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Global phylodynamics of Echovirus 30 revealed differential behavior among viral lineages

Cristina Lema[a,*], Carolina Torres[b], Sabine Van der Sanden[c], Daniel Cisterna[a], María Cecilia Freire[a], Ricardo M. Gómez[d,**]

[a] Neurovirosis Service at Virology Department, INEI-ANLIS, Dr. Carlos G. Malbran Institute, Argentina
[b] Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina
[c] Department of Vector Development, Arthrogen, Netherlands
[d] Institute of Biotechnology and Molecular Biology, CONICET-UNLP, 1900 La Plata, Argentina

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ABSTRACT

Echovirus 30 (E30) is an important causative agent of aseptic meningitis worldwide. Despite this, the global and regional dispersion patterns, especially in South America, are still largely unknown. We performed an in-depth analysis of global E30 population dynamics, by using the VP1 sequences of 79 strains isolated in Argentina, between 1998 and 2012, and 856 sequences from GenBank. Furthermore, the $3D^{pol}$ regions of 329 sequences were analyzed to study potential recombination events. E30 evolution was characterized by co-circulation and continuous replacement of lineages over time, where four lineages appear to circulate at present and another four lineages appear to have stopped circulating. Five lineages showed a global distribution, whereas three other lineages had a more restricted circulation pattern. Strains isolated in South America belong to lineages E and F. Analysis of the $3D^{pol}$ region of Argentinean strains indicated that recombination events occurred in both lineages.

1. Introduction

Echovirus 30 (E30) belongs to the species *Enterovirus B* (EV-B) of the *Picornaviridae* family. This species includes more than 60 serotypes (Adams et al., 2016) that are ubiquitous in nature and may cause different clinical symptoms. Some serotypes show epidemic circulation patterns, whereas others circulate endemically (Pallansch et al., 2013). E30 is one of the EV-B viruses most frequently detected in clinical specimens. It is associated with large, global outbreaks, but also with sporadic cases of severe infection (Bailly et al., 2009; dos Santos et al., 2011; Oberste et al., 1999a; Palacios et al., 2002; Savolainen-Kopra et al., 2011; Yarmolskaya et al., 2015).

E30 has been frequently associated with meningitis in the pediatric population (Centers for Disease, C., Prevention, 2003; Tao et al., 2014). Less frequently, E30 may be also associated with meningoencephalitis and multiorgan infection that can rapidly lead to a critical status (Bailly et al., 2000; Pino-Ramirez et al., 2008). Despite its clinical impact and its high global prevalence, little is known about the genetic and geographic population dynamics of E30. Continuous molecular epidemiological surveillance is important to help identify newly emerging strains and to better understand trends in viral circulation.

On the basis of nucleotide sequence diversity in the VP1 capsid gene, at least four different classifications of E30 have been proposed (Bailly et al., 2009; Oberste et al., 1999a; Palacios et al., 2002; Savolainen et al., 2001); they agree with each other, with minor differences in some clusters and in the names of lineages. In general, a succession of the different lineages through time is observed, but not an association with geographical region.

Genome recombination is a common event among EVs. Mosaic recombinant genomes emerge by intra- and/or inter-species modular genetic exchanges that may result in new circulating recombinant lineages (Korotkova et al., 2017; Lukashev et al., 2005; Mirand et al., 2007; Muslin et al., 2015; Nikolaidis et al., 2019; Oberste et al., 2004). Recombination mostly occurs in the region encoding non-structural genes, including the $3D^{pol}$ gene, and can be recognized by identifying phylogenetic incongruence between the VP1 and $3D^{pol}$ gene regions (Lindberg et al., 2003; Lukashev et al., 2005; Oberste et al., 2004). McWilliam Leitch et al. described discrete clustering of E30 with other EV-B viruses in the $3D^{pol}$ region (McWilliam Leitch et al., 2009). These descriptions are in line with the observed frequency of recombination

* Correspondence to: INEI-ANLIS-Malbrán, Avenida Vélez Sarsfield 563, 1281 CABA, Argentina.
** Correspondence to: IBBM, Calle 49 y 115, 1900 La Plata, Argentina.
E-mail addresses: clema@anlis.gov.ar (C. Lema), rmg@biol.unlp.edu.ar (R.M. Gómez).

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and the evolution separated into modules of structural and non-structural regions in EV-B and other EVs (Mirand et al., 2007). Although not proven, recombination has been proposed to shape EV lineage replacement through time: recombinant forms emerge, dominate, and disappear over a period of 3–5 years (McWilliam Leitch et al., 2009). The enormous diversity (in both the VP1 and $3D^{pol}$ region) of the EVs that circulate worldwide at any time emphasizes the need to use both VP1 and $3D^{pol}$ genome regions for the epidemiological investigation of E30.

Phylodynamic analyses have been used previously to elucidate epidemic episodes in viral evolution and transmission (Grenfell et al., 2004). However, despite of the large number of E30 studies performed worldwide, detailed information about E30 types circulating in South America is still missing. Only two studies on the molecular epidemiology of E30 in South America (Brazil and Argentina) have been reported (Castro et al., 2009; dos Santos et al., 2011; Farias et al., 2011; Gomes Mde et al., 2007; Mistchenko et al., 2006; Pinto Junior et al., 2009). In particular, the patterns of global and regional dispersion are still unknown, and a comprehensive analysis including all the lineages under flexible demographic scenarios has not yet been carried out.

Therefore, the aim of this work was to analyze the population dynamics of E30 and recombination patterns of strains circulating in Argentina in the context of patterns observed globally.

2. Materials and methods

2.1. Specimen collection and ethics statement

The 79 samples included in this study were collected during the years 1998–2012 (Supplementary Table 1) by medical centers located in distinct areas of Argentina, as part of the routine and mandatory virologic diagnostics of suspected cases of meningitis.

Samples were processed by the Neurovirosis Service at the Virology Section of the INEI-ANLIS Dr. Carlos G. Malbran Institute. At least two strains isolated during periods of elevated virus circulation in two different regions, and all sporadic isolated strains, were selected for this study. Confluent rhabdomyosarcoma (RD) cells were used to isolate and/or amplify virus strains (Bell and Cosgrove, 1980; Perez-Ruiz et al., 2003). Viruses were isolated from 64 cerebrospinal fluid (CSF) samples, 14 stool samples (SS), and one throat sample. This study was performed according to the principles expressed in the Declaration of Helsinki. All samples were de-identified and analyzed anonymously.

2.2. Viral RNA extraction, RT-PCR, and sequencing

RNA extraction was performed using TRIzol (Invitrogen) according to the manufacturer's instructions. Supplementary Table 2 shows the primers used to amplify the VP1 and 3D regions (Chua et al., 2001; Mirand et al., 2007; Oberste et al., 1999b). Amplicon size analysis was performed using gel electrophoresis. Purification of the PCR product was performed using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Sequencing of PCR products was performed using a Big Dye Terminator Cycle Sequencing v3.1 kit (Perkin Elmer Biosystems) in Hitachi 3500 apparatus (Applied Biosystems). The full-length VP1 sequences of 876 nts, corresponding to positions 2460–3335 of Bastianni strain (GenBank: AF311938), and sequences of the $3D^{pol}$ gene of 571 nts, corresponding to positions 5631–6202 of Bastianni strain (GenBank: AF311938), of the 79 Argentinean isolates were deposited in GenBank under accession numbers MK410118 - MK410196 (VP1) and MK410197 - MK410275 ($3D^{pol}$).

2.3. Phylogenetic analysis

To evaluate the genetic relationship among globally circulating E30 strains, a phylogenetic analysis was performed, using VP1 nucleotide sequences (876 nts). The sequences analyzed (n = 935) included 79 strains from Argentina and 619 sequences kindly provided by Bailly [609 from GenBank isolated before 2011 (Bailly et al., 2009) and 10 unpublished sequences], together with 237 GenBank sequences isolated between 2011 and 01/04/2014. The sequences analyzed in this work were retrieved from samples collected between 1956 and 2014. It is worth mentioning that 71% of the sequences were obtained during to the last 20 years. Sixty-six sequences 851 nts long were included because they were the only strains available from Brazil (Latin America). The prototype E21 Farina (AY302547) strain was used as the outgroup (data not shown). Sequences were aligned with BIOEDIT v7.0 (Hall, 1999). Neighbor-joining (NJ) and maximum-likelihood (ML) phylogenetic trees were constructed using MEGA v6.0 (Tamura et al., 2013). Reliability of the trees was tested by a bootstrap analysis (1000 replicates) and RAXML software with a rapid bootstrap algorithm (Stamatakis, 2006; Stamatakis et al., 2008) implemented in the CIPRES Science Gateway server (Miller et al., 2010), respectively.

2.4. Phylodynamic analysis

Bayesian coalescent analyses were performed to study spatio-temporal dynamics along with the substitution rate and the time to the most recent common ancestor (tMRCA) for a selection of E30 lineages. To be able to perform phylodynamic analysis in realistic computational times, a subset of 224 VP1 sequences (out of the 937 available VP1 sequences) representing all branches of the phylogenetic tree was included. The best-fitting nucleotide substitution model was determined with JMODELTEST v2.1 software, using the Akaike information criterion. The selected model was GTR + G4. Phylodynamic analyses were subsequently performed, using the uncorrelated lognormal (UCLN) molecular clock model and a GMRF Bayesian Skyride coalescent model (Drummond et al., 2006, 2012; Minin et al., 2008; Posada, 2008; Rambaut et al., 2013), implemented in the BEAST v1.8.4 software package and carried out on the CIPRES Science Gateway server (Miller et al., 2010).

In addition, a spatial diffusion process was modelled on temporal-scale genealogies of currently circulating lineages (A, E, F, and H). Calibrations were performed by using sampling time and applying an asymmetric discrete phylogeographical model with the country of origin as a state (Lemey et al., 2009). In all cases, convergence was assessed by effective sample size (ESS) values higher than 200 using TRACER v1.6 software (Rambaut et al., 2013), and 10% of the sampling was discarded as burn-in. The uncertainty in parameter estimates was evaluated in the 95% highest posterior density (HPD95%) interval. Visualization of diffusion processes was generated with SPREAD v1.0.7 software, and the geographic centre of each country was taken as the reference coordinate (Bielejec et al., 2011).

2.5. Recombinant analysis

To identify recombination events, the genetic diversity of E30 strains in the $3D^{pol}$ region was studied, using a 379-nt fragment corresponding to positions 5825–6202 of the Bastianni reference strain AF311938, and compared to that in the VP1 region. The analysis included 79 strains from Argentina, all strains (n = 268) included in previously published studies in which a comprehensive recombinant analysis has been performed (McWilliam Leitch et al., 2009), 56 reference strains belonging to EV-B, and five representatives of EV-C and EV-D used as an outgroup. A Maximum Likelihood phylogenetic tree was constructed with the PhyML v3.1 software (Guindon et al., 2010), and confidence was evaluated using the bootstrap method (1000 replicates). The best-fitting nucleotide substitution model was determined with JMODELTEST v2.1 software, using the Akaike information criterion. The selected model was TrN + G4. Tree searching strategy included a BioNJ tree and five random trees as initial points, and the best SPR and NNI branch swapping strategy.

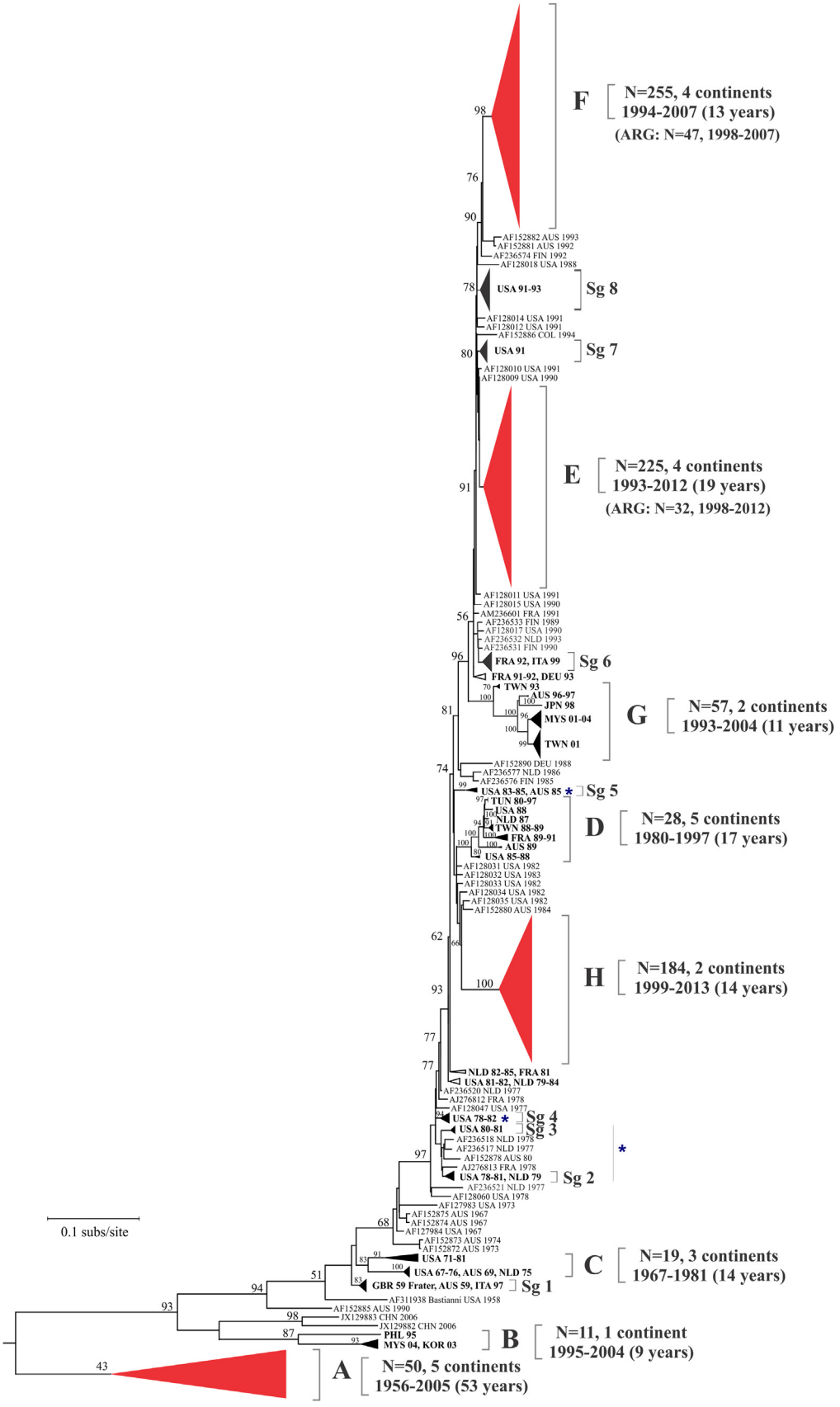

Fig. 1. Phylogenetic analysis by maximum likelihood (ML) of VP1 complete sequences (876 nts) of E30. The bootstrap values are shown at nodes for relevant groups. Lineages further studied are indicated by red triangles, and the small groups are indicated by black triangles. The sequences that were considered part of lineage D (Bailly et al., 2009) are marked with asterisks. Unassigned sequences are shown with white triangles for visualization purposes. The countries of the strains, abbreviated to three capital letters, and the last two digits of the year are indicated. The Farina strain of E21 AY302547 (not shown) was used as an outgroup. Sg: small group. Country codes: ARG: Argentina, AUS: Australia, AUT: Austria, AZE: Azerbaijan, BEL: Belgium, BGD: Bangladesh, BHR: Bahrain, BLR: Belarus, BRA: Brazil, CAF: Central African Republic, CHE: Switzerland, CHN: China, COL: Colombia, DEU: Germany, DNK: Denmark, EST: Estonia, FIN: Finland, FRA: France, GBR: United Kingdom, GEO: Georgia, IND: India, ISR: Israel, ITA: Italy, JAM: Jamaica, JPN: Japan, KGZ: Kyrgyzstan, KOR: Korea, LTU: Lithuania, LVA: Latvia, MAR: Morocco, MDA: Moldova, MYS: Malaysia, NLD: Netherlands, PHL: Philippines, POL: Poland, PRI: Puerto Rico, RUS: Russia, SWE: Sweden, TUN: Tunisia, TWN: Taiwan, UKR: Ukraine, USA: United States, UZB: Uzbekistan, VNM: Vietnam. Strains from Russia were assigned to Europe, because most of them belong to the European part of the country.

3. Results

3.1. Phylogenetic analysis of E30

Phylogenetic reconstruction of E30 sequences (Fig. 1) clustered most of them (88.7%) into eight previously described monophyletic groups or lineages (A to H), with (except for group A) significant bootstrap support (values higher than 70%). Within each lineage, subgroups of geographically and temporally linked strains were observed with high bootstrap values. In addition, eight small groups were formed, that consisted of a few sequences (3–9 sequences) or by sequences belonging to the same country (up to 11 sequences). Finally, 59 sequences did not cluster with any lineage or group already described (Fig. 1). In general, the ML tree suggested an epochal evolution with the continued replacement of prevailing subgroups. Especially in the lower part of the tree, a ladder-like structure in the evolution of new lineages is observed (one lineage evolves from the other over time) which complicates the assignment of intermediate strains to specific lineages. Some strains that were previously assigned to lineage D (3) are clustered differently (asterisks in Fig. 1). Lineages differed in the number of strains, in their genetic variability, in the time span, and in the geographical regions in which they have circulated (Table 1). Regarding the geographical distribution, lineages A, C, D, E, and F presented a wide global distribution (three or more continents), whereas lineages B, G, and H showed a more restricted geographic distribution (one or two continents). The sequences belonging to the small groups and the individual ones have been detected in three and four continents, respectively (Table 1).

Phylogenetic lineages and subgroups showed a temporal distribution, with multiple subgroups sometimes co-circulating at the same time. The most recently isolated strains (between 2007 and 2013) belonged to lineages A, E, F, and H, whereas lineages B, C, D, and G stopped circulating in 2004, 1981, 1997, and 2004, respectively.

A phylogeographic analysis was performed for lineages A, E, F, and H in order to better understand the evolutionary behavior of recently circulating E30 strains. Interestingly, lineage B, with 11 representative strains, was only detected for a short period of time (9 years) in three Asian countries, whereas lineage G, with 57 strains, circulated in three Asian countries for 11 years and in Australia for 1 year. In addition, lineages C and D, with wide geographic distribution, were detected in three and six countries, respectively, but they circulated in different continents. Strains that did not belong to lineages A–H were detected in America, Europe, Asia, and Oceania before 2007 (Table 1).

3.2. Phylodynamic analysis of E30

A phylodynamic analysis was performed with a subset of 224 VP1 nucleotide sequences (876 nts) representing all lineages, groups, subgroups, and single sequences defined in the phylogenetic analysis (n = 935). According to this analysis (Fig. 2), the MRCA of the E30 strains analyzed would have started its diversification in 1937 (HPD95% = 1930–1942), with a global substitution rate of 4.5×10^{-3} substitutions/site/year (s/s/y) (HPD95% = 4.2–4.9×10^{-3}).

Lineage A showed a worldwide distribution (five continents) and is also the oldest group detected with a tMRCA estimated in 1948 (estimated year with the complete data set 1943, Fig. 3) and strains isolated over a 53-year time span. In contrast, other lineages like E and F acquired their diversity and geographic distribution in a significantly shorter period of time (from the 1990s to the present). Moreover, the broadly distributed lineages C and D and the more circumscribed B and G were estimated to have started diversifying in 1961, 1982, 1984, and 1990, respectively. Lineage H, currently detected in Asia, would have started its diversification in 1994, similar to lineages E and F (Fig. 2). Lineage D appears to be the ancestor of lineage H, with a narrow distribution and with the oldest strains seeming to have found their niche in two countries of Eastern Europe (Belarus and Ukraine) and five countries of Asia (lineage H). On the contrary, lineage G, distributed in only four countries (Taiwan, Malaysia, Japan, and Australia), could be the ancestor of lineages E and F, having the largest number of strains distributed worldwide.

3.3. Phylogeographic and demographic reconstruction of E30 lineages A, E, F, and H

In order to compare the evolutionary trajectory of recently circulating strains, the phylogeographic and demographic patterns of lineages A, E, F, and H were further analyzed. The analysis of lineage A (Fig. 3) showed that the most probable ancestral location would be the USA (ppas = 1), and its diversification would have begun in 1943 (HPD95% = 1937–1948), with a substitution rate estimated as 7.5×10^{-3} s/s/y (HPD95% = 6.3–8.8×10^{-3}). The highest posterior probability of this group (pp = 1) contributes to the statistical support to consider it as a defined lineage, despite the low bootstrap value obtained in the phylogenetic analysis by ML. This lineage showed two subgroups (Fig. 3a), with a probable origin in 1951 and 1947, respectively. Subgroup 1 includes a cluster with strains exclusively from the USA, and a single strain from Puerto Rico isolated in 1959. This subgroup seems to be extinct. Subgroup 2 showed an apparently extinct cluster with old strains from Australia (1956–1959), and a second cluster possibly originated in 1963 in the USA, of which the most recent strain was isolated in 2009. This cluster showed a wider geographical dispersion and includes strains from Africa, Eastern Europe, Colombia, and, since 2009, India (representing the only reported E30 sequences from India until now). Although there is an apparent period of several years without reported isolation of strains that belong to this cluster (1977–1997), in the following years, multiple strains have been isolated in different regions of the world. However, constant viral diversity of the lineage over time was observed (Fig. 3b), indicating a cryptic circulation of this lineage.

The analysis of lineage E (Fig. 4) showed that the most probable ancestral location would be the USA (ppas = 0.89), and its diversification would have begun in 1991 (HPD95% = 1990–1992), with a substitution rate estimated at 6.2×10^{-3} s/s/y (HPD95% = 5.5–7.1 × 10^{-3}). The process of spatial and temporal diffusion of lineage E is complex and, over time, it has spread to distant geographic regions, uni- and bidirectionally. This lineage presented three subgroups with a probable origin in 1992 and 1994. In each subgroup, internal clusters with diversification at different times were observed (Fig. 4a). The first strains of subgroup 1 may have originated between 1993 and 1996, then, probably from the USA, they arrived in Brazil, where different outbreaks occurred between 1998 and 2002 (Pinto Junior et al., 2009). Later, strains of the same cluster were detected in Jamaica and Australia in 2005. The second cluster circulated in China, Vietnam, and Korea between 2005 and 2010. Subgroup 2 showed a high support value (pp = 0.94) and two clusters. Strains of the first cluster were isolated in Europe (France, Italy, the Netherlands, and Russia, among others) from 1994 to 2005. The second cluster, with an apparent origin in France (pp = 1), comprised sequences that circulated in South America. First, it was detected in Argentina from 1998 until 2004; then it circulated in Brazil where it caused outbreaks in 2005 and 2008, and sporadic cases in 2003, 2006, and 2007. Finally, the most recent strains were detected in different regions of Argentina in 2011 and 2012 (Fig. 4). Subgroup 3, with few representative sequences, separated into two clusters. One of them circulated in France during 1997, the other in Europe and Asia between 1997 and 2000, and later in 2006–2007 in Australia. The Argentine strain of 2008 (BA66/08) was genetically closely related to the Australian strains. In summary, lineage E presented three subgroups that co-circulated in different regions of the world and presented internal clusters with diversification processes at different times. South American strains were all found in the same cluster of subgroup 2, except for a single strain (BA66/08) found in subgroup 3. Peaks of high diversity of lineage E strains in 1997–1999,

Table 1
Summary of the number of strains, year and country for lineages, small groups and unassigned sequences found in the phylogenetic analysis of E30. For Country code, see caption in Fig. 1.

Lineages	N° samples	Year	Country	Continent
A	**50**	**53 years (circulate)**	**14 countries**	**5 (wide)**
		1956–1959	AUS	Oceania
		1957–1977, 1995	USA, PRI, COL	America
		1996–2005	TUN, MAR, CAF	Africa
		2000–2009	BGD, UZB, KGZ, IND	Asia
		2003–2005	GEO, UKR, RUS	Europe
B	**11**	**9 years (apparently extint)**	**3 countries**	**1 (limited)**
		1995–2004	PHL, KOR, MYS	Asia
C	**19**	**14 years (apparently extint)**	**3 countries**	**3 (wide)**
		1967–1981	USA	America
		1969	AUS	Oceania
		1975	NLD	Europe
D	**28**	**17 years (apparently extint)**	**6 countries**	**5 (wide)**
		1985–1988	USA	America
		1987–1991	NLD, FRA	Europa
		1980–1997	TUN	Africa
		1988–1989	TWN	Asia
		1989	AUS	Oceania
E	**225**	**19 years (circulate)**	**20 countries**	**4 (wide)**
		1993–2012	USA, JAM, ARG, BRA	America
		1993–2010	BHR, KOR, CHN, VNM, TWN	Asia
		1994–2006	FRA, ITA, BEL, NLD, DNK, CHE, MDA, UKR, RUS, BLR	Europe
		2005–2007	AUS	Oceania
F	**255**	**13 years (circulate)**	**22 countries**	**4 (wide)**
		1994–2007	FRA, ITA, NLD, DNK, DEU, CHE, FIN, AUT, LTU, SWE, POL, LVA, EST, BEL, BLR, RUS	Europe
		1997–2007	ISR, KGZ	Asia
		1998–2007	ARG, BRA, JAM	America
		1999–2006	TUN	Africa
G	**57**	**11 years (apparently extint)**	**4 countries**	**2 (limited)**
		1993–2004	TWN, MYS, JPN	Asia
		1996–1997	AUS	Oceania
H	**184**	**14 years (circulate)**	**7 countries**	**2 (limited)**
		1999–2004	BLR, UKR, RUS, GEO	Europe
		1999–2013	AZE, CHN, TWN	Asia
Small groups	**47**	**38 years (apparently extint)**	**6 countries**	**3 (wide)**
Sg 1	3	1959–1997	GBR, ITA, AUS	Europe, Oceania
Sg 2	9	1978–1981	USA, NLD	America, Europe
Sg 3	4	1980–1981	USA	America
Sg 4	11	1978–1982	USA	America
Sg 5	5	1983–1985	USA, AUS	America, Oceania
Sg 6	4	1992–1999	FRA, ITA	Europe
Sg 7	3	1991	USA	America
Sg 8	8	1991–1993	USA	America
Sequences unassigned	**59**	**48 years (apparently extint)**	**8 countries**	**4 (wide)**
		1958–1994	USA, COL	America
		1967–1993	AUS	Oceania
		1977–1993	NLD, FRA, DEU, FIN	Europe
		2006	CHN	Asia

2002–2003, 2005, and 2007–2008 correlated with increased viral activity in Argentina (Fig. 4b). It should be noted that in recent years, 2011–2012, the increase in E30 circulation in this country did not coincide with an increase in viral diversity.

The phylogeographic analysis of lineage F (Fig. 5) showed that the most likely ancestral location for sequences included would be the Netherlands (ppas = 0.98), and its tMRCA was dated in 1994 (HPD95% = 1993–1994) with a substitution rate estimated at 5.6×10^{-3} s/s/y (HPD95% = 4.6–6.4 $\times 10^{-3}$). The geographic and temporal dispersion process of this lineage is simpler than that of lineage E. Strains of lineage F circulated in both Europe and Asia, arriving in Central and South America from Europe. This lineage was not detected in North America or Oceania (Fig. 5a). Lineage F also presented three subgroups with high support, all of which are likely to have originated in the Netherlands (ppas = 0.99, 0.99, and 1, respectively). The origin of the diversification of these groups was estimated to be in 1994 for subgroup 1 and in 1995 for subgroups 2 and 3 (Fig. 5a). Subgroup 1 mainly circulated in several European countries until 2000. Subgroup 2 was detected between 1996 and 1998 in Central European countries, and subsequently between 2000 and 2004 in Russia and Belarus. Circulation of subgroups 1 and 2 seemed to be replaced by circulation of subgroup 3 strains, of which strains from the Netherlands (1996–1997) and Argentina (1998, an outbreak in Mendoza province) could be the ancestor. This subgroup presented two branches (A and B) with strains that co-circulated and showed an epochal evolution characterized by cluster displacement. Branch A comprised mainly sequences from France isolated between 2000 and 2006 along with strains from South America. The strains that circulated in South America (Fig. 5a) appeared to have their origin in France in 1999 (ppas = 1). The Argentine strains in this branch were isolated from sporadic cases in 2001. During 2003–2004, there was a significant increase in viral activity in Argentina, and two clusters were detected: one major one with a more restricted geographic distribution, and a smaller cluster with few representative sequences but with a wider geographic dispersion since it was found in Brazil in 2006 and again in Argentina in 2007. Branch B was composed of strains from Europe, North Africa, and Asia, isolated between 2001 and 2008. No strains of the F lineage were detected after 2008. High viral diversity for lineage F was observed in 1995–1997,

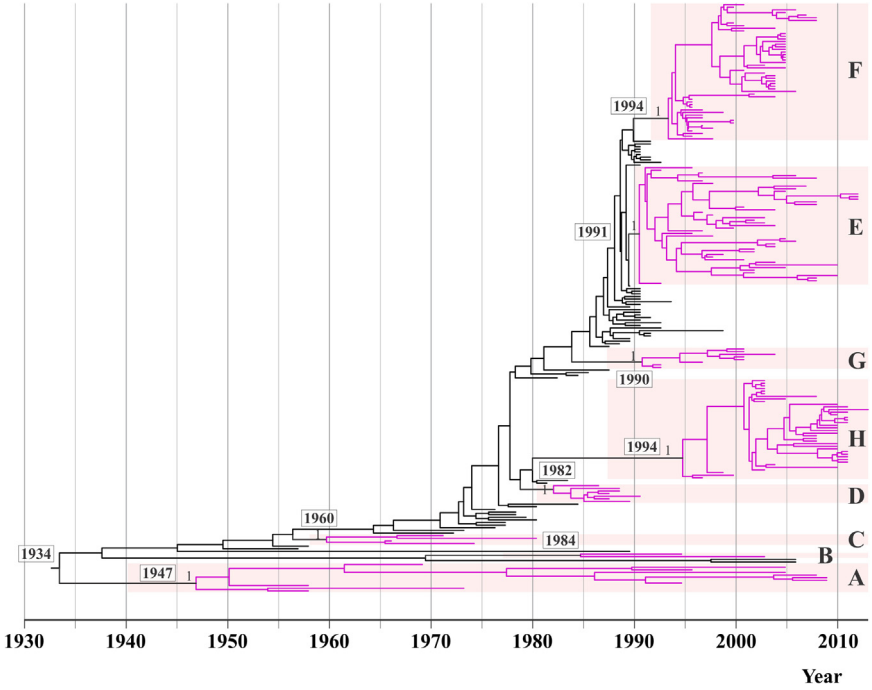

Fig. 2. Maximum clade credibility tree for Bayesian coalescent analysis from 224 sequences representing all branches of the E30 phylogenetic tree. The previously defined lineages A-H are marked by a colored box. Branches are in time scale (year). Posterior probabilities are shown at nodes.

2000, 2002–2003, and 2005 (Fig. 5b). The succession in time of subgroups and clusters correlated with increases of higher viral diversity followed by periods of a marked decrease.

Analysis of lineage H (Fig. 6) showed that Belarus would be the most likely ancestral location, although with a low probability (ppas = 0.42), followed by Georgia (ppas = 0.25), suggesting that the information available is not sufficient to solve the geographical origin of this lineage. The tMRCA was dated in 1996 (HPD95% = 1995–1997), with an estimated substitution rate of 6.3×10^{-3} s/s/y (HPD95% = 5.2–7.4 $\times 10^{-3}$). This lineage (Fig. 6a) is formed by sequences from Eurasian countries isolated in 1997–2000 that could have been the ancestors of a large cluster of strains isolated in China between 2003 and 2011, with a probable origin in Azerbaijan (ppas = 0.76). Two sequences from Russia from 2004 and from Taiwan from 2005 were genetically interspersed with the Chinese strains in this cluster. Within this cluster, a replacement of viral variants over time was observed. The viral diversity (Fig. 6b) of this cluster presented two peaks around 2008 and 2011, when multiple sub-clusters coexisted.

3.4. Recombination analysis of E30

To analyze the potential role of recombination in the evolution of Argentine strains, a phylogenetic analysis of the $3D^{pol}$ region was performed, and clustering in this region was compared to that in VP1 (Figs. 7 and 8). In total, 408 strains for which the $3D^{pol}$ sequence was available (79 from Argentina and 329 from other countries) were included to study the genetic relationship between the Argentine strains and those from other parts of the world. The Argentinian strains were the sole strains from Latin America for which a $3D^{pol}$ region was available.

A dissimilar clustering from that obtained in the VP1 region was observed (Figs. 7 and 8). Three major groups (one group formed by F2 strains, a second group composed of E1-E2-F2 strains, and a third group with E1-F1-F2-E4 strains) and two unique sequences were identified (Figs. 7 and 8).

The first group, called F2 in our work, belonged to lineage F in VP1 (Fig. 8) and was formed by one Argentine strain from 2001 and 39 strains from an outbreak that occurred in Argentina between 2003 and 2004 (Fig. 8). These strains grouped with the strains of recombinant form P (RF-P) strains already described (McWilliam Leitch et al., 2009), which were a predominant variant in Europe in 2001. The second group (Figs. 7 and 8) contained strains of four different groups: F1 (composed of two strains from Mendoza, isolated in 1998), a single strain of the F2 group from the year 2006 (Fig. 7), E1 (two strains from Mendoza, 1998), and E4 (18 strains that circulated in 2011 and 2012) (Figs. 7 and 8). This group showed a high bootstrap value and was associated with the described strain D (McWilliam Leitch et al., 2009). The third group contained strains of three groups: E1 (the five strains from Buenos Aires province from 1998), E2 (five strains from 2001 to 2004) (Figs. 7 and 8), and F2 (four strains from 2006 to 2007) (Figs. 7 and 8), and clustered with the previously reported recombinant forms AO strain from 2007 and H from 2002. The strains BA66-ARG08 (E3) and Chaco151-ARG12 (E4) clustered separately from the other Argentine strains, and BA66-ARG08 is grouped with AI strain.

In summary, two groups are made up of Argentine strains of lineages E and F defined in the phylogenetic tree of the VP1 gene. This suggests that recombination events occurred between the strains of both lineages. In addition, within the lineages defined in VP1, the different groups of VP1 are grouped with different recombinant forms (F2 strains are found that are grouped with the strains named P, D, and the third group AO and H, and sequences of E1 are grouped with D and AO), suggesting that recombination events also occurred within lineages.

3.5. Regional strains

The first strains detected in Argentina dated from 1998 and were isolated from two outbreaks, one located in the province of Buenos Aires and the other in the province of Mendoza. The strains from Mendoza belonged to lineages E and F (Figs. 7 and 8). The ancestor of the strains of lineage E (subgroup 2, cluster 2) would have been circulating in France in 1995, and those of F (subgroup 3) in the Netherlands in 1995. However, in the $3D^{pol}$ region, these strains grouped together in the group named as D before (McWilliam Leitch et al.,

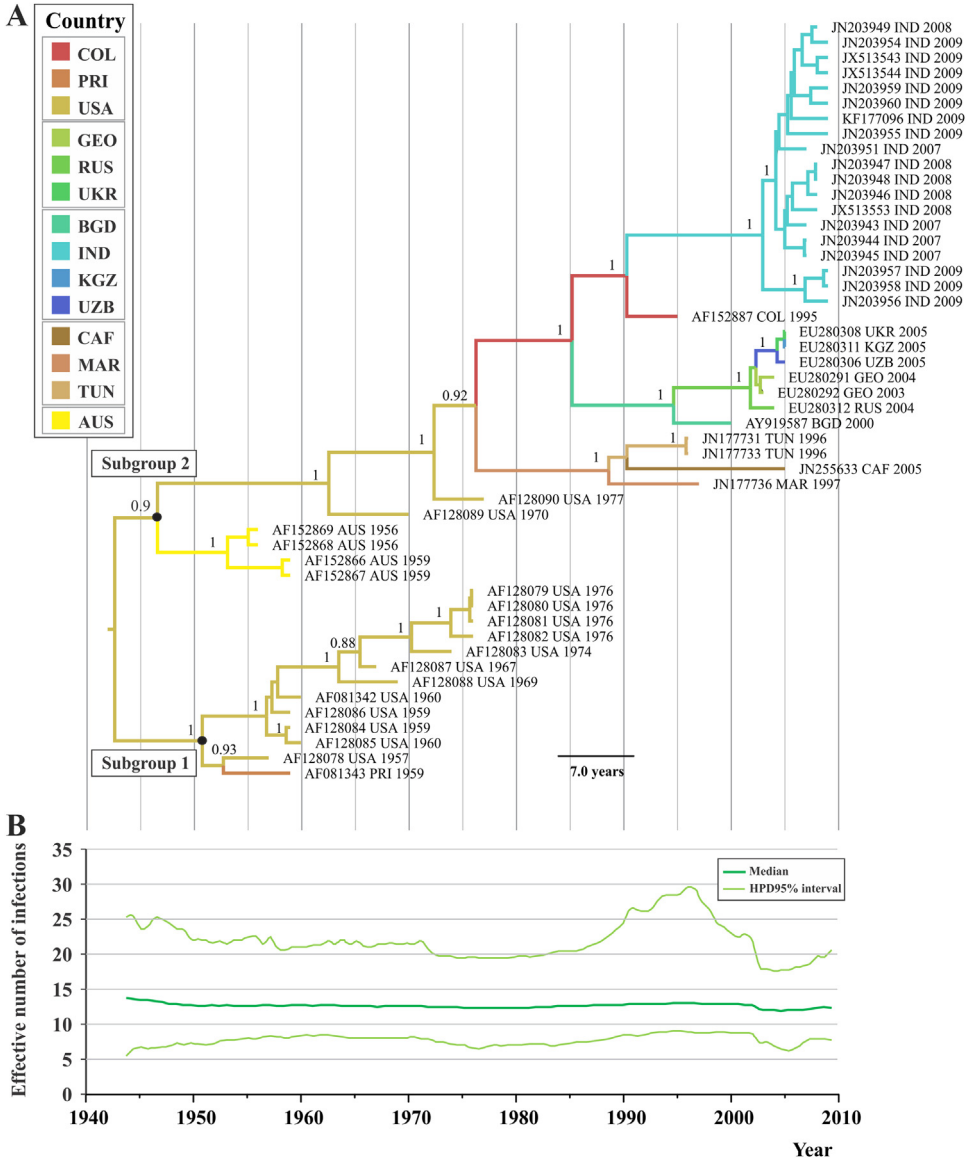

Fig. 3. Maximum clade credibility tree (a) and demographic reconstruction (b) of lineage A. The color of the branches represents the most probable country of origin. The temporal scale of the tree coincides with that of demographic reconstruction. Posterior probability values are shown at nodes for relevant groups. Nodes for proposed subgroups have been marked with black dots. For country codes, see caption for Fig. 1.

2009), which would mean that at least two lineages (in VP1) of European circulation shared the same $3D^{pol}$ sequence, and both also circulated in Argentina. Subsequently, the outbreak with the largest number of cases caused by E30 in Argentina occurred in 2003–2004. Most of these strains belonged to lineage F and were genetically closely related to the FR-P group in the $3D^{pol}$ region. The strains of FR-P group circulated predominantly in Europe a couple of years earlier (2001). Some strains isolated in Argentina during this outbreak belonged to lineage E and clustered differently in the $3D^{pol}$ region (AO, H). In the following years, sporadic cases were detected in Argentina, and isolated strains all belonged to lineage F and to two different groups in the polymerase region, except for one strain from 2008 that was not related to any other strain that circulated in the country. Finally, the last outbreak recorded between 2011 and 2012 corresponded to strains of lineage E, and all but one grouped together in the polymerase region. Therefore, in Argentina, the co-circulation of lineages and different possible recombinants was observed even within the same outbreak.

4. Discussion

This study describes a comprehensive analysis of the molecular epidemiology and population dynamics of E30 worldwide, including, for the first time, strains isolated in South America. In line with previous studies, this work showed that E30 lineages differ in their spatiotemporal dynamics. Lineages A, C, D, E, and F present a global distribution, whereas lineages B, G, and H present a more restricted circulation. Strains that were isolated in more recent years belong to lineages A, E, F, and H. Phylodynamic analysis using the available VP1 sequences estimated that the MRCA of E30 began to diversify in 1934. This viral diversification which was also associated with a wide geographical dispersion could be related to the more frequent human movements since that period. Differences in geographical distribution were observed. Lineages B and G were detected in short periods of time, mainly in Asian countries, meanwhile lineages C and D spread in different continents.

The current study shows that lineage B had a low prevalence and has not been detected since 2004. However, other authors found that

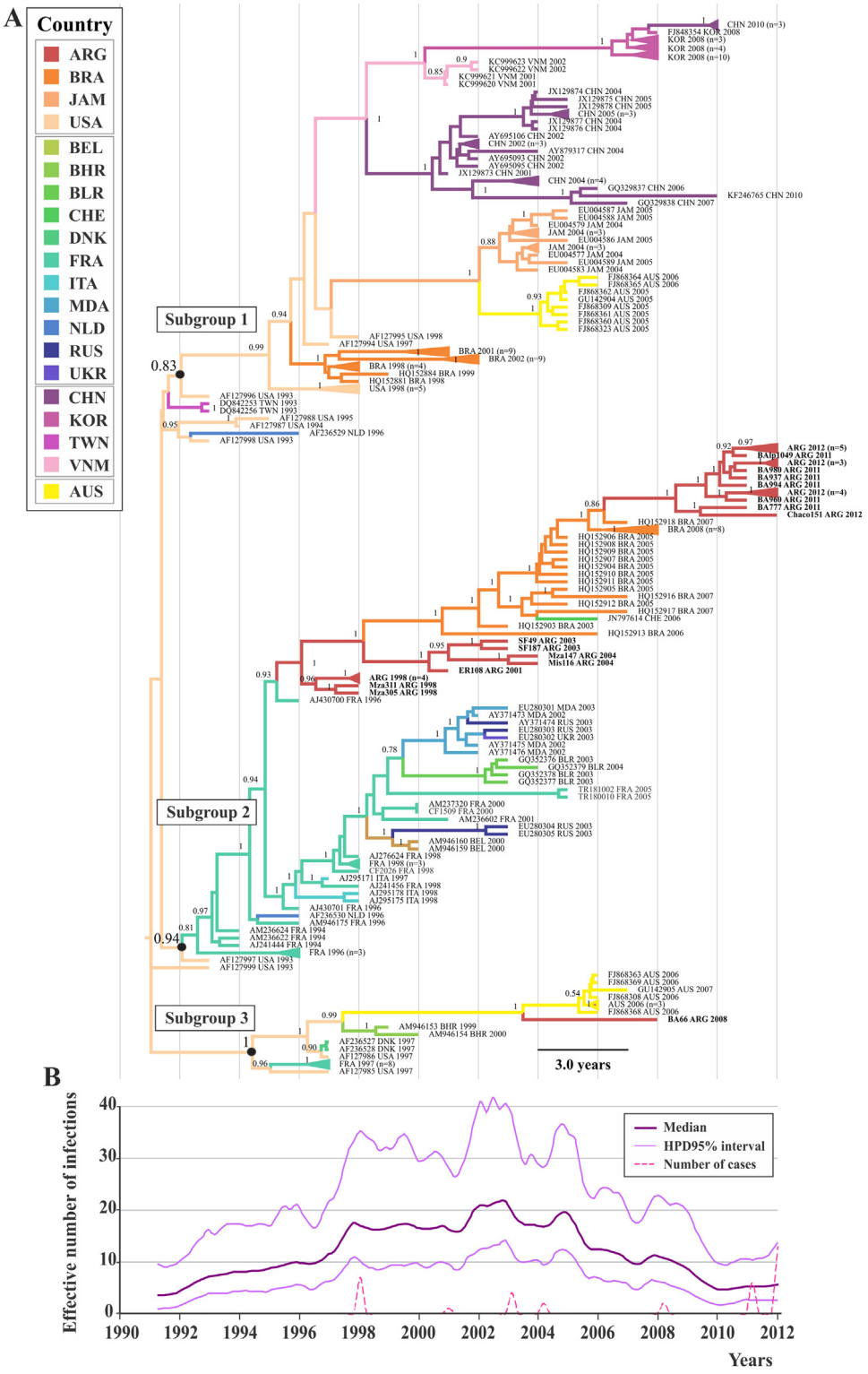

Fig. 4. Maximum clade credibility tree (a) and demographic reconstruction (b) of lineage E. The color of the branches represents the most probable country of origin. The temporal scale of the tree coincides with that of demographic reconstruction. Posterior probability values are shown at nodes for relevant groups. Argentine strains are shown in bold. The number of cases was plotted superimposed on viral demographics. Nodes for proposed subgroups have been marked with black dots. For country codes, see caption for Fig. 1.

strains of E30 obtained from sewage samples and from two clinical cases (gastroenteritis and upper respiratory illness) in 2010 grouped monophyletically with those belonging to the lineage B from Korea, Malaysia, and Japan, suggesting that this lineage continued to circulate at least until 2010 (Iwai et al., 2011). These strains could not be included in the analyses performed in the current study as only partial VP1 sequences were available. Lineage G circulated in Taiwan in 1993 and 2001, in Australia in 1997, and in Malaysia in 2001 and 2004 (Choi et al., 2010; Ke et al., 2011; Oberste et al., 1999a; Wang et al., 2002; Xiao et al., 2013; Zhao et al., 2006), indicating a limited geographic

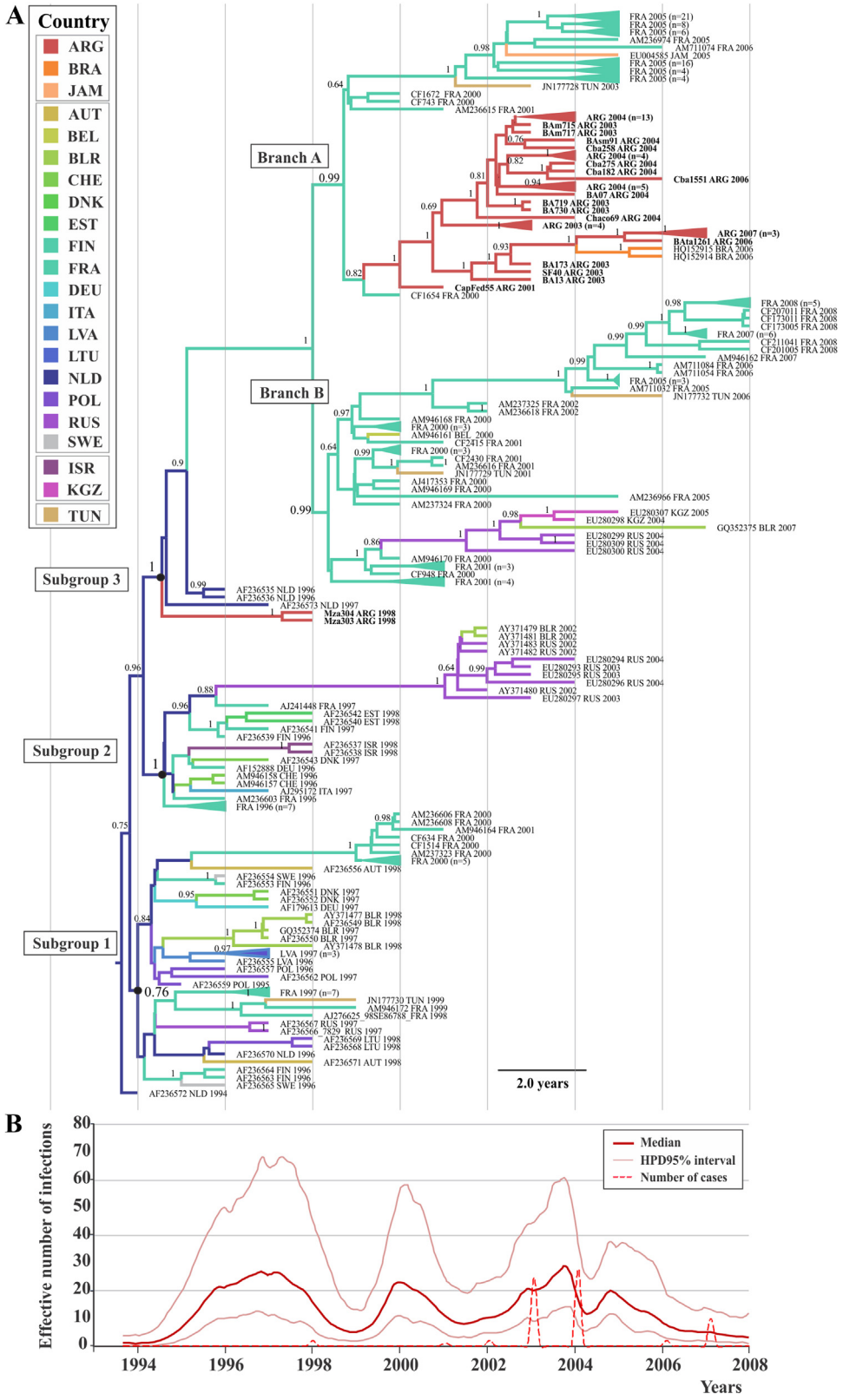

Fig. 5. Maximum clade credibility tree (a) and demographic reconstruction (b) of lineage F. The color of the branches represents the most probable country of origin. The temporal scale of the tree coincides with that of demographic reconstruction. Posterior probability values are shown at nodes for relevant groups. Argentine strains are shown in bold. The number of cases was plotted superimposed on viral demographics. Nodes for proposed subgroups have been marked with black dots. For country codes, see caption for Fig. 1.

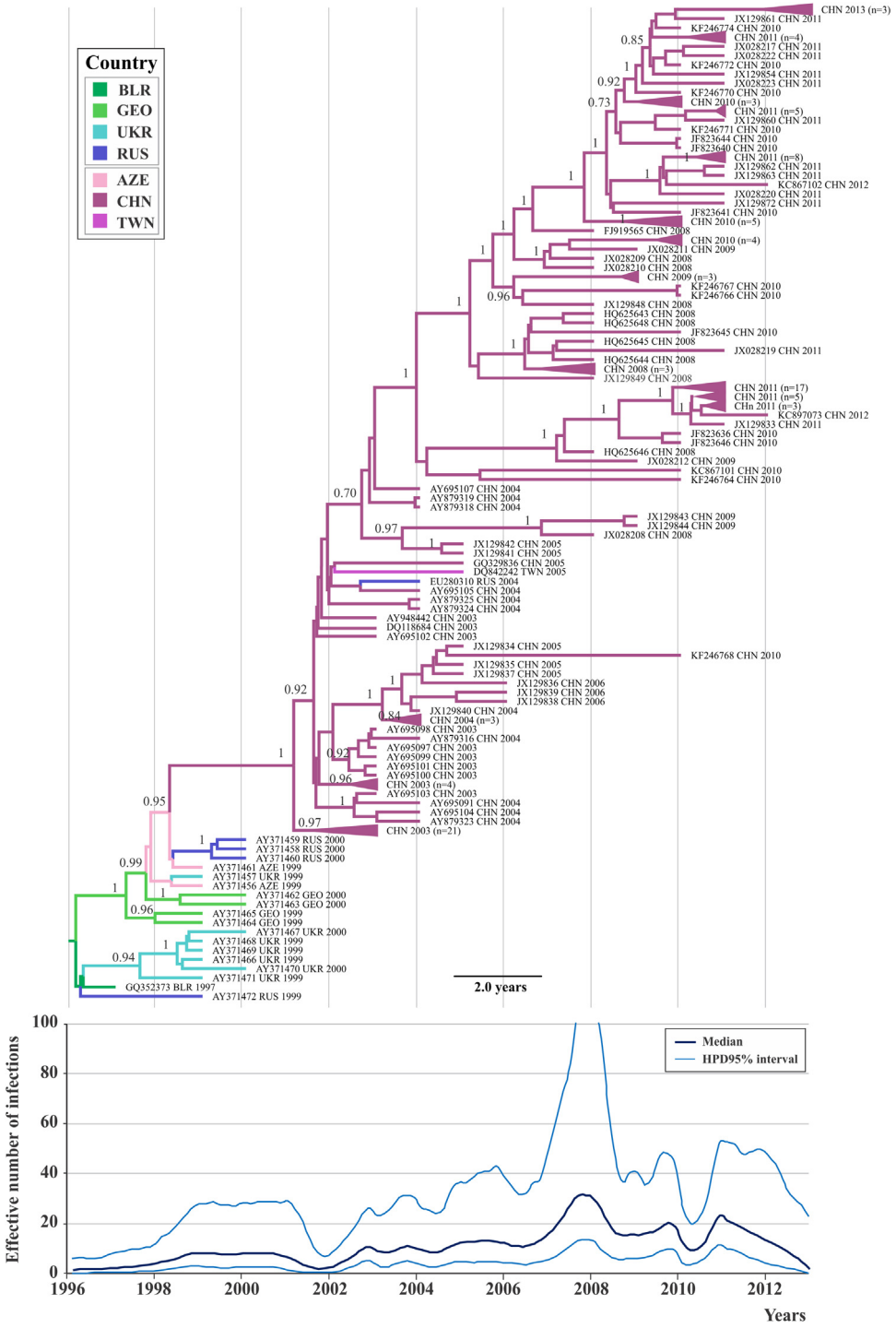

Fig. 6. Maximum clade credibility tree (a) and demographic reconstruction (b) of lineage H. The color of the branches represents the most probable country of origin. The temporal scale of the tree coincides with that of demographic reconstruction. Posterior probability values are shown at nodes for relevant groups. For country codes, see caption for Fig. 1.

distribution in Asia. Lineages G, H, and B mainly circulated in Asia, and only H still circulates. Lineages C and D were described by Ke et al. (Ke et al., 2011), showing that lineage C has high diversity and a long period of distribution (1958–1981), although, unlike this work, they included the Bastianni strain in this group. Lastly, lineage D with few representatives became apparently extinct (1992), and the ancestor was estimated in 1982. This lineage was detected in North Africa, and its contribution in Asia is scarce (TWN 88–89).

For those in active circulation, a phylodynamic analysis that included phylogeographical estimations was performed. According to the current study, the most probable ancestors of each lineage started circulating in the USA (lineages A and E), the Netherlands (lineage F), and Belarus (lineage H). However, these results should be interpreted with caution as the enterovirus surveillance in the USA and the Netherlands has been more systematic than in other regions, which might have affected the estimation of the most probable ancestral locations.

Lineage A has been previously described (Ke et al., 2011; Lukashev et al., 2008; Oberste et al., 1999a; Palacios et al., 2002; Yarmolskaya et al., 2015), but in this work, it was shown that it presented a different behavior to the other E30 lineages described, being the oldest lineage

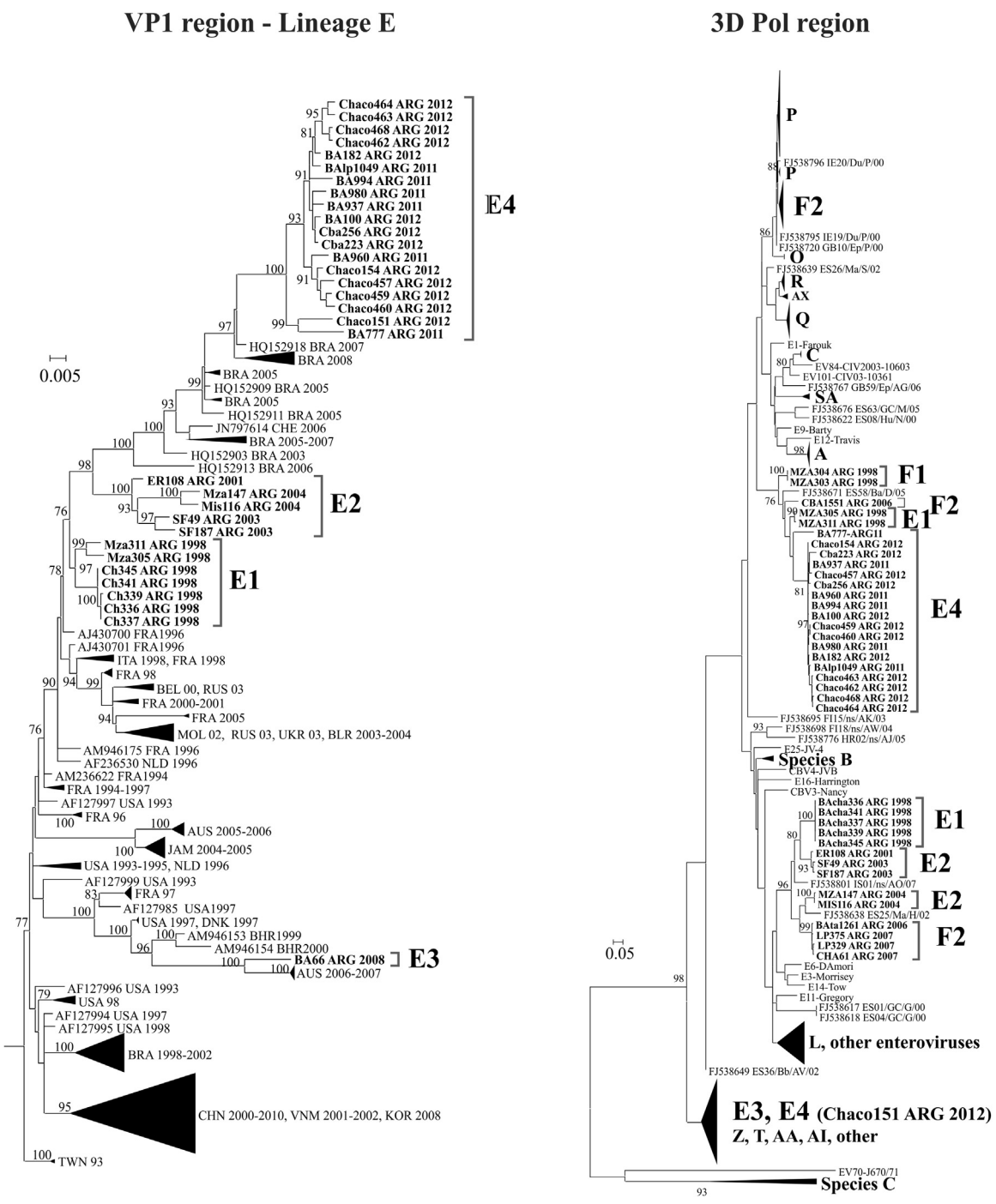

Fig. 7. Phylogenetic analysis by maximum likelihood of VP1 and $3D^{pol}$ sequences of lineage E E30. The bootstrap values are shown at nodes for relevant groups.

that has been circulating for more than 53 years, with worldwide distribution. In contrast to lineages E, F, and H, the Bayesian skyline plot of lineage A showed a constant effective number of infections over time, suggesting an endemic circulation, as previously suggested (Yarmolskaya et al., 2015). This behavior has been shown to differ from that observed for other lineages like E, F, and H which showed a more epidemic circulation. The most modern strains of this lineage (1997–2009, cluster 2 of subgroup 2) had not been described so far, dismissing the conclusion that lineage A is not currently circulating. The comprehensive analysis of the phylogeny of E30, which included sequences not previously analyzed, showed the circulation of this lineage in low proportions in comparison with the number of strains of the other lineages, and its spread to distant geographic areas such as North Africa and Asia. Shorter sequences previously reported as related to those classified as Genotype I (Palacios et al., 2002) also support the current circulation and the change in its distribution pattern (McWilliam Leitch et al., 2009; Savolainen-Kopra et al., 2011). It is not known if this genotype has an evolutionary advantage that allowed it a wider and longer circulation than the other lineages, or if it presents a different clinical behavior.

Lineage E has been described previously (Choi et al., 2010; dos Santos et al., 2011; Ke et al., 2011; Kim et al., 2012; Lukashev et al., 2008; Takamatsu et al., 2013; Tao et al., 2014; Wang et al., 2002; Yang et al., 2013; Zhao et al., 2006), but this is the first work in which all available sequences (n = 225) have been included. Although clusters with concomitant diversification processes were observed at different

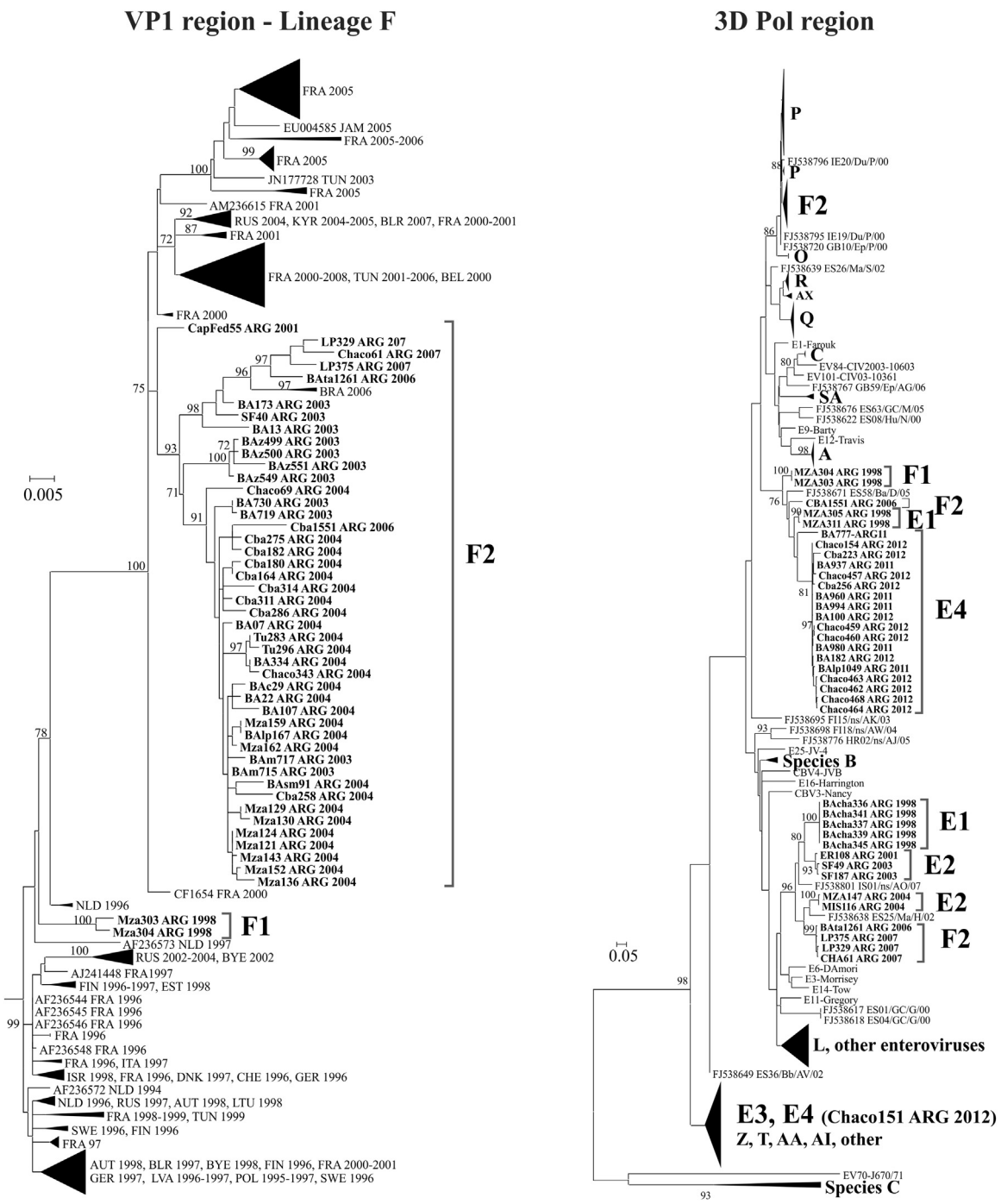

Fig. 8. Phylogenetic analysis by maximum likelihood of VP1 and $3D^{pol}$ sequences of lineages F E30. The bootstrap values are shown at nodes for relevant groups.

times, periods without virus detection were observed. The absence of detection of virus descendant from some ancestral strains could be due to the fact that they could have become extinct during the evolution of this lineage, being replaced by other strains, or they may have circulated in a smaller proportion than other strains and could not have been detected and sequenced, preventing their inclusion in the analyses. It is possible that a larger sampling, especially of older strains, allows higher precision in the estimates of these analyses.

The analysis of lineage F (dos Santos et al., 2011; Ke et al., 2011; Lukashev et al., 2008), composed of 255 sequences, showed that although there was a concomitant and more ancient circulation of subgroups 1 and 2, these appear to have been displaced by subgroup 3, with some overlaps. A decrease in the circulation of this lineage is suggested since there are no strains detected after 2008. The behavior of this lineage is different from that observed with lineage E that presented a more diverse structure in which different subgroups coexisted at different times, and although there were periods of greater diversity (1997–2000, 2002, 2004) they were not as marked as in lineage F. In Argentina, there is no formal epidemiological surveillance system and only E30 strains from suspected cases of meningitis are isolated and characterized. Circulation of strains belonging to lineages other than E and F might therefore have gone unnoticed. The E and F strains that were isolated in Argentina are closely related to those isolated in the Netherlands and France, indicating a global distribution of those lineages.

Lineage H (Choi et al., 2010; Ke et al., 2011; Lukashev et al., 2008;

Tao et al., 2014; Xiao et al., 2013; Yang et al., 2013; Zhao et al., 2006) clearly showed a widespread geographical distribution between strains, and it was mostly formed by strains from China with probable ancestors located in Russia and the Commonwealth Independent States.

This demonstrates that some E30 lineages do show a geographic association, as was previously proposed (Yarmolskaya et al., 2015). According to our analysis, the strains from South East Asia clustered in five lineages: B, D, E, G, and H. It should be mentioned that all strains from China belonged to lineages E and H, which is in line with previous observations (Xiao et al., 2013; Yang et al., 2013; Zhao et al., 2006). The lineages with limited geographical distribution (B, G, and H) were detected almost exclusively in South East Asia; lineage G was also detected in Australia (only strains from 1996 and 1997), and lineage H was also found in Russia, Belarus, and Ukraine.

Within lineage A, subgroup 1 and/or cluster 1 of subgroup 2 would appear to be the ancestors of cluster 2 of subgroup 2. Lineage C could have co-circulated with the ancestors of the lineages D–H and could have been replaced by them in the 1980s. Lineage D – apparently extinct – may have been replaced by lineage H, and lineages E and F co-circulated with lineage G – also apparently extinct – but then they replaced it and were distributed globally. More recently, within lineage F, subgroups 1 and 2 also appear to have become extinct, giving rise to subgroup 3 with two branches in current circulation. Moreover, lineage E showed co-circulation of three subgroups. This behavior is similar to that observed for other enteroviruses of clinical importance (van der Sanden et al., 2010).

The phylodynamic analysis allowed us to estimate the substitution rates for the currently circulating E30 lineages. All estimates were in the order of 10^{-3} s/s/y, similar to the rates estimated by other authors that ranged from 6.2 to 8.8×10^{-3} s/s/y (McWilliam Leitch et al., 2009; Savolainen-Kopra et al., 2011; Yarmolskaya et al., 2015). The differences between the overall rates estimated in the present work and by other studies could be due to the subsets of analyzed data. It is expected that the overall rate estimated here is lower than those estimated in other studies since it contains sequences that comprise a larger time lapse (57 years) and represents different lineages, small groups, and individual sequences.

The comprehensive study performed here revealed that a global consensus is needed to carry out the typing of these viruses to facilitate future analyses, as was previously proposed (Yang et al., 2013; Yarmolskaya et al., 2015). In addition, in the GenBank database (up to 01/04/2014), there were more than 2246 VP1 sequences but only 792 (35%) were complete VP1 gene sequences, making comparison difficult among isolates from different studies. Typing of these viruses with the complete VP1 fragment would be recommended, allowing performance of phylogenetic and phylodynamic analyses with more confidence than with partial regions. It should be considered that the conclusions drawn by the present study might be biased by the selection of solely complete VP1 sequences.

Finally, this is the first report on genetic diversity in the $3D^{pol}$ region of South American E30 strains. Clustering in the $3D^{pol}$ region differed from that observed in the VP1 region, suggesting that recombination events occurred between the strains of both lineages found (E and F). Besides that, the results also suggest that recombination events also occurred within these lineages. In addition, our results showed that Argentinean strains, despite the geographical distance, are related to strains that circulated in Europe and did not form a separate cluster as was observed for strains from South East Asia (McWilliam Leitch et al., 2009).

5. Conclusion

This is the first phylodynamic study of E30 with strains from South America. Evolution of E30 is characterized by co-circulation and replacement of lineages and clusters, giving rise to the current circulation of lineages A, E, F, and H and the apparent extinction of lineages B, C, D, and G. In addition, a different distribution was observed: lineages A, C, D, E, and F were distributed globally while lineages B, G, and H presented a more restricted circulation. In particular, lineages E and F reached their worldwide distribution in a short period of time, suggesting that they could have some evolutionary advantage over others. Hopefully, future studies will identify the molecular basis of such an advantage.

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Declarations of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.02.012.

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