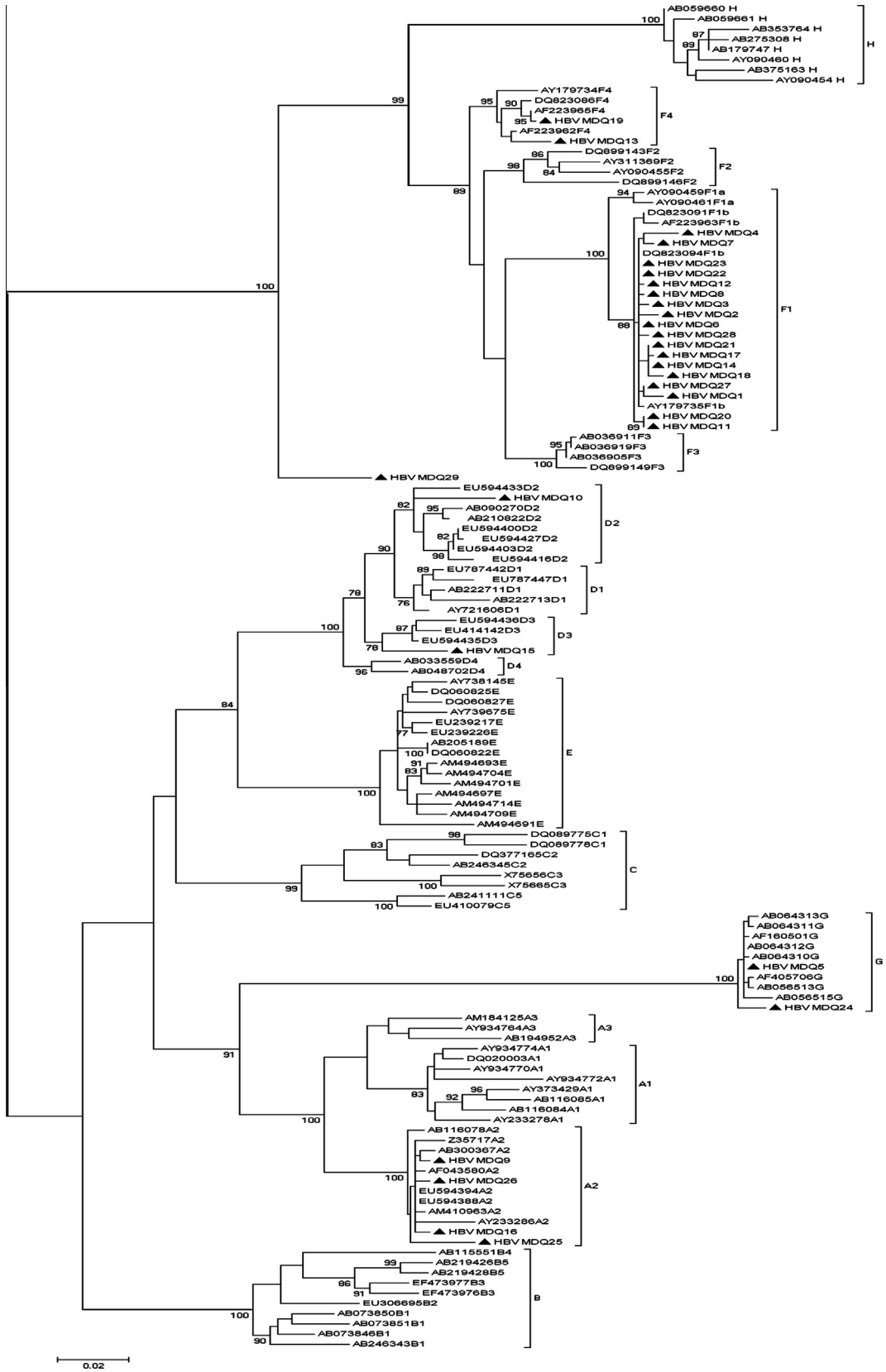

Fig. 1. Phylogenetic analysis. Maximum likelihood phylogenetic tree using a sequence of 1330 nt resulting from the concatenated sequences of S gene (nt 77–793) and X gene/BCP-preCore regions (nt 1281–1957) of twenty-nine HBV (triangles) and reference seuqences from each genotype available at GenBank. The numbers at each node correspond to bootstrap values obtained with 1000 replicates (higher than 70%). The scale bar indicates the genetic distances.

2. Materials and methods

2.1. Samples

Serum samples of 36 patients (collected between 2008 and 2010), belonging to different public health care centers of Mar del Plata, were studied. HBV serological markers: HBsAg, anti HBsAg antibodies (anti-HBs), total anti core antibodies (anti-HBc), anti core IgM antibodies (anti-HBc IgM), HBeAg and anti HBeAg antibodies were analyzed by microparticle enzyme immunoassay (MEIA) (Axsym, Abbott Laboratories).

The patients included in the study are permanent residents of Mar del Plata, not transient tourists. They do not have epidemiological link and they were selected based on the positivity of anti-HBc and HBsAg markers, from a large number of samples tested at the laboratories of the institutions during the mentioned period.

The research was performed according to the World Medical Association Declaration of Helsinki. Written informed consent to participate in this study was obtained from all patients.

2.2. DNA extraction and PCR amplification

HBV DNA was extracted from 200 μl of serum using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions.

A 716 bp fragment of the S gene and a 677 bp fragment including the X gene, BCP and preCore regions, were amplified by nested-PCR using primers described in Table 1. The amplified DNAs were sequenced in both senses, using the internal PCR primers by Macrogen, Inc. (Seoul, Korea).

2.3. Full genome amplification

To determine the complete nucleotide sequence of HBV isolates, six overlapping fragments were amplified by nested PCR after a first round of amplification. All the used primers are described in Table 1. The amplicons were sequenced in both senses, using the internal primers by Macrogen, Inc. (Seoul, Korea).

2.4. Identification of HBV genotypes, subgenotypes and serotypes

Genotype and subgenotype assignation was based on the phylogenetic analysis of the concatenated S gene sequences plus X gen/BCPprecore regions.

The numbering of the aminoacidic positions was established according to subgenotype A2 small S and P ORFs. Serotypes were deduced from the aminoacid sequences, as previously described (Purdy et al., 2007).

2.5. Phylogenetic analysis

The phylogenetic analysis was performed using a 1330 nt sequence resulting from the concatenated sequences of S gene (nt 77–793) and X gene/BCP-preCore regions (nt 1281–1957) of the 29 analyzed samples, and HBV reference sequences for each genotype available at GenBank. Sequences were aligned using the Clustal X program (Thompson et al., 1997). Phylogenetic trees were constructed using the Maximum Likelihood (ML) method. Likelihood analysis was performed with the PhyML v2.4.4 program (Guindon and Gasguel, 2003). Evolutionary models were inferred according to the Akaike Information Criterion (AIC) statistics (Akaike, 1974) obtained with the Modeltest 3.7 program (Posada and Crandall, 1998). The robustness of the reconstructed phylogenies was evaluated by bootstrap analysis (1000 replicates).

2.6. Recombination analysis

Recombination was assessed using SimPlot software (http://sray.med.som.jhmi.edu/SCRoftware/SimPlot). The window and step sizes were set to 350 bp and 20 bp, respectively. The results obtained in the recombination analysis were confirmed using bootscanning analysis (Salminen et al., 1995).

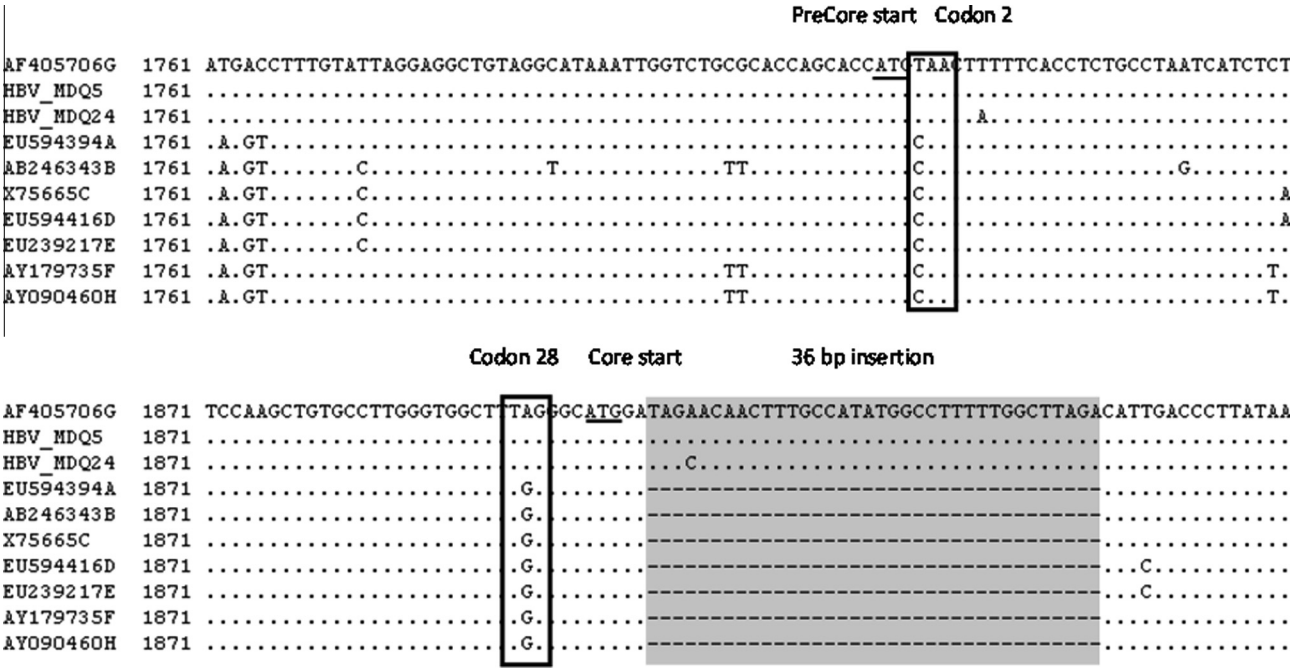

Fig. 2. Characteristics of genotype G isolates. Nucleotide sequences (1761 to 1970) of the two genotype G isolates (HBV_MDQ5 and HBV_MDQ24) aligned with reference sequences from GenBank of genotypes A (EU594394), B (AB246343), C (X75665), D (EU594416), E (EU239217), F (AY179735), G (AF405706) and H (AY090460). The 36 bp insertion in the core gene and two stop codons in the preCore region are shown.

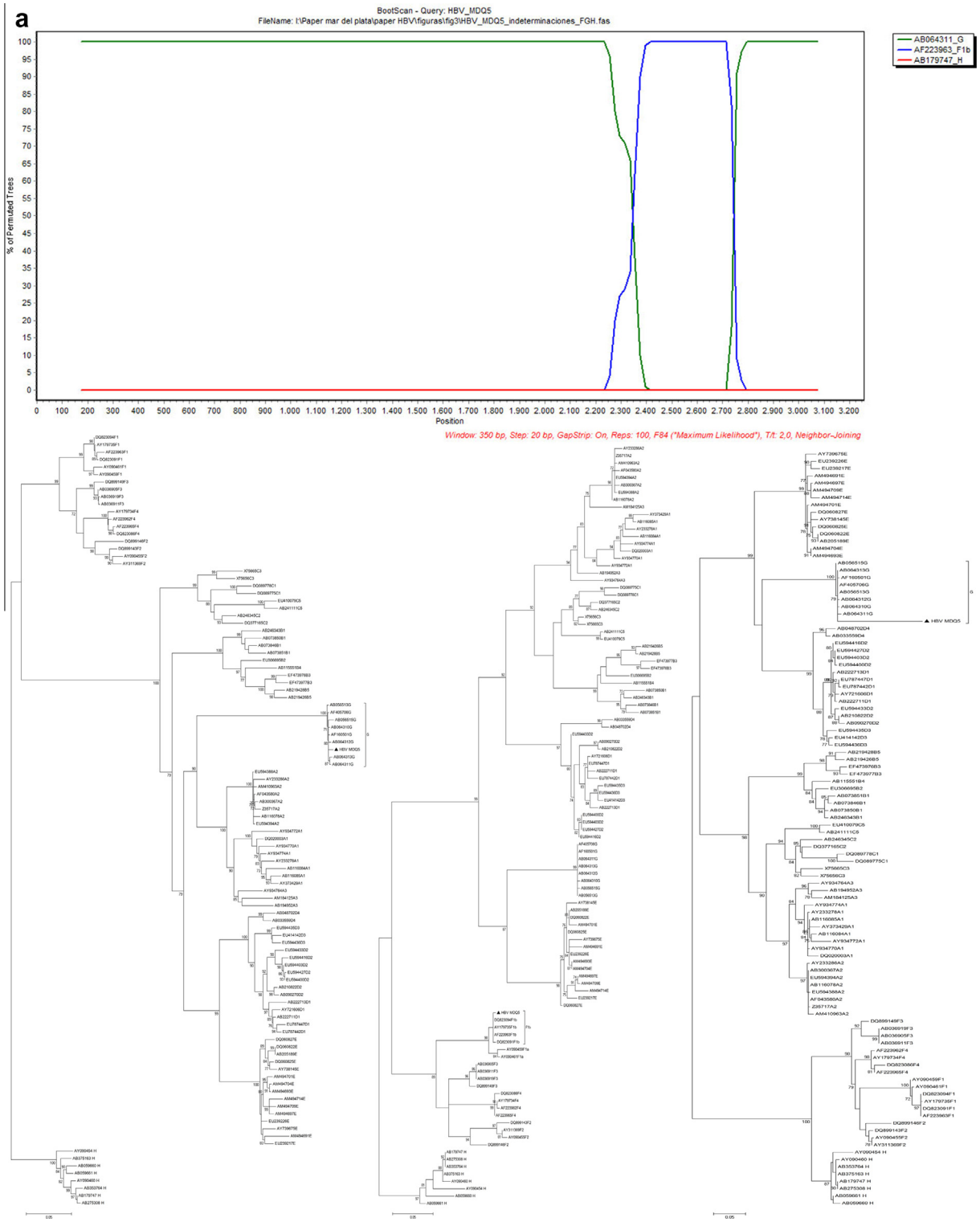

Fig. 3. Genotype G recombinants. a) and b) Bootscan plots (350 bp window size, 20 bp step size and 100 bootstrap replicates) showing the likelihood of clustering of the putative G/F1b recombinant sequence with genotypes G, F and H (outgroup, red, AB179747 and AY090454) reference sequences, along with the maximum likelihood phylogenetic trees generated using each part of the recombined sequences.

2.7. Coalescent analysis

A Bayesian coalescent analysis was performed to estimate the population dynamics and the time to the most recent common ancestor (tMRCA) for the most prevalent group (sgt F1b). Owing to the lack of temporal structure of data (sequences were isolated in a short period of time), calibration was carried out calculating a substitution rate for the concatenated of S gene and X gene/BCP-preCore regions and for the S gene alone, obtained from complete genome sequences subgenotype F1b with an available year of isolation from GenBank database. Analysis was carried out using an appropriate substitution model estimated with the jModeltest v0.1.1 program (Posada, 2008) according to the Akaike Information Criterion. The uncorrelated

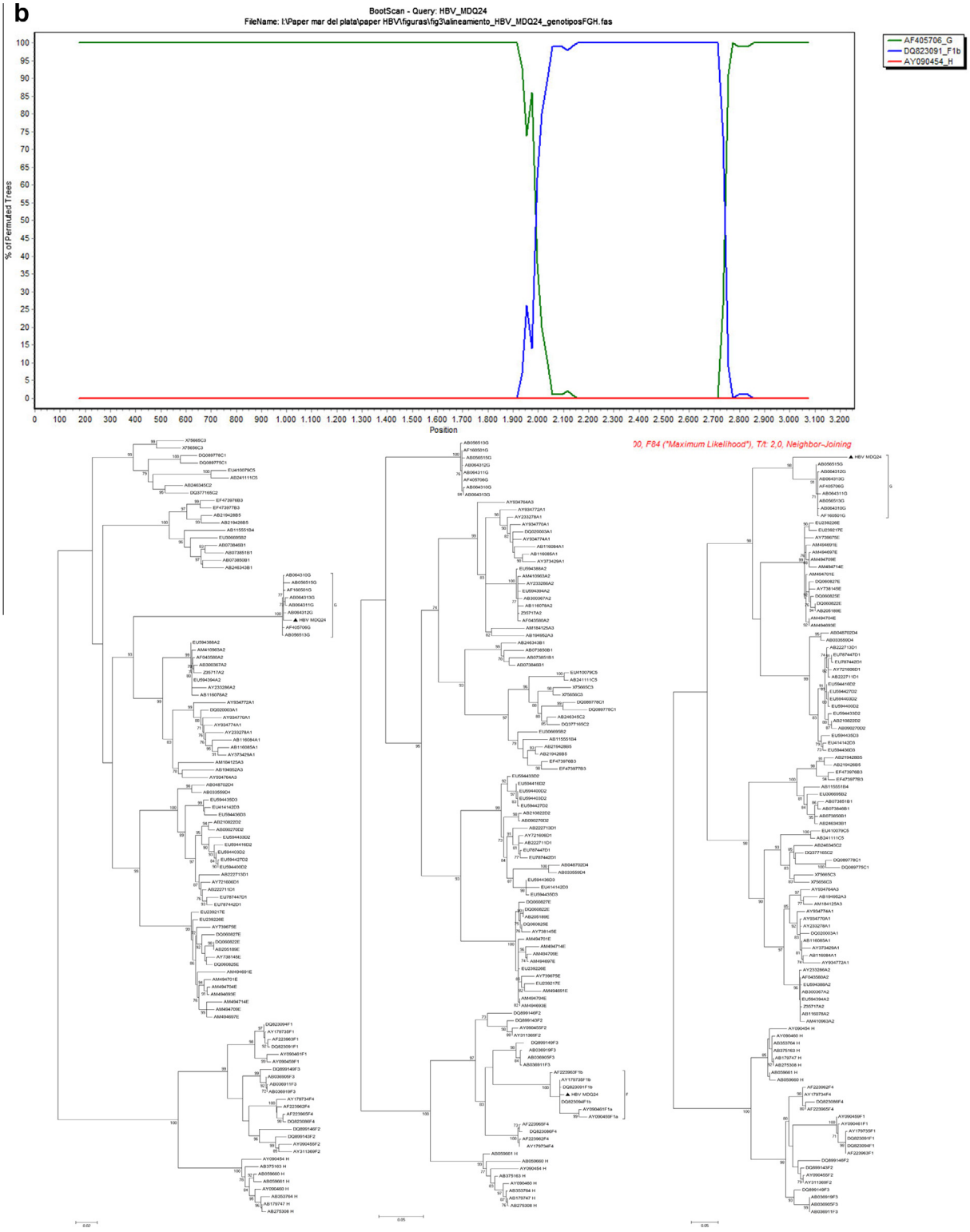

Fig. 3 (*continued*)

lognormal (UCLN) molecular clock model (Drummond et al., 2006) and different demographic models (constant population size, exponential and the Bayesian skyline plot (BSP) coalescent model (Drummond and Rambaut, 2007) implemented in the BEAST v1.7.4 software package (Drummond et al., 2005) were tested. Convergence was assessed by effective sample sizes (ESS) values higher than 200% and 10% of the sampling was discarded as burn-in. Uncertainty in parameter estimates was evaluated in the 95% highest posterior density (HPD95%) interval. The Bayes Factor was used to select the model that better fits to the data (Suchard et al., 2001). According to this analysis, the selected model was the coalescent population size with an

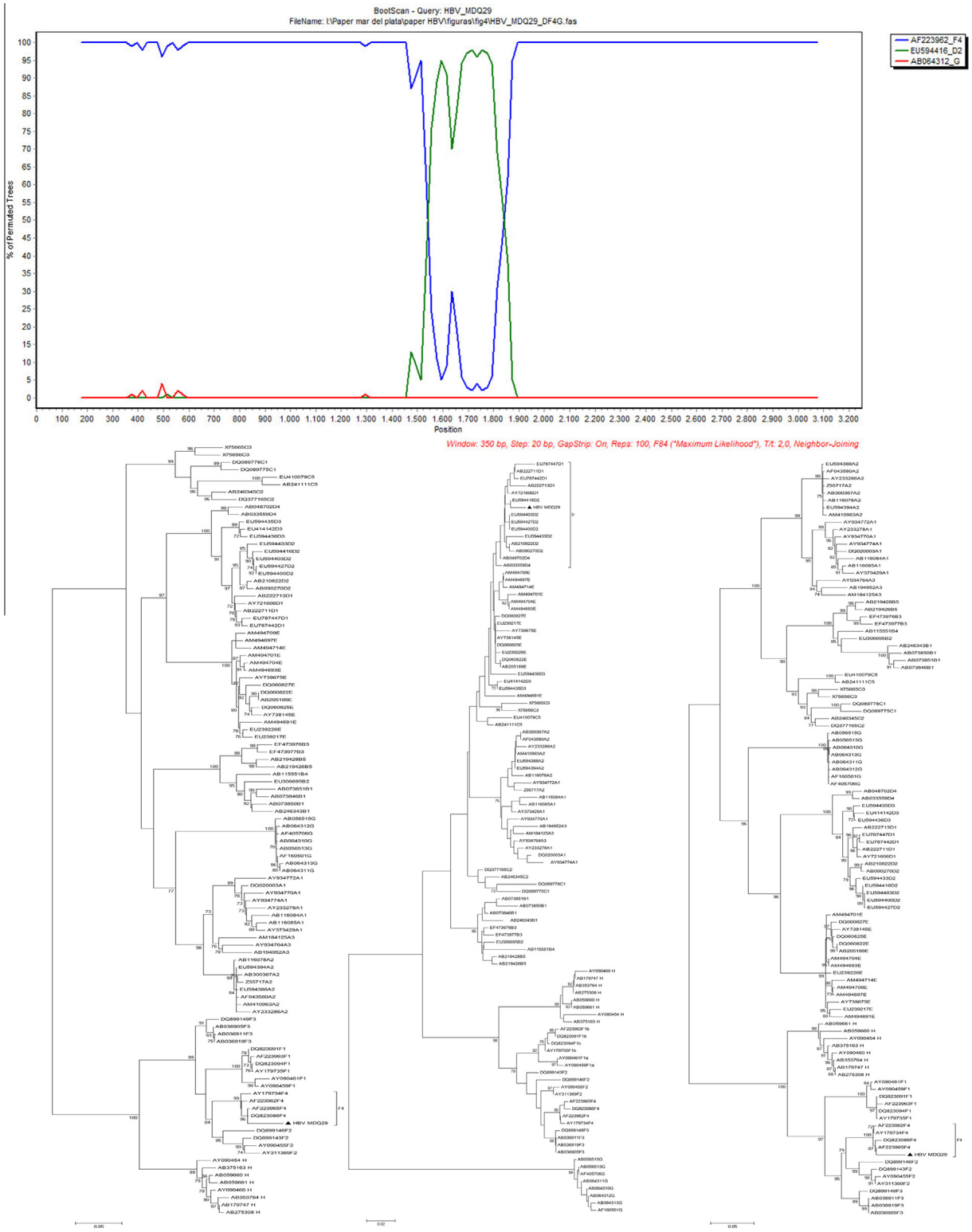

Fig. 4. HBV_MDQ29 recombinant. Bootscan plots (350 bp window size, 20 bp step size and 100 bootstrap replicates) showing the likelihood of clustering of the putative F4/D2 recombinant sequence with genotypes F, D, and G (outgroup, red, AB064312) reference sequences, along with the maximum likelihood phylogenetic trees generated using each part of the recombined sequences(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

estimated substitution rate of 2.27×10^{-4} substitution per site per year (s/s/y) (HPD95% = 7.06×10^{-5}–3.85×10^{-5}) for the concatenated region, and of 2.54×10^{-4} s/s/y (HPD95% = 7.79×10^{-5}–4.63×10^{-4}) for the S gene alone, which have been used to calibrate the analysis of F1b sequences from this work. Analysis was performed as previously. A maximum clade credibility tree (MCCT) was constructed with the TreeAnnotator v1.7.4 program (part of the BEAST package) after discarding 10% of the sampling, and visualized with the FigTree v1.3.1 program (available at http://tree.bio.ed.ac.uk/soft-ware/figtree/).

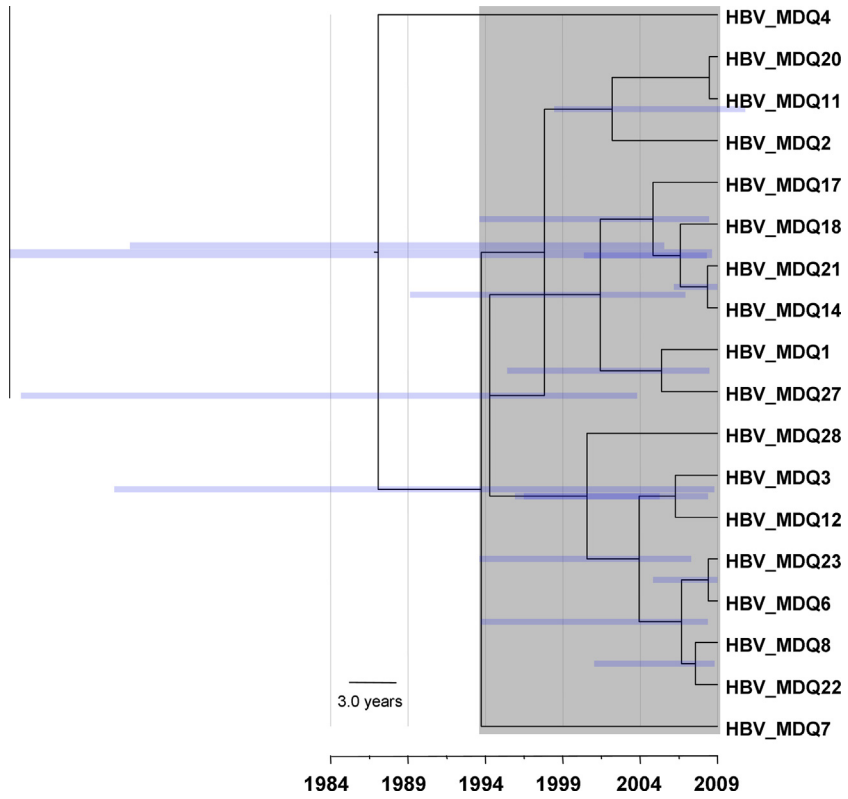

Fig. 5. Maximum clade credibility tree of F1b sequences from Mar del Plata. The HPD95% values for tMRCAs are shown in the horizontal lines. For the tMRCA of the entire group, upper limit of the HPD95% interval was cut off for clarity purpose. Shaded area shows the period of viral diversification.

2.8. Nucleotide sequence accession number

Nucleotide sequences analyzed in this work were deposited at GenBank under accession numbers: JQ272860 to JQ272885 for the S gene; JQ272889 to JQ272914 for the X gene, BCP and preCore region and JQ272886 to JQ272888 for the full length genomes.

3. Results

3.1. HBV phylogenetic and recombination analysis

In order to assess HBV genetic variability in Mar del Plata city, a phylogenetic analysis was performed on 36 HBsAg and anti-HBc positive serum samples. HBV DNA was detected in 29 (29/36, 81%) of these isolates by nested-PCR. Serological markers of the 29 samples are shown in Table 2. For anti-HBc IgM, 16 out of the 29 samples were positive, five samples were negative and eight samples were not analyzed.

The phylogenetic analysis was performed using a 1330 nt sequence resulting from the concatenation of S gene (nt 77–793) and BCP-preCore regions (nt 1281–1957) of the 29 samples (Fig 1). Most of the sequences grouped as sgt F1b (18/29, 62.1%), followed by sgt A2, (4/29, 13.8%), sgt F4 and gt G (2/29, 6.9% each) and two gt D (sgt D2 and sgt D3). One sample, HBVMDQ29, remained unclassified in this analysis.

A further discrimination in relation to the course of infection revealed that among anti-HBc IgM positive samples, sgt F1b was the most prevalent (13/16, 81.2%), followed by sgt A2, sgt F4 and sgt D3 (1/16, 6.2% each, Table 2).

In order to deeply characterize the two genotype G isolates (HBV_MDQ5 and HBV_MDQ24), the full length HBV genomes were sequenced. As it was previously reported for this genotype, both genomes were found to be 3248 bp long, the preCore region has two translational stops at codon 2 (TAA instead of CAA) and codon 28 (TAG instead of TGG), and the core region presented a nucleotide insertion of 36 bp, after the fifth nucleotide following the core translation initiation point (Fig 2). In addition, both gt G samples showed similarities with genotype F in the core and polymerase regions, suggesting a recombination event. The recombination analyses showed regions of higher similarity to gt G alternating with regions of higher similarity to sgt F1b for both isolates. The recombination breakpoints were estimated at positions 2320 and 2735, for HBV_MDQ5 isolate, and in nucleotides 1975 and 2375 for HBV_MDQ24 isolate, which are located in the core and polymerase ORFs, respectively (Fig. 3a and b).

On the other hand, sample HBV_MDQ29 that remained unclassified after phylogenetic analysis, was also analyzed for recombination. Similarity plots showed regions of higher similarity to sgt F4 alternating with regions of higher similarity to sgt D2. The recombination breakpoints were estimated at nt 1540 and 1835, which are located within the X ORF (Fig. 4).

3.2. Coalescent analysis

The population dynamics and the time to the most recent common ancestor for the most prevalent group (sgt F1b) were analyzed through a Bayesian coalescent analysis. The analysis was calibrated using a substitution rate estimated for the concatenated S gene and X gene/BCP-preCore regions and for the S gene alone. Despite a constant population size model was selected as the best for the sgt F1b sequences from Mar del Plata (data available upon request), most of diversification events were located in mid ‘90s (Fig. 5) according to the results obtained for the concatenated region. In addition, under this model, the tMRCA was estimated in

Table 3
Characterization of HBsAg and polymerase variants, and basal core promoter and preCore mutations.

Sample	HBsAg (1–212 aa)			Polymerase (rt1–rt230)			BCP-preCore (nt)		HBV-X (130–131 aa)
	Mutation description previously reported in literature			Mutation description previously reported In literature			1762–64	1896	
HBV_MDQ1	S64F		yes				A-G	G	K-V
	Q101H	a	yes						
HBV_MDQ2				rtE8K		yes	A-G	G	K-V
HBV_MDQ3							A-G	G	K-V
HBV_MDQ4	N3H		yes	rtE8K		yes	A-G	G	K-V
	T23I		yes	rtQ11P		no			
	Q30K		yes	rtT38K		yes			
				rtG127R		no			
				rtL151F		no			
HBV_MDQ5							T-A	A	M-T
HBV_MDQ6							A-G	G	K-V
HBV_MDQ7				rtL151F		no	A-G	G	K-V
HBV_MDQ8							A-G	G	K-V
HBV_MDQ9	I68T	d	yes	rtE39K		no	A-G	G	K-V
HBV_MDQ10				rtA7T		yes	A-G	A	K-V
				rtL91I		yes			
				rtR120G		yes			
HBV_MDQ11							A-G	G	K-V
HBV_MDQ12							A-G	G	K-V
HBV_MDQ13	G47E		no	rtL151F		no	A-G	A	K-V
HBV_MDQ14							A-G	G	K-V
HBV_MDQ15	M198I	b, c	yes	rtS21A		yes	T-A	G	M-I
	I208T	c	yes	rtI162V		yes			
				rtL164M		yes			
				rtV207M		yes			
HBV_MDQ16							A-G	G	K-V
HBV_MDQ17							A-G	G	K-V
HBV_MDQ18							A-G	G	K-V
HBV_MDQ19	N3S		yes				A-G	G	K-V
HBV_MDQ20							A-G	G	K-V
HBV_MDQ21							A-G	G	K-V
HBV_MDQ22							A-G	G	K-V
HBV_MDQ23							A-G	G	K-V
HBV_MDQ24	N207S		yes				T-A	A	M-T
HBV_MDQ25	F8L		yes	rtI16T		yes	A-G	G	K-V
	S45A	b	yes	rtA38T		yes			
				rtI53S		yes			
				rtM129V		yes			
HBV_MDQ26			yes				A-G	G	K-V
HBV_MDQ27							A-G	G	K-V
HBV_MDQ28	**D144E**	b	yes				A-G	G	K-V
HBV_MDQ29	T23I		yes	rtE1D		yes	A-G	G	K-V
	G44E		yes	rtY122H		yes			
	I195M	b, c	yes	rtD123H		yes			
	I208T	c	yes	rtQ149K		yes			
				rtL151F		no			
				rtL180M	e	yes			
				rtM204V	e	yes			

Nucleotide and aminoacid substitutions in HBsAg, polymerase and basal core promoter (BCP)-preCore regions. HBsAg: hepatitis B virus surface antigen; aa: aminoacid; rt: aminoacidic position on the retrotranscriptase domain of the polymerase gene; nt: nucleotide; HBV-X: hepatitis B virus X protein. Mutations associated with, a: failure in diagnostic assays, b: immune escape, c: antiviral treatment, d: development of hepatocellular carcinoma, e: Lamivudine resistance. In bold: mutation in the "a" determinant.

1987 (HPD95% = 1914–2002). Similar estimates were obtained using the S gene (Supplementary material, Table S1 and Fig. S1).

3.3. Serotype assignation

According to their deduced aminoacid sequences, all genotype F samples were adw4. Genotype A and G samples were adw2, while genotype D samples were ayw2 (D3) and adw3 (D2) (Table 2).

3.4. Characterization of circulating HBsAg and polymerase variants

The analysis of the aminoacidic substitutions within the HBsAg (1–212 aa) was performed by comparison of the 29 HBV sequences with each genotype/subgenotype consensus sequences. Mutations were detected in 10 out of the 29 (34.5%) of the analyzed samples. Nine of these changes occurred outside the "a" determinant (S45A, I68T Q101H D144E, I195M, M198I I208T) and only one (HBV_MDQ28) within the "a" determinant (D144E) (Table 3).

In the region where the S gene overlaps with the coding sequence for the reverse transcriptase domain of the polymerase (rt1–rt230), nine samples (31%) showed aminoacidic substitutions (Table 3). Lamivudine resistance mutations (rtL180 M and rtM204 V), were detected in one of the patients (HBV_MDQ29).

3.5. Basal core promoter and preCore mutations

We also investigated the presence of mutations associated with the HBeAg phenotype in this cohort. In the BCP region, most of the samples (26/29, 89.7%) showed the *wild type* sequence and only three samples (10.3%) presented the A1762T/G1764A mutations.

Within the preCore region, 86.2% (25/29) of the isolates presented the *wild type* sequence, while the nucleotide mutation G1896A, that introduces a stop signal at codon 28, was found in four isolates (13.8%;Table 3).

In summary, most of the samples showed *wild type* sequences at BCP/PC but three of them presented both A1762T/G1764A and G1896A mutations (10.3%, HBV_MDQ5, HBV_MDQ13 and HBV_MDQ24). In addition, HBV_MDQ10 presented only the G1896A mutation.

4. Discussion

In this work we describe the genotype distribution and genetic diversity of HBV in Mar del Plata city, Argentina.

The phylogenetic analysis based on the concatenated S gene plus X/BCP/core region showed high proportion of genotype F among HBV infected people from Mar del Plata (72.4%). This genotype has been described as the indigenous representative of the virus in the Americas, since it is almost exclusive among diverse Amerindian groups (Blitz et al., 1998; Livingston et al., 2007; Devesa et al., 2008). In Argentina, a high proportion of genotype F is observed in Northern Provinces in accordance with the major aboriginal background described for the population in this region. In contrast, the Metropolitan Region of Buenos Aires presents a more homogeneous distribution of genotypes (F, D and A), related to the European component of Buenos Aires population (França et al., 2004; Piñeiro y Leone et al., 2008; Torres et al., 2011).

Since its origin, Mar del Plata's population is characterized by an important influence of internal and external migrations and has been populated due to the migration of people from different origins. By year 1895 the 40% of the population was foreign, predominantly from italian and spanish origin. While the demographic growth was slow at the beginning, between 1960 and 2010, population increased from 200,000 to 700,000 inhabitants, approximately.

The important economic and tourist activities led to a large influx of people especially from the Northern Provinces and neighboring countries, resulting in a significant change in the population composition that currently differs from the former population of the city (Europeans) (Lucero, 2003). These migratory waves with an important native component, could explain the high proportion of sgt F1b (autochthonous genotype from Latin America) detected in this study.

In addition, sgt F1b was the principal genotype in anti-HBc IgM (+) infections in agreement with previously reported results by Pezzano et al. (2010), suggesting that this sgt is the current responsible for HBV new infections in Mar del Plata. The coalescent analysis of the predominant sgt F1b revealed that the HBV recent diversification of Mar del Plata samples mainly occurred in mid 90s. These times are compatible with the recent diversification proposed for other subgenotypes or genotypes in circumscribed areas (Zehender et al., 2008; Forbi et al., 2010; Alvarado Mora et al., 2010; Zehender et al., 2012a,b, Ciccozzi et al., 2013).

Then, the sgt F1b would have arrived to Mar del Plata by recent migratory processes and its diversification in the last 50 years would be responsible for the current high proportion in HBV infections.

Furthermore, the phylogenetic analysis also revealed that two of the analyzed samples belong to HBV genotype G. This is one of the first reports of patients infected with this genotype in Argentina. HBV genotype G is frequently presented as a minority but despite the apparent low prevalence throughout the world, this genotype appears to circulate globally, as it has been reported in several European countries (Jardi et al., 2008; Lacombe et al., 2006; Vieth et al., 2002), North and South America (Kato et al., 2004; Sanchez et al., 2007), Nigeria (Olinger et al., 2006) and Asia (Suwannakarn et al., 2005; Shibayama et al., 2005; Toan et al., 2006). The two G isolates share the characteristic features of genotype G previously reported.

Strong evidence suggests that genotype G is frequently co-infecting with other genotypes (Suwannakarn et al., 2005; Kato et al., 2002). The co-infecting genotype is the highly prevalent within the geographical region, thus suggesting that genotype G is capable of co-infecting with any other HBV genotype (Osiowy et al., 2008). Results obtained in the present study show that the two isolates were G/F1b recombinants, being two of the first reported worldwide. Other G/F recombinants were recently reported by Araujo et al. (2013) in HIV co-infected patients in this region. The estimated recombination breakpoints occurred near the DR1 region and the encapsidation signal of the HBV pregenome. The DR1 region covers a hot spot (nt 1885–1915) for genomic recombination and is frequently integrated into the host genome. It has been proposed as a candidate site for intragenic recombination between HBV isolates of different genotypes (Hino et al., 1991; Sugauchi et al., 2002; Yang et al., 2006).

Moreover, the recombination analysis of the full length genome of the HBV_MDQ29 isolate revealed that is a F4/D2 recombinant. This is the first report of recombination between these two subgenotypes.

The fact that 3 out of the 29 analyzed samples were recombinants (10.3%) revealed that the intergenotype recombination is not a rare event in HBV infection, at least in this cohort. Recombination is supposed to result from co-infection of different genotypes (Yang et al., 2006) and further investigation is required to analyze the clinical significance of these recombinants.

Mutations at HBsAg may influence important clinical aspects of HBV infection. The analysis of aminoacid substitutions revealed that most of the changes occurred outside the "a" immunodeterminant, being some of these mutations associated with diagnosis detection (Q101H) and antiviral treatment failures (I195M, M198I, I208T), immune escape (S45A, D144E, I195M, M198I) and hepatocellular carcinoma (I68T). MDQ_MDQ4 sample, that presented three previously described mutations in the HBsAg gene, also showed positive HBsAg and anti-HBs markers. These mutations facilitate HBsAg escape from antibodies, avoiding generation of immunocomplexes and leaving free HBsAg that can be detected by the enzyme immunoassay. The HBV_MDQ28 sample, that was the only one with a substitution within the "a" determinant (D144E), also presented positive HBsAg and anti-HBs antibodies. This mutation has been reported to markedly reduce the binding to antibodies and play an important role in immune escape (Torresi et al., 2002).

Other analyzed mutations involve the region where the S gene overlaps the reverse transcriptase domain of the polymerase. Aminoacid substitutions in the polymerase gene related to antiviral resistance (rtM204V and rtL180M) were identified in one of the isolates (HBV_MDQ29). This patient had previously received antivirals treatment because of an HIV co-infection, favoring the selection of resistant HBV mutants. It must also be standed out that some of the changes detected in this work in the polymerase gene have not been reported before in the literature, being these substitutions found mainly in genotype F isolates.

5. Conclusions

The phylogenetic and coalescent analyses showed that the sgt F1b would have arrived to Mar del Plata by recent migratory processes and its diversification in the last 50 years would be responsible for the current high proportion in HBV infections. In addition, it has been shown the presence of gt G in Argentina, the detection

of new recombinants and the circulation of clinically important mutations in Mar del Plata city.

Acknowledgments

This work was funded by Agencia Nacional de Promoción Científica y Tecnológica (PICT 2006-00285 and PICT 2006-25355), Universidad de Buenos (UBACyT 2008-B601) and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2009-112-200801-01169).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.07.007.

References

Akaike, H., 1974. A new look at the statistical model identification. IEEE T. Automat. Contr. 19, 716–723.
Alvarado Mora, M.V., Romano, C.M., Soares Gomes-Gouvea, M., 2010. Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. J. Gen. Virol. 91, 501–508.
Araujo, N.M., Araujo, O.C., Silva, E.M., et al., 2013. Identification of novel recombinants of hepatitis B virus genotypes F and G in human immunodeficiency virus-positive patients from Argentina and Brazil. J. Gen. Virol. 94, 150–158.
Arauz-Ruiz, P., Norder, H., Visoná, K.A., et al., 1997. Molecular epidemiology of hepatitis B virus in Central America reflected in the genetic variability of the small S gene. J. Infect. Dis. 176, 851–858.
Arauz-Ruiz, P., Norder, H., Robertson, B.H., et al., 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J. Gen. Virol. 83, 2059–2073.
Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.
Blitz, L., Pujol, F.H., Swenson, P.D., et al., 1998. Antigenic diversity of hepatitis B virus strains of genotype F in Amerindians and other population groups from Venezuela. J. Clin. Microbiol. 36, 648–651.
Cao, G.W., 2009. Clinical relevance and public health significance of hepatitis B virus genomic variations. World J. Gastroenterol. 15, 5761–5769.
Carman, W.F., 1997. The clinical significance of surface antigen variants of hepatitis B virus. J. Viral Hepatitis 4 (Suppl. 1), 11–20.
Chauhan, R., Kazim, S.N., Kumar, M., et al., 2008. Identification and characterization of genotype A and D recombinant hepatitis B virus from Indian chronic HBV isolates. World J. Gastroenterol. 14, 6228–6236.
Ciccozzi, M., Babakir-Mina, M., Lo Presti, A., 2013. Molecular analysis of hepatitis B virus in Bulgaria. J. Med. Virol. 85, 49–54.
Coleman, P.F., 2006. Detecting Hepatitis B Surface Antigen Mutants. Emerg. Infect. Dis. 12, 198–203.
Cui, C., Shi, J., Hui, L., et al., 2002. The dominant hepatitis B virus genotype identified in Tibet is a C/D hybrid. J. Gen. Virol. 83, 2773–2777.
Devesa, M., Loureiro, C.L., Rivas, Y., et al., 2008. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. J. Med. Virol. 80, 20–26.
Di Lello, F.A., Piñeiro y Leone, F.G., Muñoz, G., et al., 2009. Diversity of Hepatitis B and C Viruses in Chile. J. Med. Virol. 81, 1887–1894.
Drummond, A.J., Rambaut, A., Shapiro, B., et al., 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Mol. Biol. Evol. 22, 1185–1192.
Drummond, A.J., Ho, S.Y., Phillips, M.J., et al., 2006. Relaxed phylogenetics and dating with confidence. PLoS Biol. 4, e88.
França, P.H., González, J.E., Munné, M.S., et al., 2004. Strong association between genotype F and hepatitis B virus (HBV) e antigen-negative variants among HBV-infected Argentinean blood donors. J. Clin. Microbiol. 42, 5015–5021.
Forbi, J.C., Vaughan, G., Purdy, M.A., et al., 2010. Epidemic history and evolutionary dynamics of hepatitis B virus infection in two remote communities in rural Nigeria. PLoS One 5, e11615.
González, J.,2009 Informe Epidemiológico Número 10. Programa Nacional de Control de Hepatitis Virales en Argentina.
Guindon, S., Gasguel, O., 2003. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. Syst. Biol. 52, 696–704.
Hino, O., Tabata, S., Hotta, Y., 1991. Evidence for increased in vitro recombination with insertion of human hepatitis B virus DNA. Proc. Natl. Acad. Sci. U. S. A. 88, 9248–9252.
Jardi, R., Rodríguez-Frias, F., Schaper, M., et al., 2008. Analysis of hepatitis B genotype changes in chronic hepatitis B infection: influence of antiviral therapy. J. Hepatol. 49, 695–701.
Kato, H., Orito, E., Gish, R., et al., 2002. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). J. Virol. 76, 6131–6137.
Kato, H., Gish, R., Bzowej, N., et al., 2004. Eight genotypes (A–H) of hepatitis B virus infecting patients from San Francisco and their demographic, clinical, and virological characteristics. J. Med. Virol. 73, 516–521.
Kurbanov, F., Tanaka, Y., Fujiwara, K., et al., 2005. A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. J. Gen. Virol. 86, 2047–2056.
Lacombe, K., Massari, V., Girard, P., et al., 2006. Major role of hepatitis B genotypes in liver fibrosis during coinfection with HIV. AIDS 20, 419–427.
Lai, C.L., Ratziu, V., Yuen, M.F., et al., 2003. Viral hepatitis B. Lancet 362, 2089–2094.
Livingston, S.E., Simonetti, J.P., McMahon, B.J., et al., 2007. Hepatitis B virus genotypes in Alaska Native people with hepatocellular carcinoma: preponderance of genotype F. J. Infect. Dis. 195, 5–11.
Lucero, P., 2003. Dinámica demográfica y configuración territorial de Mar del Plata y el Partido de General Pueyrredón en la segunda mitad del siglo XX. Universidad Nacional de, Mar del Plata.
Morozov, V., Pisareva, M., Groudinin, M., 2000. Homologous recombination between different genotypes of hepatitis B virus. Gene 260, 55–65.
Norder, H., Couroucé, A.M., Magnius, L.O., 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198, 489–503.
Olinger, C.M., Venard, V., Njayou, M., et al., 2006. Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. J. Gen. Virol. 87, 1163–1173.
Osiowy, C., Gordon, D., Borlang, J., et al., 2008. Hepatitis B virus genotype G epidemiology and co-infection with genotype A in Canada. J. Gen. Virol. 89, 3009–3015.
Owiredu, W.K., Kramvis, A., Kew, M.C., 2001. Molecular analysis of hepatitis B virus genomes isolated from black African patients with fulminant hepatitis B. J. Med. Virol. 65, 485–492.
Paraná, R., Almeida, D., 2005. HBV epidemiology in Latin America. J. Clin. Virol. 34 (Suppl. 1), 130–133.
Pezzano, S.C., Torres, C., Fainboim, H.A., et al., 2010. Hepatitis B virus in Buenos Aires, Argentina: genotypes, virological characteristics and clinical outcomes. Clin. Microbiol. Infect. 17, 223–226.
Piñeiro y Leone, F.G., Mbayed, V.A., Campos, R.H., 2003. Evolutionary history of hepatitis B virus genotype F: An in-depth analysis of Argentine isolates. Virus Genes 27, 103–110.
Piñeiro y Leone, F.G., Pezzano, S.C., Torres, C., et al., 2008. Hepatitis B virus genetic diversity in Argentina: Dissimilar genotype distribution in two different geographical regions; description of hepatitis B surface antigen variants. J. Clin. Virol. 42, 381–388.
Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.
Posada, D., 2008. JModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.
Purdy, M.A., Talekar, G., Swenson, P., et al., 2007. A new algorithm for deduction of hepatitis B surface antigen subtype determinants from the amino acid sequence. Intervirology 50, 45–51.
Salminen, M.O., Carr, J.K., Burke, D.S., et al., 1995. Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. AIDS Res. Hum. Retroviruses 11, 1423–1425.
Sánchez, L.V., Tanaka, Y., Maldonado, M., et al., 2007. Difference of hepatitis B virus genotype distribution in two groups of Mexican patients with different risk factors. Intervirology 50, 9–15.
Shibayama, T., Masuda, G., Ajisawa, A., et al., 2005. Characterization of seven genotypes (A to E, G and H) of hepatitis B virus recovered from Japanese patients infected with human immunodeficiency virus type 1. J. Med. Virol. 76, 24–32.
Stuyver, L., De Gendt, S., Van Geyt, C., et al., 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J. Gen. Virol. 81, 67–74.
Suchard, M.A., Weiss, R.E., Sinsheimer, J.S., 2001. Bayesian selection of continuous time Markov chain evolutionary models. Mol. Biol. Evol. 18, 1001–1013.
Sugauchi, F., Orito, E., Ichida, T., et al., 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J. Virol. 76, 5985–5992.
Sugauchi, F., Orito, E., Ichida, T., et al., 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 124, 925–932.
Suwannakarn, K., Tangkijvanich, P., Theamboonlers, A., et al., 2005. A novel recombinant of Hepatitis B virus genotypes G and C isolated from a Thai patient with hepatocellular carcinoma. J. Gen. Virol. 86, 3027–3030.
Thompson, J.D., Gibson, T.J., Plewniak, F., et al., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Toan, N.L., Song, L.H., Kremsner, P.G., et al., 2006. Impact of the hepatitis B virus genotype and genotype mixtures on the course of liver disease in Vietnam. Hepatology 43, 1375–1384.
Torres, C., Piñeiro y Leone, F.G., Pezzano, S.C., et al., 2011. New perspectives on the evolutionary history of hepatitis B virus genotype F. Mol. Phylogenet. Evol. 59, 114–122.
Torresi, J., Earnest-Silveira, L., Deliyannis, G., et al., 2002. Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in

the overlapping polymerase gene that are selected by lamivudine therapy. Virology 293, 305–313.
Vieth, S., Manegold, C., Drosten, C., et al., 2002. Sequence and phylogenetic analysis of hepatitis B virus genotype G isolated in Germany. Virus Genes 24, 153–156.
Wang, Z., Liu, Z., Zeng, G., et al., 2002. A new intertype recombinant between genotypes C and D of hepatitis B virus identified in China. J. Gen. Virol. 86, 985–990.
Wu, C., Zhang, X., Tian, Y., et al., 2010. Biological significance of amino acid substitutions in hepatitis B surface antigen (HBsAg) for glycosylation, secretion, antigenicity and immunogenicity of HBsAg and hepatitis B virus replication. J. Gen. Virol. 91, 483–492.
Yang, J., Xing, K., Deng, R., et al., 2006. Identification of Hepatitis B virus putative intergenotype recombinants by using fragment typing. J. Gen. Virol. 87, 2203–2215.
Zehender, G., De Maddalena, C., Giambelli, C., et al., 2008. Different evolutionary rates and epidemic growth of hepatitis B virus genotypes A and D. Virology 380, 84–90.
Zehender, G., Shkjezi, R., Ebranati, E., et al., 2012a. Reconstruction of the epidemic history of hepatitis B virus genotype D in Albania. Inf. Gen. Evol. 12, 291–298.
Zehender, G., Ebranati, E., Gabanelli, E., Shkjezi, R., Lai, A., Sorrentino, C., Lo Presti, A., Basho, M., Bruno, R., Tanzi, E., et al., 2012b. Spatial and temporal dynamics of hepatitis B virus D genotype in Europe and the Mediterranean Basin. PLoS One 7, e37198.