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Molecular epidemiology of echovirus 30 in Europe: succession of dominant sublineages within a single major genotype

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Summary. The genetic relationships between 131 echovirus type 30 (E-30) field isolates were studied using phylogenetic analysis of three genomic intervals: VP4/VP2 (420 nt), the entire VP1 and VP1/2A (150 nt). The strains had been isolated between 1975–1998, in different European countries, and in Israel and Japan. The maximum genetic variation was 15.7% in the VP4/VP2 region, 21.3% across the VP1/2A junction and 16.7% in the VP1-gene. The clustering patterns were very similar in all three regions. Two distinct genotypes were observed among the European strains, one of which was prevailing, spanning most of the investigated period. The same genotype was previously described to be the most prevalent circulating lineage of E-30 in Northern America. Interestingly, the two other genotypes comprising the prototype strain Bastianni and the oldest European isolates circulating before 1976, respectively, had apparently disappeared. Furthermore, the oldest lineages of the prevailing genotype had likewise disappeared and the recently isolated strains in the prevailing genotype were genetically quite homogenous, even when isolated in geographic regions far apart. These results indicate that the genetic variability of echovirus 30 is significantly lower than that of other previously characterized enteroviruses. Furthermore, one single, major genotype showed epidemic spread across two continents. Interestingly, despite the low nucleotide variability, maximum amino acid sequence variability in VP1 was surprisingly high, 8.0%, suggesting possible antigenical differences.

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Introduction

Echovirus 30 is a member of the *Enterovirus* genus in the family of *Picornaviridae*. Enteroviruses, known to cause infection in humans, comprise 64 immunologically distinct serotypes [23]. They are currently classified, mainly based on their genetic features, in polioviruses and human enterovirus species A to D (HEV A–D). Echoviruses belong to species HEV B. In addition, at least 28 animal enterovirus serotypes are known [15]. Enteroviruses are usually transmitted through the feco-oral or oral-oral route. Infections are often subclinical, mild or "flu-like" at the most. Enterovirus infections may, however, cause acute febrile illness or neurological complications, e.g., paralysis, encephalitis, or meningitis. Enterovirus infections can also cause symptoms of myocarditis, pleurodynia, conjunctivitis, or a neonatal multi-system disease [23]. Of all cases of viral meningitis in children, echoviruses account for 80–90% [3]. Echovirus serotype 30 (E-30) is one of the most frequently isolated of these echovirus serotypes [12, 19, 29, 32]. An increased amount of aseptic meningitis outbreaks due to E-30 have been reported during the last decade including Japan, 1990 [24]; USA, 1992 [9]; Spain, 1995 [18]; Poland, 1995 and 1996 [20]; Finland, 1996 [30]; Germany, 1997 [39]; France, 1997 [21]. It is the recent strikingly increased epidemic activity that has prompted the present study. The virus-encoded RNA-dependent RNA-polymerase shows a high error frequency, which is due to the lack of a proof-reading mechanism [10]. As a result, viruses exist as mixtures of genetic variants or quasispecies [10]. Under non-selective environmental conditions the equilibrium of the virus population may remain unchanged [5]. Any change in the environmental conditions that gives an opportunity to any variant to compete with the dominant virus may shift the equilibrium and, thus, drive virus evolution. In addition, homologous intertypic and intratypic recombination occurs frequently between RNA strands of the same or different enterovirus serotype (reviewed in [1]). As a result, numerous genetic lineages, so called genotypes, of any given enterovirus serotype circulate worldwide. Genotypes of poliovirus, another enterovirus, have originally been defined as clusters of strains with less than 15% sequence divergence in the VP1/2A junction region [40]. In the global Poliomyelitis Eradication Initiative [44], partial genome sequencing has successfully been used to establish typical geographic distribution of distinct genetic lineages, and to identify genetic sources of outbreaks and sporadic cases of poliomyelitis [14].

Despite its clinical importance, knowledge about the evolutionary and molecular epidemiological behaviour of E-30 has been limited. Previous studies have elucidated variability of E-30 isolated in distinct areas [8]. Our aim was to study genetic relationships and sequence variability between European echovirus 30 isolates in several genomic intervals including VP4/VP2, as well as the VP1/2A junction region, and the entire VP1. These regions have been used previously in similar studies of other enteroviruses [27]. While this work was in progress, two other groups published studies on E-30 carried out with a similar approach but focusing on different geographical areas [29, 32].

Materials and methods

Virus samples

131 echovirus type 30 field isolates were used in this study. The strains had been isolated in Austria, Byelorussia, Denmark, Estonia, Finland, France, Israel, Japan, Latvia, Lithuania, Poland, Russia, Sweden and The Netherlands (Table 1). In addition, the American Type Culture Collection prototype strain Bastianni, isolated from a stool sample of a child with aseptic meningitis in New York, 1958 [34] was included.

Virus propagation in cell culture

Freezer stocks were passaged once in human rhabdomyosarcoma (RD) cells (1:10 dilution in cell culture medium (MEM) with 5% FCS), cultures with full cytopathic effect were freeze-thawed three times and clarified by centrifugation for 10 min at 235 g. The supernatant was collected and stored at −70 °C. The serotype of the isolates was confirmed using a monovalent neutralizing antiserum [12]. Virus isolates from sewage water samples [12] were plaque purified to resolve possible mixtures of different viruses according to a method previously described [26].

RNA isolation

The viral RNA was isolated from 100 µl tissue culture supernatant using the RNeasy Total RNA kit (Qiagen GmbH, Hilden, Germany). Upon purification, RNA was eluted from the columns with 30 µl diethyl pyrocarbonate treated H_2O and subsequently stored in aliquots at −70 °C.

Reverse transcription polymerase chain reaction (RT-PCR)

Several combinations of different primer pairs were used for cDNA synthesis and RT-PCR (Table 2). For the VP4/VP2 region four primer pair combinations were used: 71693-72438, 9895-9565, 580-72438 and 580-81294 (Table 2), for VP1/2A junction region primer pair 82138-82139 and for VP1 primer pair combinations 8839-82139, 8839-8841, 8840-73124

Table 1. The numbers of echovirus 30 strains from different countries

Country	Number of strains
Austria	2
Denmark	5
Estonia	5
Finland	43
France	6
Israel	2
Japan	1
Latvia	8
Lithuania	4
Poland	13
Russia and Byelorussia	5
Sweden	2
The Netherlands	35

Table 2. Primers used in this study

Primer	Position[a]	Orientation	Sequence 5′ → 3′
71693	0449-0470	F	CCT CCG GCC CCT GAA TGC GGC
9895	0538-0565	F	GGG ACC AAC TAC TTT GGG TGT CCG TGT
580	0591-0609	F	GGC TGC TTA TGG TGA CAA T
71693	0449-0470	F	CCT CCG GCC CCT GAA TGC GGC
9565	1191-1217	R	GCA TCI GGY ARY TTC CAC CAC CAN CC
72438	1211-1192	R	GGC AAC TTC CAC CAC CAC CC
81294	1211-1195	R	GGC AAC TTC CAC CAC CA
8839	2388-2407	F	TGC TTT GTG TCA GCA TGC AA
82138	2626-2645	F	TAC CAC ACC AGA TCA GAG TC
8840	2884-2906	F	ACA CAC CAA ATA ATG TAC GTG CC
81494	2935-2958	F	AAC AGT TAC AGC TGG CAG ACA TC
8841	2993-2974	R	GCA TTG CCC TCT GTC CAA AA
82139	3120-3100	R	CAA GTG TCC CAT GTT GTT CAA
73124	3418-3437	R	TCC CAC ACG CAA TTT TGC CA

[a]Primer positions according to the orientation of the primer, either forward (F) or reverse (R)-sense; I = inosine; N = A, C, G, T; R = A, G; Y = C, T. Numbering according to strain Bastianni, GenBank AF162711

or 81494-73124, producing two overlapping fragments covering the entire VP1 and including the flanking sequences. cDNA synthesis and PCR were carried out as described previously [27]. The PCR products were visualized after electrophoresis on an ethidium bromide stained 2% agarose gel.

Sequence analysis

PCR products were purified either directly using the PCR Purification Kit (QIAquick, Qiagen) in the case of a single band in the electrophoresis, or excised from gel and purified using the Gel Extraction Kit (QIAquick, Qiagen) in the case of multiple bands. The purified products were eluted with 30–50 μl 10 mM Tris-HCl (pH 8.5) and subsequently stored at −20 °C. Cycle sequencing reactions were performed using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Espoo, Finland) using the same forward and reverse primers, as in the RT-PCR. Both forward and reverse reactions were performed to resolve possible ambiguities. An automated DNA sequencer was used for sequencing (ABI PRISM, model 377). Sequence data was analyzed using Sequencing Analysis (version 3.1) and Sequence Navigator (version 1, PE Applied Biosystems) for pairwise comparisons. Multiple sequence alignments were made using PileUp, part of the GCG program suite (version 10, Genetics Computer Group, Inc., USA).

The lengths of the studied genomic intervals in E-30 Bastianni were 420 (207 nt. in VP4, 213 nt. in VP2) in VP4/VP2, 150 (90/60) nt. in VP1/2A and 876 nt. in VP1. Distance matrices were estimated using the DNAdist program, part of the PHYLIP (Phylogeny Inference) package (version 3.572c; [7]), using the maximum likelihood model of nucleotide substitution. Dendrograms were drawn using the UPGMA option in Neighbor (PHYLIP), and were visualized using NJplot or Tree View (version 1.5.3). Bootstrap analysis was performed using Seqboot (PHYLIP) and quartet puzzling using Puzzle (version 4.0, [43]), both with 1000 replicates. GenBank accession numbers for E-30 sequences derived from this work are AF236388-AF236635.

Results

General observations

Sequences in one or more genomic intervals were obtained from a total of 131 E-30 isolates (Table 1). The maximum genetic variation between E-30 isolates was 15.7% in the VP4/VP2 region, 16.7% in the VP1-gene and 21.3% in the VP1/2A junction region. The rank of difference maxima of the different genomic regions was similar to that of coxsackievirus B4 [27] but the overall range of variation was definitely smaller in the case of E-30. Based on this and the distance matrices generated for each region (data not shown) the genotype demarcation in the VP4/VP2 and VP1 region was defined at 12%.

Sequence variation and clustering in the VP4/VP2 region

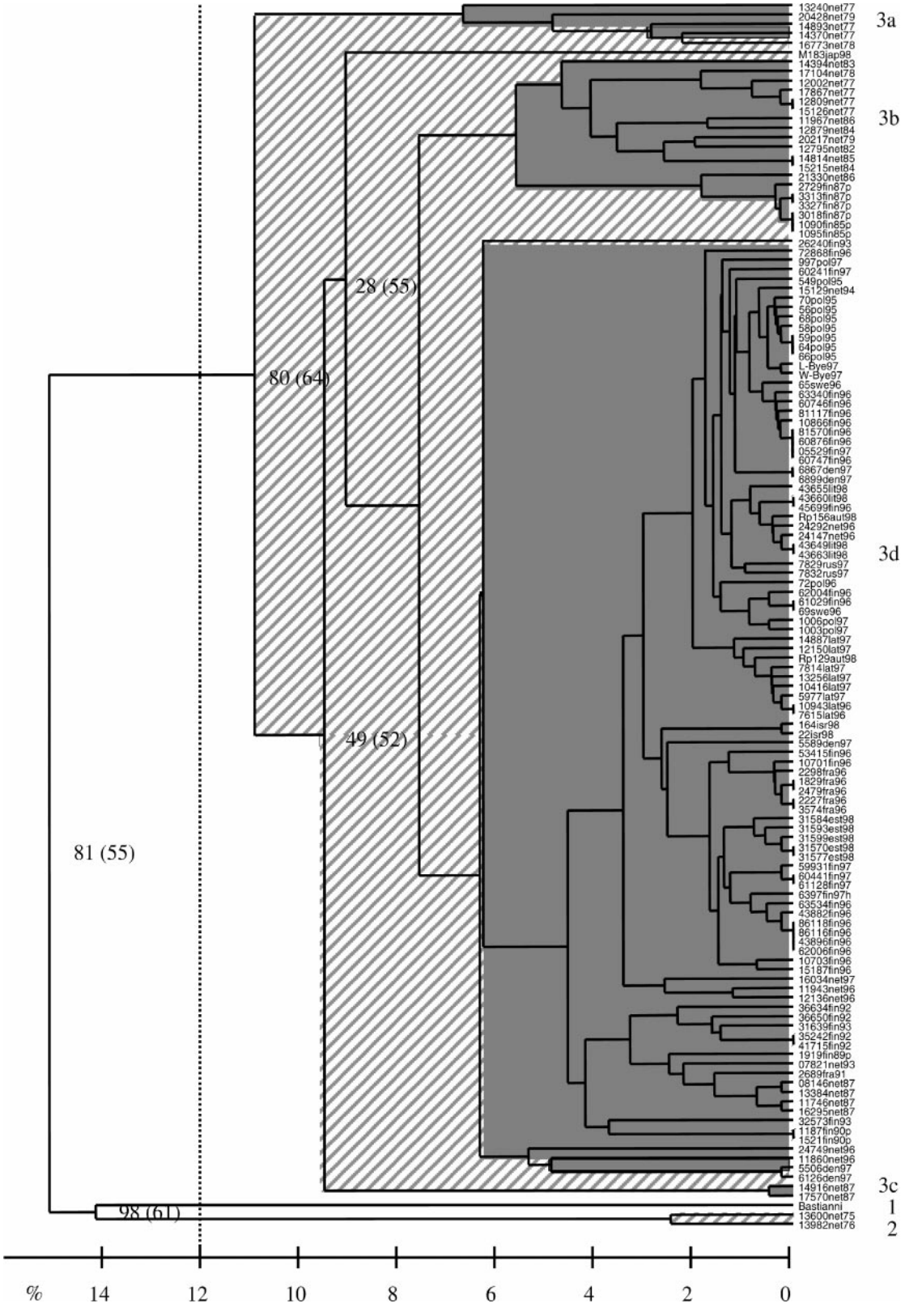
Sequences were obtained from a total of 131 E-30 strains in the VP4/VP2 region, including the prototype strain Bastianni. The Bastianni sequences obtained in different genomic regions from our own laboratory strain were identical to the GenBank sequence (accession number AF162711). Three distinct genotypes could be observed (Fig. 1, 1–3), one of which is prevailing. The branching was supported by reliable bootstrap and quartet puzzling values (Fig. 1). The prototype strain Bastianni did not cluster with any of the sequences and therefore represents a genotype of its own. In the second genotype there were two Dutch strains isolated in 1975 and 1976.

The prevailing genotype contained strains from all areas studied. In addition, the only Japanese strain analyzed (M183jap98) belonged to this genotype. The strains belonging to this genotype had been isolated almost throughout the entire observed period, 1977–1998. Among the European strains, four distinct subclusters (Fig. 1, cluster 3 a–d) with the average genetic variation of approximately 7% or more could be found.

The first subcluster (Fig. 1, cluster 3a) was composed of strains isolated before 1979. Subcluster 3b (Fig. 1) comprised strains from 1977–1987. The two strains in subcluster 3c were from 1987 and the vast majority of strains comprising the subcluster 3d from 1987–1998. Based on the clustering in the VP4/VP2 region, subsets of strains, representative of each cluster, were selected for further sequence analysis in the VP1 gene and the VP1/2A junction region.

Sequence clustering in the VP1-gene

63 strains were sequenced in the entire genomic region of VP1. The dendrogram based on the alignment of the nucleotide sequences of this region is shown in Fig. 2. The observed maximum genetic variation, 16.7%, was slightly higher than that of the VP4/VP2 region, 15.7% (distance matrix not shown). Clustering patterns in this region were very similar to that of the VP4/VP2 region. Two distinct genotypes could be observed among the European strains and the Japanese strain from 1998 clustered with the prevailing European genotype. Temporal subclustering of the strains of the prevailing genotype was seen here, too, although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15126net77
11967net86
12879net84
20217net79
12795net82
14814net85
15215net84
21330net86
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3327fin87p
3018fin87p
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997pol97
60241fin97
548pol95
15129net94
70pol95
56pol95
68pol95
58pol95
59pol95
64pol95
66pol95
L-Bye97
W-Bye97
65swe96
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10701fin96
2298fra96
1829fra96
2479fra96
2227fra96
3574fra96
31584est98
31593est98
31599est98
31570est98
31577est98
59931fin97
60441fin97
61128fin97
6397fin97h
63534fin96
43882fin96
86118fin96
86116fin96
43896fin96
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41715fin92
1919fin89p
07821net93
2689fra91
08146net87
13384net87
11746net87
16295net87
32573fin93
1187fin90p
1521fin90p
24749net96
11860net96
5506den97
6126den97
14816net87
17570net87
Bastianni
13600net75
13982net76
3a
3b
3d
3c
1
2
28 (55)
80 (64)
49 (52)
81 (55)
98 (61)
%
14
12
10
8
6
4
2
0

the distinction between the subclusters was less clear than in the VP4/VP2 region (Fig. 2).

Genetic variation in the VP1/2A junction region

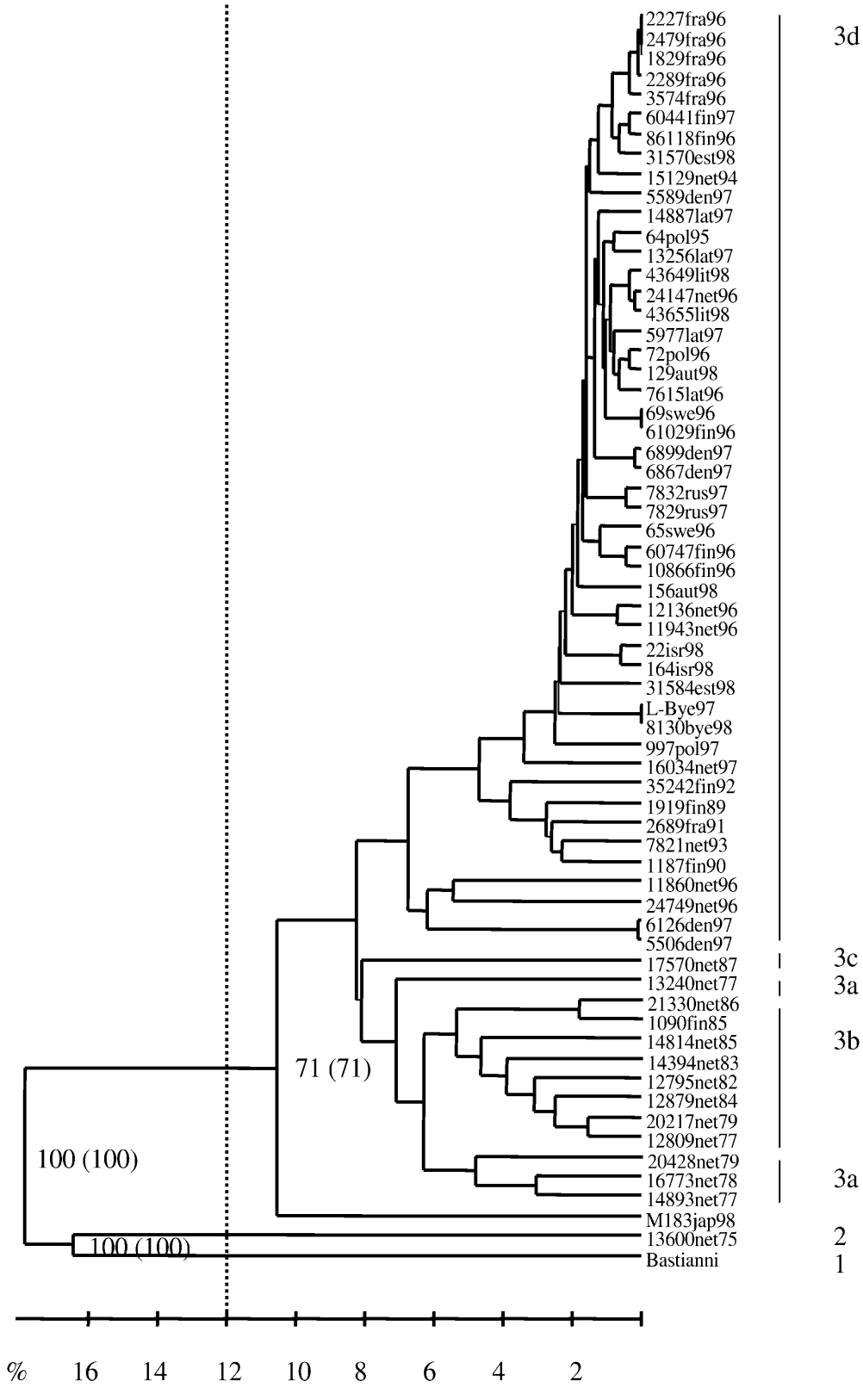
Sequences were obtained from 63 E-30 strains in the 150 nt. VP1/2A junction region, including the prototype strain Bastianni. Clustering of the strains was a little different from that in the two other genomic regions. In the major genotype there were again actually all European strains from years 1977–1998. Within this genotype the strains could also be divided into four subclusters (Fig. 3, cluster 3 a–d) with an average genetic variation of 9% or more. However, the branching order differed from that of the VP4/VP2 region. The older subclusters could not be distinguished, but formed a single subcluster, and, moreover, three recently isolated strains (24749net96, 6126den97 and 5506den97) differed from the large majority of strains isolated after 1989.

The prototype strain Bastianni and a Dutch strain from 1975 (13600net75) differed from all the other European isolates well beyond the genotype demarcation of 15% introduced by Rico-Hesse and co-workers for this region (Fig. 3, 1–2). In contrast to the situation in the two other regions, the Japanese strain from 1998 (M183jap98) was an outlier in this region.

5′ *non-coding region*

The length of the hypervariable region in the 5′NCR was 105 nt. based on the alignment of the E-30 sequences. Compared to polioviruses, 100–104 nt. [37] and coxsackievirus B4, around 100 nt. [13, 27] the hypervariable region of E-30 is of approximately the same length, but shorter than that reported for coxsackie A viruses [35]. The maximum nucleotide variation in the hypervariable region was 45.7%, for poliovirus 3 was reported to be 56% [37]. No AUG codons preceeding the initiation codon were found in the sequenced part of the 5′NCR. The sequence flanking the translation initiation codon was AAA**AUG**G in most of the strains, also GAA**AUG**G was observed. These sequences are almost the same as the sequence favoured by eukaryotic ribosomes for the initiation of protein synthesis [17] and the first sequence exactly identical with that found in coxsackievirus B4 strain J.V.B. [13].

Fig. 1. Genotype distribution of echovirus 30 in the 420 nt. region coding for VP4/VP2. Numbers indicate bootstrap values of each branch as calculated using the PHYLIP package (1000 replicates). Numbers between brackets are the corresponding quartet puzzling support values. The 12% line indicates genotype demarcation. A distance matrix was calculated using DNAdist (maximum likelihood) and the dendrogram was calculated with Neighbor (UPGMA). The shaded regions indicate genotypes, marked with numerals 1–3, and subclusters within the prevailing genotype, letters a–d. Bastianni is the ATCC-prototype strain [34]. Country abbreviations: aut: Austria, bye: Byelorussia, den: Denmark, est: Estonia, fin: Finland, fra: France, isr: Israel, jap: Japan, lat: Latvia, lit: Lithuania, net: The Netherlands, pol: Poland, rus: Russia, swe: Sweden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2479fra96
1829fra96
2289fra96
3574fra96
60441fin97
86118fin96
31570est98
15129net94
5589den97
14887lat97
64pol95
13256lat97
43649lit98
24147net96
43655lit98
5977lat97
72pol96
129aut98
7615lat96
69swe96
61029fin96
6899den97
6867den97
7832rus97
7829rus97
65swe96
60747fin96
10866fin96
156aut98
12136net96
11943net96
22isr98
164isr98
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L-Bye97
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14394net83
12795net82
12879net84
20217net79
12809net77
20428net79
16773net78
14893net77
M183jap98
13600net75
Bastianni
3d
3c
3a
3b
3a
2
1
71 (71)
100 (100)
100 (100)
%
16
14
12
10
8
6
4
2

Amino acid sequence variability

The conserved length of the VP4 gene was 207 nt. The putative cleavage site was determined according to studies on other picornaviruses [13, 44]. The deduced amino acid sequence of the putative cleavage site of VP4/VP2 was PALN/S and was conserved among all strains. The maximum amino acid sequence variability in the sequenced VP4/VP2 region was 4.4%. The positions of variable amino acids are shown in Fig. 4.

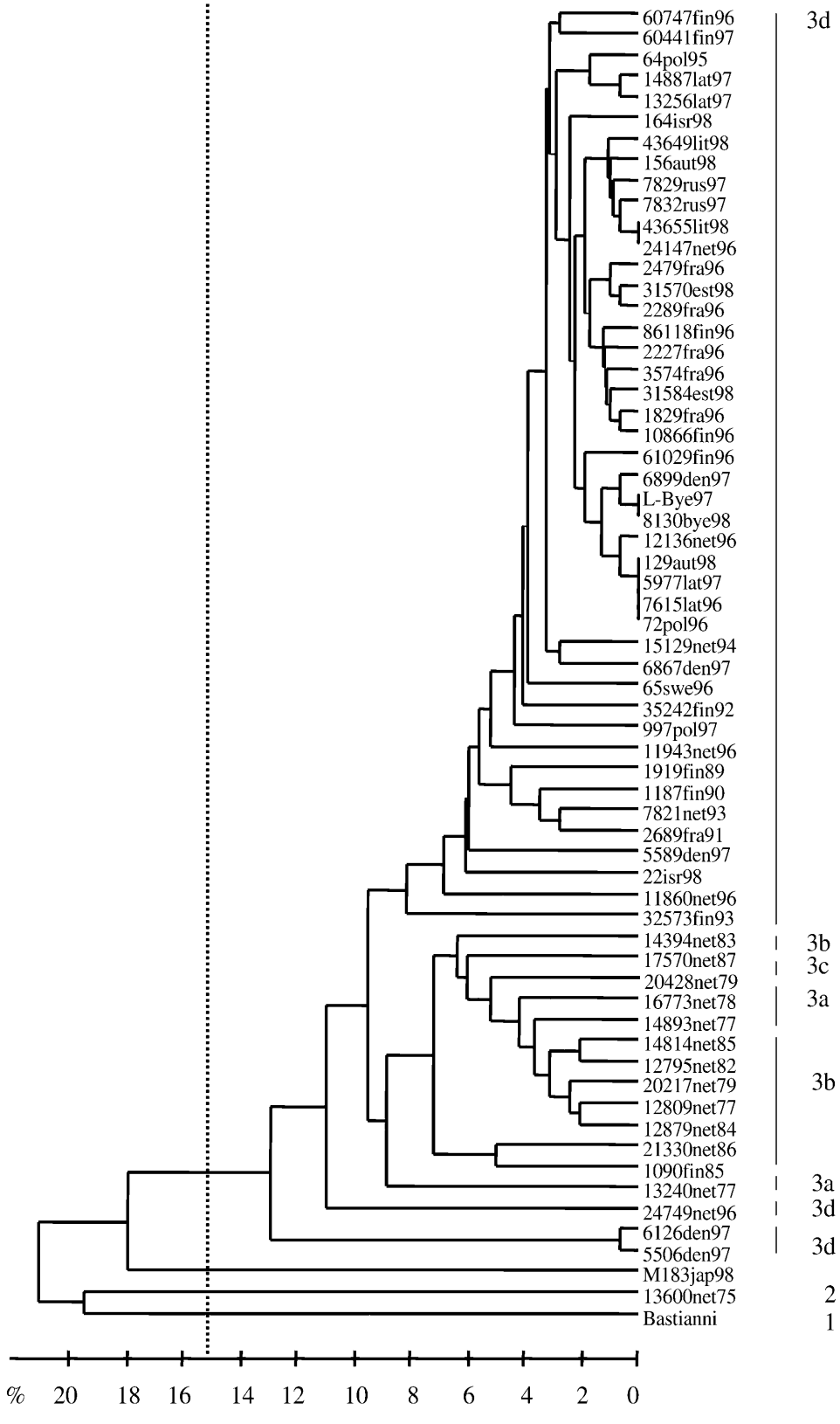
The conserved length of VP1 was 292 amino acids. The deduced amino acid sequence in the putative cleavage site between VP3 and VP1, as judged from an alignment with other enteroviruses [13, 44] was ALYQ/N, and it was conserved among all strains. In contrast, some variation was seen in the corresponding site between VP1 and 2A (cf. [16, 27] in poliovirus and coxsackievirus B4, respectively). The amino acid sequence was LSNT/G in most of the echovirus 30 strains that we studied; six times it was LLNT/G (14814net85, 2689fra91, 15129net94, 61029fin96, 68swe96 and 60441fin97), once LSKS/G (59771lat97), once LTNT/G (17570net87) and once VLTT/G (Bastianni). The maximum amino acid variation in VP1 was 8.0%. The distribution of variable amino acid positions is shown in Fig. 4. The majority of variable positions were located at the end of VP1 and the beginning of 2A (Fig. 4). In the C-terminal VP1 (30 amino acids) the maximum amino acid sequence variability was 23.3% and in the N-terminal 2A (20 amino acids) 50.4%. The C-terminus of VP1 is known to contain an antigenic site in several other enteroviruses [22, 36].

Discussion

We studied the genomic variation and genotype distribution of 131 echovirus 30 field isolates from different parts of Europe by partial nucleotide sequencing. The isolates covered a period of more than two decades (1975–1998). Genetic clustering of the strains was assessed according to the principles of Rico-Hesse and co-workers [40] and the later studies on polioviruses [14, 25] and coxsackievirus B4 [27]. We found that compared with other enteroviruses analyzed echovirus 30 shows much less genetic variation as exemplified by the fact that since the late 1970s only a single genotype has apparently been circulating in Europe.

The genotype composed of the two oldest isolates (1975–76) under study seems to have disappeared and representatives of the prototype Bastianni cluster were not seen among the European strains. The genotype containing sequences after 1977 could be divided in subclusters. These subclusters showed a temporal relationship with each other. The currently circulating lineage

Fig. 2. UPGMA dendrogram based on sequence alignment of a subset of echovirus 30 strains in the VP1 gene (for abbreviations and additional information see legend to Fig. 1)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156aut98
7829rus97
7832rus97
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31570est98
2289fra96
86118fin96
2227fra96
3574fra96
31584est98
1829fra96
10866fin96
61029fin96
6899den97
L-Bye97
8130bye98
12136net96
129aut98
5977lat97
7615lat96
72pol96
15129net94
6867den97
65swe96
35242fin92
997pol97
11943net96
1919fin89
1187fin90
7821net93
2689fra91
5589den97
22isr98
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32573fin93
14394net83
17570net87
20428net79
16773net78
14893net77
14814net85
12795net82
20217net79
12809net77
12879net84
21330net86
1090fin85
13240net77
24749net96
6126den97
5506den97
M183jap98
13600net75
Bastianni
3d
3b
3c
3a
3b
3a
3d
3d
2
1
% 20 18 16 14 12 10 8 6 4 2 0

displaced older ones in the late 1980s. Members of the last subcluster had been collected from different parts of Europe, which indicates an efficient transmission capacity of this genetic lineage. This pattern is clearly different from that of other enteroviruses. For coxsackievirus B4 at least seven distinct genotypes were found to circulate simultaneously [27]. It is not known how the large number of intraserotypic genotypes have been generated, but it is tempting to assume that they result from repeated "bottleneck transmission" events [6] rather than being due to selection. The same distribution of E-30 has been found in the United States and Canada where the extinction of genotypes prevailing before 1981 was also observed [32].

The most recent subcluster was found to be dominant on both continents and also present in Australia as can be seen in Fig. 5. The close relatedness of strains from different continents can also be seen in Fig. 5. The separate genotype formed by the Japanese strain in VP1/2A with genetic difference of 18%, and two other genotypes represented by a Colombian and a Philippino isolate in VP1 (Fig. 5), could indicate that outside Europe and Northern America the situation may be different. More strains from these regions should, however, be analysed before proper conclusions can be drawn. The designated genotypes of poliovirus strains have been shown to typically circulate only in geographically restricted regions [25, 40] Echovirus 30 does not seem to be similarly geographically restricted, as a given genotype can circulate uninterrupted in many distinct areas for decades. On the other hand, the apparent restriction of poliovirus circulation might result from the vaccination efforts bringing about an inhibition of natural transmission.

The reasons for a dominance of a single global genotype could be various. The environmental conditions may have changed to favour a certain variant, and the latter may have found an ecological niche, best suited for it in the present conditions. The lineage may have been virulent enough in terms of transmission, without having to produce new variants. It appears that, within Europe, echovirus 30 has separated out into two independently evolving lineages before 1977. Obviously, the currently prevailing genotype has been more fit to the environment and the other has apparently become extinct. A similar displacement pattern is apparently being reproduced in the intra-genotype variation. Subclusters of genotype 3 appeared to follow each other and not co-circulate for extended periods. This pattern is reminiscent of the evolution of influenzaviruses A and B, where prevailing lineages are selected on the basis of immune escape [42]. We did not study potential antigenic differences between the echovirus 30 clusters.

Fig. 3. UPGMA dendrogram based on sequence alignment of a subset of echovirus 30 strains in the 150 nt. VP1/2A junction region. Sequences of the French isolates from Caro, 2000, GenBank accession numbers AJ279174, AJ279190-AJ279192, AJ279196 and AJ279197 (for abbreviations see legend to Fig. 1)

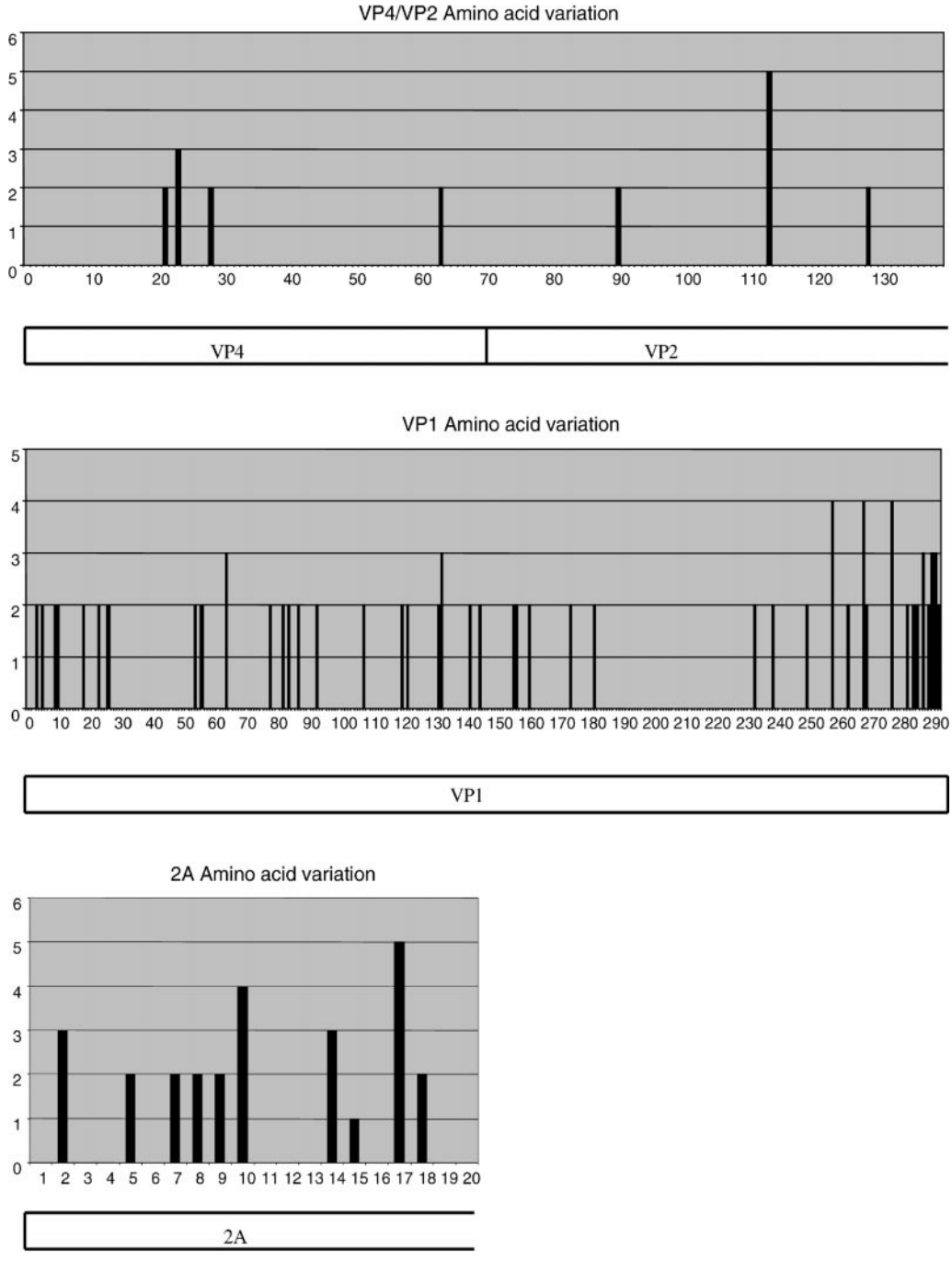

Fig. 4. Distribution of variable amino acid positions in the genomic regions VP4/VP2, VP1 and 2A. The height of the columns at position indicates the number of different amino acid substitutions observed. Note that in the VP4/VP2 region (140 aa) 131 strains were analysed while only 63 strains were used in the VP1 gene (292 aa) and 2A region (20 aa)

Table 3. Comparison of maximum nucleotide and amino acid variations in different genomic regions of echovirus 30 and coxsackievirus B4. CV-B4 values from [27]

		VP1/2A	VP1	VP4/VP2
CV-B4	max nt var	27.3%	19.3%	20.7%
	max aa var	22.0%	4.3%	5.7%
E-30	max nt var	21.3%	16.7%	15.7%
	max aa var	21.1%	8.0%	4.4%

Echovirus 30 also seems to differ from the other analysed enterovirus serotypes in the low overall genetic variation. For echovirus 30 the average genetic variation in the VP1/2A junction region was 21.3%. For coxsackievirus B4 variation in this region was 27.3% [27] for coxsackievirus B5 32.0% (Mulders, M., Salminen, M., Lindberg, M. and Hovi, T., unpublished results) and for coxsackievirus A9 34% [41]. Maximum variation in the VP1/2A junction region of poliovirus 1 isolates was 22.6% [40].

In the entire VP1 gene the variation of E-30 was 16.7%, also lower than that found for coxsackie B4, 19.3% [27] and enterovirus 71, 19.7% [2]. In spite of the relatively low genetic variation of E-30, the maximum variation of the deduced amino acid sequences in different regions was quite similar to that found in coxsackievirus B4 [27]. As a matter of fact, in VP1 the maximum amino acid variation was twice as much as found for coxsackievirus B4, although the maximum nucleotide variation was lower (Table 3). This might suggest that strains of E-30 also differ antigenically, favouring the idea of an ecological niche, where new immunological variants are no longer recognized by human hosts with pre-existing antibodies against the older variant.

The clustering of E-30 strains in genomic regions VP4/VP2, VP1 and VP1/2A was found to be very similar. Previously, VP4/VP2 or VP2 have been stated not to be suitable regions for genetic typing [27, 31] because of the limited demarcation between inter- and intratypic variation. However, the use of VP4/VP2 in basic molecular analysis has been suggested and that is also supported by our findings in this study, as the variation in the VP4/VP2 region did not essentially differ from that in the VP1. In contrast, the VP1/2A junction region revealed a different segregation pattern due to large variation of 2A. This part of the genome, however, has no direct link with serotyping, since it codes for a non-structural protein.

In conclusion, echovirus 30 differs from other enteroviruses analysed so far with respect to genetic variability and geographic distribution of its genotypes. Only one genotype appears to be circulating at the present in Europe and Northern America. The reasons and significance of this behaviour remain to be elucidated.

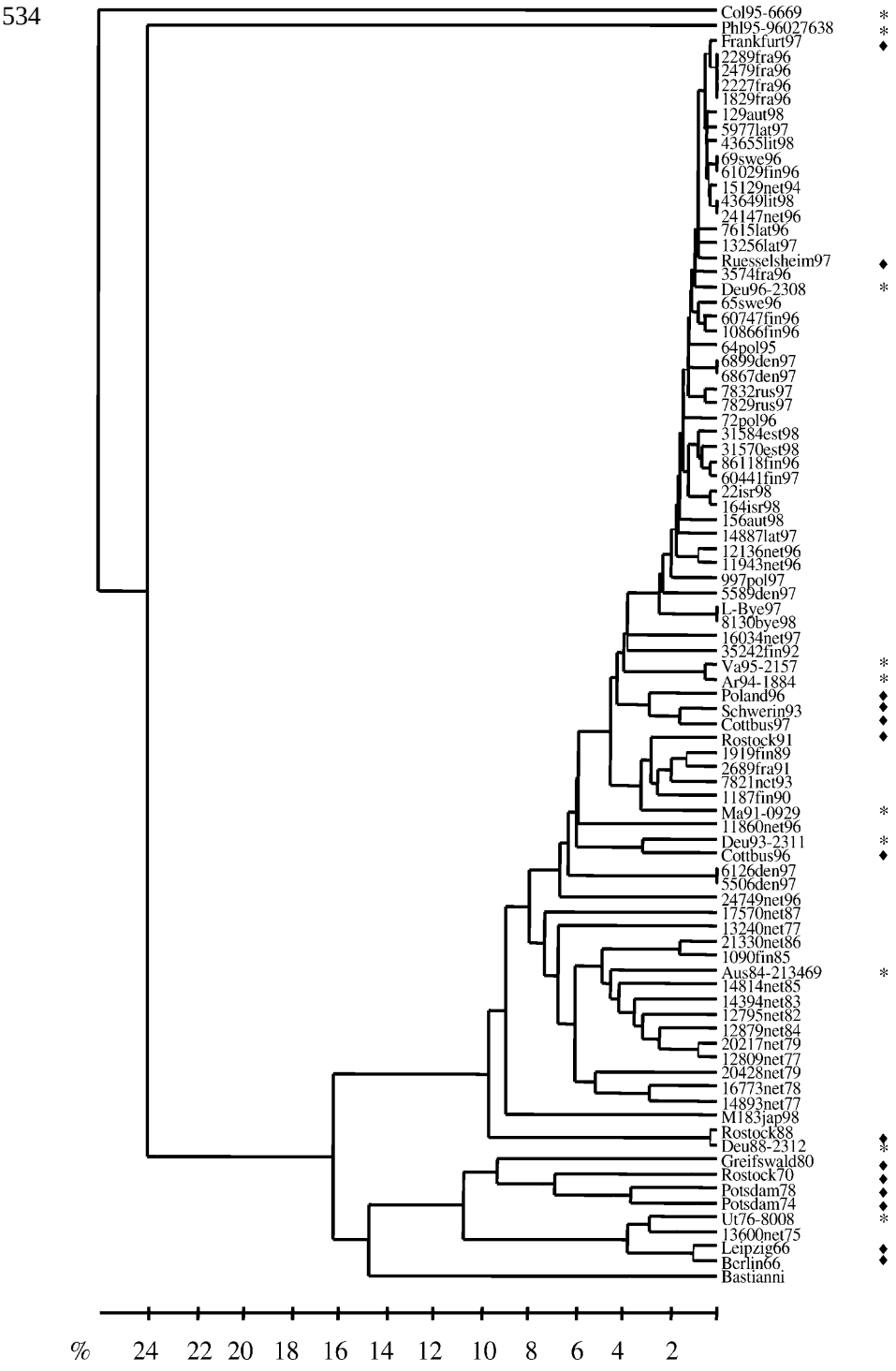

Fig. 5. UPGMA dendrogram based on sequence alignment of 414 nucleotides in VP1. Sequences marked with ∗ have been published previously by [32] (GenBank accession numbers AF127987, AF127988, AF128014, AF128064, AF152880, AF152887-AF152891). Sequences marked with ◆ are available in GenBank by Künkel and Schreier (accession numbers AF061395, AF061397-AF061399, AF061400, AF067071-AF067079)

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References

1. Agol VI (1997) Recombination and other genomic rearrangements in picornaviruses. Semin Virol 8: 77–84
2. Brown BA, Oberste MS, Alexander JP, Kennett ML, Pallansch MA (1999) Molecular epidemiology and evolution of enterovirus 71 strains isolated from 1970 to 1998. J Virol 73: 9969–9975
3. Diedrich S, Driesel G, Schreier E (1995) Sequence comparison of echovirus type 30 isolates to other enteroviruses in the 5′ noncoding region. J Med Virol 46: 148–152
4. Domingo E, Sabo D, Taniguchi T, Weissman C (1978) Nucleotide sequence heterogeneity of an RNA phage population. Cell 13: 735–744
5. Domingo E, Holland JJ (1994) Mutation rates and rapid evolution of RNA viruses. In: Morse SS (ed) Evolutionary biology of viruses. New York, Raven Press, pp 161–183
6. Domingo E, Escarmis C, Sevilla N, Moya A, Santiago FE, Quer J, Novella IS, Holland JJ (1996) Basic concepts in RNA virus evolution. FASEB J 10: 859–864
7. Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle
8. Gjøen K, Bruu A-L, Orstavik I (1995) Intratypic genome variability of echovirus type 30 in part of the VP4/VP2 coding region. Arch Virol 141: 901–908
9. Helfand RF, Khan AS, Pallansch MA, Alexander JP, Meyers HB, DeSantis RA, Schonberger LB, Andersson LJ (1994) Echovirus 30 infection and aseptic meningitis in parents of children attending a child care center. J Infect Dis 169: 1133–1137
10. Holland JJ, De La Torre JC, Steinauer DA (1992) RNA virus populations as quasispecies. Curr Top Microbiol Immunol 176: 1–20
11. Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S (1982) Rapid evolution of RNA genomes. Science 215: 1577–1585
12. Hovi T, Stenvik M, Rosenlew M (1996) Relative abundance of enterovirus serotypes in sewage differs from that in patients: clinical and epidemiological implications. Epidemiol Infect 116: 91–97

13. Jenkins O, Booth JD, Minor PD, Almond JW (1987) The complete nucleotide sequence of coxsackievirus B4 and its comparison to other members of the picornaviridae. J Gen Virol 68: 1835–1848
14. Kew OM, Mulders MN, Lipskaya GY, da Silva EE, Pallansch MA (1995) Molecular epidemiology of enteroviruses. Semin Virol 6: 401–414
15. King AMQ, Brown F, Christian P, Hovi T, Hyypiä T, Knowles NJ, Lemon SM, Minor PD, Palmenberg AC, Skern T, Stanway G (2000) Picornaviridae. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Calisher CH, Carsten EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (eds) Virus Taxonomy. Seventh Report of the International Committee for the Taxonomy of Viruses. Academic Press, New York San Diego, pp 657–678
16. Kitamura N, Semler BL, Rothberg PG, Larsen GR, Adler CJ, Dorner AJ, Emini EA, Hanecak R, Lee JL, van der Werf S, Anderson CW, Wimmer E (1981) Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature 291: 547–553
17. Kozak M (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eucaryotic ribosomes. Cell 44: 283–292
18. Lopez Alcala MI, Rodriguez Priego M, de la Cruz Morgado D, Barcia Ruiz R (1997) Outbreak of meningitis caused by echovirus type 30. Anales Espanoles de Periatria 46: 237–240
19. Maguire HC, Atkinson P, Sharland M, Bendig J (1999) Enterovirus infections in England and Wales: laboratory surveillance data: 1975 to 1994. Commun Dis Public Health 2: 122–125
20. Majda-Stanislawska E, Kuydowicz J, Bartczak D (1997) Epidemic of ECHO type 30 virus meningitis in children from the Lodz region in the years 1995 and 1996. Przeglad Epidemiol 51: 267–274
21. Maisonneuve L, Esteve V, Scart G, Lusina D, Le Pennec MP, Baledent F, Bingen E (1999) PCR SSCP study of an echovirus 30 meningitis outbreak. Pathol Biol 47: 534–538
22. Mateu M (1995) Antibody recognition of picornaviruses and escape from neutralization: a structural view. Virus Res 38: 1–24
23. Melnick JL (1996) Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Fields BN, Knipe DM, Howley PM et al. (eds) Fields Virology, 3rd ed. Lippincott-Raven, Philadelphia, pp 655–712
24. Mori I, Matsumoto K, Hatano M, Sudo M, Kimura Y (1995) An unseasonable winter outbreak of echovirus type 30 meningitis. J Infect Dis 31: 219–223
25. Mulders MN, Lipskaya GY, van der Avoort HGAM, Koopmans MPG, Kew OM, van Loon AM (1995) Molecular epidemiology of wild poliovirus type 1 in Europe, the Middle-East and the Indian subcontinent. J Infect Dis 171: 1399–1405
26. Mulders MN, Reimerink JHJ, Stenvik M, Alaeddinoglu I, van der Avoort HGAM, Hovi T, Koopmans MPG (1999) A Sabin vaccine-derived field isolate of poliovirus type 1 displaying aberrant phenotypic and genetic features, including a deletion in antigenic site 1. J Gen Virol 80: 907–916
27. Mulders MN, Salminen M, Kalkkinen N, Hovi T (2000) Molecular epidemiology of coxsackievirus B4 and disclosure of the correct VP1/2A^{PRO} cleavage site – evidence for high genomic diversity and long-term endemicity of distinct genetic lineages. J Gen Virol 81: 803–812
28. Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD (eds) (1995) Classification and Nomenclature of Viruses. Sixth Report of the International Committee on Taxonomy of Viruses. Springer, Wien New York (Arch Virol [Suppl] 10)

29. Nairn C, Clements GB (1999) A study of enterovirus isolations in Glasgow from 1977 to 1997. J Med Virol 58: 304–312
30. NPHI (1997) Infectious diseases in Finland 1996. National Public Health Institute Publications, KTL B6/1997
31. Oberste MS, Maher K, Pallansch A (1998) Molecular phylogeny of all human enterovirus serotypes based on comparison of sequences at the 5′ end of the region encoding VP2. Virus Res 58: 35–43
32. Oberste MS, Maher K, Kennett ML, Campbell JJ, Carpenter MS, Schnurr D, Pallansch MA (1999) Molecular epidemiology and genetic diversity of echovirus type 30 (E30): genotypes correlate with temporal dynamics of E30 isolation. J Clin Microbiol 37: 3928–3933
33. Olive DM, Al-Mufti S, Al-Mulla W, Khan MA, Pasca A, Stanway G, Al-Nakib W (1990) Detection and differentiation of picornaviruses in clinical samples following genomic amplification. J Gen Virol 71: 2141–2147
34. Plager H, Decher W (1962) A newly-recognized enterovirus isolated from cases of aseptic meningitis. Am J Hyg 77: 26–28
35. Pulli T, Koskimies P, Hyypiä T (1995) Molecular comparison of coxsackie A virus serotypes. Virology 212: 30–38
36. Pulli T, Roivainen M, Hovi T, Hyypiä T (1998) Induction of neutralizing antibodies by synthetic peptides representing the C-terminus of coxsackievirus A9 capsid protein VP1. J Gen Virol 79: 2249–2253
37. Pöyry T, Kinnunen L, Kapsenberg J, Kew O, Hovi T (1990) Type 3 poliovirus/Finland/1984 is genetically related to common Mediterranean strains. J Gen Virol 71: 2535–2541
38. Pöyry T, Kinnunen L, Hovi T (1992) Genetic variation in vivo and proposed functional domains of the 5′ noncoding region of poliovirus RNA. J Virol 66: 5313–5319
39. Reintjes R, Pohle M, Vieth U, Lyytikäinen C, Timm H, Schreier E, Petersen L (1999) Community wide outbreak of enteroviral illness caused by echovirus 30: a cross-sectional survey and a case-control study. Pediatr Infect Dis J 18: 104–108
40. Rico-Hesse R, Pallansch MA, Nottay BK, Kew OM (1987) Geographic distribution of wild type poliovirus type 1 genotypes. Virology 160: 311–322
41. Santti J, Harvala H, Kinnunen L, Hyypiä T (2000) Molecular epidemiology and evolution of coxsackievirus A9. J Gen Virol 81: 1–12
42. Scholtissek C (1996) Molecular evolution of influenza viruses. Virus Genes 11: 209–215
43. Strimmer K, von Häseler A (1996) Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. Mol Biol Evol 13: 964–969
44. Toyoda H, Kohura M, Kataoku Y, Suganuma T, Omata T, Imura N, Nomoto A (1984) Complete nucleotide sequences of all three poliovirus serotype genomes. J Mol Biol 174: 561–585
45. World Health Assembly (1988) Global eradication of poliomyelitis by the year 2000. Resolution WHA41.28

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