# Phylogenetic analysis of infectious pancreatic necrosis virus in Ireland reveals the spread of a virulent genogroup 5 subtype previously associated with imports.

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**Abstract**

Infectious pancreatic necrosis is a significant disease of farmed salmonids resulting in direct economic losses due to high mortality and disease management costs. Significant outbreaks of the disease occurred in farmed Atlantic salmon in Ireland between 2003 and 2007, associated with imported ova and smolts. As the virus was known to occur in the country since the development of aquaculture in the 1980's, this study examined archived samples to determine whether these older isolates were associated with virulent forms. The study showed that two genotypes of IPNV were present in the 1990's, genotype 3 and genotype 5. A more virulent subtype of the virus first appeared in 2003 associated with clinical outbreaks of IPN and this subtype is now the most prevalent form of IPNV found in the country. The data also indicated that IPNV in Ireland is more related to Scottish and continental European isolates than Norwegian, Chilean and Australasian genogroup 5 isolates.

## Introduction

Infectious pancreatic necrosis (IPN), a significant disease of farmed fish worldwide, is caused by infectious pancreatic necrosis virus (IPNV) a member of the family *Birnaviridae* which are non-enveloped, double stranded RNA viruses with a bisegmented (A and B) genome encoding between five and six proteins [10]. The family consists of four genera, two of which are found in aquatic animals, *Blosnavirus* (type strain blotched snakehead virus) and *Aquabirnavirus* (type strain IPNV). IPN was first described in a freshwater brook trout, *Salvelinus fontinalis*, facility in North America in the 1950s [36] with the subsequent isolation of the virus reported by Wolf et al. [35]. In Europe, the disease was initially reported in freshwater rainbow trout, *Oncorhynchus mykiss*, farms in France [3], Denmark [34], Scotland [2] and Norway [13]. By the mid-1970s IPN had been reported in North America, Europe and Japan [16]. As with the other major European salmonid farming countries, IPNV has been present in Ireland for many years [20]. However serious clinical IPN has only emerged in the last decade, primarily in Atlantic salmon, *Salmo salar* farming. As the aquaculture industry developed in Europe through the 1970s, with the onset of Atlantic salmon production, the incidence of IPN disease also increased [24]. Despite widespread vaccination, IPN has remained a significant disease of farmed salmonids in the majority of countries where they are farmed, resulting in direct economic losses due to high mortality and disease management costs. In recent years the number of clinical IPN cases has declined, particularly in Norway, primarily believed to be due to successful selective breeding for resistance to IPN [19].

Genotyping of aquatic birnaviruses has led to the description of seven genogroups [5,7,9,23]. A previous study investigated the genogrouping of 55 IPNV isolates in Ireland from 2003 – 2007 [25]. It showed that all isolates belonged to genogroup 5 (as described by Blake et al. [5] but could be divided into two distinct sub-groups (subtypes). Subtype 1

consisted of a number of isolates from an Irish stock of farmed Atlantic salmon, including broodstock fish, which were all isolated from clinically healthy fish. Subtypes 2 were all isolated from salmon which were imported as ova or post-smolts from a single external source. All clinical cases within this period had been linked to isolates from subtype 2 [25,26]. The aim of this study was to analyse subsequent IPNV isolates from both clinical and non-clinical samples to monitor potential spread of the virulent isolates. In addition, archived samples going back to 1993 were also analysed to determine the genotyping of IPNV in Ireland prior to the onset of clinical IPN in 2003.

## Materials and methods

Information on the number of sites positive for IPNV and on the number of clinical cases was obtained from the database of the Fish Health Unit, Marine Institute which is the National Reference Laboratory for fish diseases in the Republic of Ireland. Information was obtained on the number of samples which tested positive for the IPN virus and cross referenced with site inspection reports to determine whether clinical IPN was observed on the site. A site was considered to have clinical IPN when elevated mortalities were reported together with histopathological observations (including pyknotic acinar cells, focal necrosis in the liver and kidney) and a positive detection of the virus by isolation in cell culture.

Fresh or archived material consisting of the original sample homogenate (heart, spleen and kidney) or the first passage material stored at -80$^{o}$C were used to isolate the virus from a selection of IPNV positive samples from 1993 – 2013 (see Table 1 for details). Each sample was inoculated (150 µl) onto monolayers of the bluegill, *Lepomis macrochirus*, fry (BF-2) cell line in Costar® 24-well plates (Corning Life Sciences). Each sample was inoculated onto the plates in triplicate giving final dilutions of $10^{-2}$, $10^{-3}$ and $10^{-4}$. Plates were incubated at

84 15$^{o}$C and monitored daily for the development of viral cytopathic effect (CPE). If no CPE
85 was observed after 7 days, media was collected from the wells and inoculated onto fresh
86 monolayers of BF-2 cells for a further 7 days. Cells were monitored for a maximum of 14
87 days in total and IPNV was confirmed by the IPNV Ag ELISA (Testline).

88 Once full CPE was observed for each sample, the supernatant was harvested and viral
89 RNA extracted using the Viral RNA Mini-Kit (Qiagen) according to the manufacturers'
90 instructions. The extracted RNA was used in a One-Step RT-PCR Kit (Qiagen) to amplify the
91 VP2 gene using two sets of primers, A1 (5'-TGAGATCCATTATGCTTCCAGA-3') and A2
92 (5'-GACAGGATCATCTTGGCATAGT-3') [4] and the genogroup 5 specific primers A-
93 Sp500F (5'-GAGTCACAGTCCTGAATC-3') and A-Sp1689R (5'-
94 AGCCTGTTCTTGAGGGCTC-3') [28]. The PCR products were separated by
95 electrophoresis on a 1.5% (w/v) agarose gel in TAE buffer (40mM Tris, 20mM acetic acid,
96 2mM ethylenedi-aminetetraacetic acid) stained with ethidium bromide and visualized with
97 the Quantity One, 1-D Analysis System software on a UV Transluminator (Bio-Rad). The
98 PCR products were purified and sequenced commercially (Sequiserve).

99 In total, 26 Irish IPNV isolates from 1993 to 2013 were analysed in this study. A previous
100 study analysed isolates from the period 2003-2007 [25] and therefore they have not been
101 included in this study with the exception of two isolates (F3108-2006 and F3202-2006) for
102 comparison purposes. The Irish isolates were also compared with 28 previously published
103 isolates from geographically distinct regions (Table 1). All Irish isolates were from Atlantic
104 salmon with the exception of one isolate from Arctic char, *Salvelinus alpinus* (F811-1994)
105 and two from rainbow trout (F484-1993 and F2017-2000). The isolates from Atlantic salmon
106 came from a range of freshwater (fry, smolt and broodstock stages) and marine sites (post-
107 smolts). One isolate (F6-2011) was from a wild Atlantic salmon returning to spawn. A 529 bp
108 region of the VP2 gene of all isolates was analysed to determine the phylogenetic

relationships. Multiple sequence alignments were performed by ClustalW analysis [33]. The pairwise distances were calculated for all isolates at the nucleotide level to evaluate the degree of similarity. The evolutionary history was inferred using the neighbour-joining method [27] and evolutionary distances computed using the Kimura 2-parameter method [18]. One thousand bootstrap replicates were performed for each analysis to assess the likelihood of the tree constructions. All phylogenetic analyses were conducted using MEGA 6 [32]. The nucleotide sequences generated in this study were deposited in GenBank and the accession numbers are shown in Table 1.

## Results and discussion

Building on from a previous study which focussed on the period of 2003-2007 [25], this study described the molecular analysis of 26 IPNV isolates from salmonid fish in Ireland between 1993 and 2013. Results from the phylogenetic analyses showed that there were two genogroups present in the country during the study period with the majority belonging to genogroup 5 as previously reported [22,25] and three isolates clustered in genogroup 3 (Fig. 1). The oldest genogroup 3 isolate (F811-1994) was isolated from Arctic char, *Salvelinus alpinus*, which had been imported from Iceland. The two remaining isolates (F1619-1998, F1703-1999) which showed 100% similarity with each other were detected in Atlantic salmon, which had also been imported from Iceland. F1619 was isolated from salmon fry in 1998 and F1703 was isolated from the same stock of fish one year later, after they had been moved to a sea site although analyses of the site inspection reports at that time do not indicate any issues with IPN. Genogroup 3 isolates have previously been reported in wild fish from Scotland, indicating that they are present in the NW Atlantic [1], however this genogroup has

not been detected in Ireland since 1999 and all subsequent isolations of IPNV have been from genogroup 5.

This study again shows the presence of two separate subtypes of Irish IPNV isolates. Interestingly, all isolations of IPNV prior to 2003, before clinical outbreaks were reported, were subtype 1 suggesting that subtype 2 isolates were not present in the country prior to 2003 and the first serious IPN outbreaks supporting previous findings [26]. Since 2007, subtype I isolates have only been detected three times, twice from farmed salmon (F81-2010; F200-2010), both from asymptomatic broodstock fish and once from a wild Atlantic salmon (F6-2011). Isolate F6-2011 was from a returning adult Atlantic salmon in the west of Ireland and the fish was not showing any signs of disease at the time. Subtype 2 isolates, first detected in 2003 have continued to be detected in association with IPN outbreaks and although the number of IPN cases/detections have decreased, this subtype now accounts for the majority of isolations of IPNV in Ireland. This study also indicates that subtype 2 isolates are now occurring on sites which previously only had subtype 1 isolates (such as sites B and H in Table 1). In 2013, IPNV was isolated only once (F106-2013), this isolate came from an Atlantic salmon broodfish and grouped with the subtype 2 isolates. Previously, all isolations from broodstock had been subtype 1, indicating that this subtype has now spread through the production cycle.

Previous studies have shown that virulent IPNV isolates from Norway have a specific amino acid motif of $T_{217}A_{221}$ [28,31]. All Irish isolates in this study had $P_{217}$ and either $T_{221}$ or $A_{221}$ (Table 2), in agreement with previously published reports [22,25]. It has been reported that the $P_{217}A_{221}$ motif is moderately virulent and $P_{217}T_{221}$ is avirulent [31]. However, in Scotland isolates with $P_{217}A_{221}$ motifs have been reported to result in significant mortalities on aquaculture sites [1,30]. In addition to this, a study from Norway showed that a higher than expected mortality was observed in a challenge trial with a $P_{217}A_{221}$ isolate (FS19)

158 expected to be of moderate to low virulence [17]. It has been stated previously that virulence
159 will also be influenced by other factors such as host responses and local environmental
160 factors and recent studies have shown that virus mutations can occur in response to the host
161 immune response and stress [11,29]. Even so, this does not rule out the possibility that even
162 higher mortalities would have occurred if viruses with the $T_{217}A_{221}$ motif had been found in
163 Ireland. The amino acid residues at position 252 were found to be quite variable, with most
164 isolates having either valine or asparagine in this position, but aspartic acid, alanine, glutamic
165 acid and isoleucine were also found (Table 2). Most isolates examined in this study had
166 isoleucine at position 314 ($I_{314}$) with the exception of the virulent Irish isolates and FS19
167 which had valine ($V_{314}$). Both valine and isoleucine are branched-chain amino acids and
168 closely resemble each other in form and function, so it is questionable whether this
169 substitution results in a significant change in the virus. Avirulent isolates from Ireland, those
170 termed subtype 2, all have a $N_{252}I_{314}$ motif. This was also found in a number of isolates from
171 continental Europe, but also in the Norwegian isolate NVI-016 and the Scottish isolate
172 Sp432-00, both of which have been reported to be of low virulence [28,30]. A more detailed
173 study would be required to determine whether the amino acid residues at position 252, in
174 combination with those at positions 217 and 221, play a role in determining virulence, or lack
175 of, in IPNV isolates.

176 Since the major genogroups of IPNV were first described [5], genogroup 5 isolates have
177 been detected in a number of Atlantic salmon producing countries where IPN has been
178 reported such as Norway [28], Scotland [1], Ireland [25] and Chile [22]. By comparing a 529
179 bp fragment of the VP2 gene of three selected Irish isolates and 26 diverse isolates it was
180 found that there were two distinct groups within genogroup 5 (Fig. 2). Australian and New
181 Zealand isolates formed a distinct subgroup (termed 5B) while all isolates of European origin
182 formed a second related subgroup, 5A. Within that group however, Chilean and Norwegian

isolates grouped with each other while the Irish isolates grouped with the Scottish and continental European isolates (see proposed subtypes 1 – 3 in Fig. 2). It has been suggested that IPN spread to Ireland from Scotland [24] and studies on the most recent outbreaks support this [25]. However, this study also suggests the possibility that the IPNV was initially introduced to Ireland from continental Europe, most likely through the trade of live rainbow trout in the 1970s and 1980s. Although infection from wild fish or local adaptation of the isolates cannot be ruled out, this study also suggests that Irish (and Scottish) IPNV isolates are more divergent from Norwegian isolates than previously thought. As suggested above in relation to virulence, a more in depth study of all IPNV isolates deposited in sequence databases such as GenBank should be performed to examine this hypothesis.

In conclusion, following outbreaks of IPN in Irish salmon hatcheries in 2006, alternative sources of salmon ova along with IPNV screening of imports were requested by the industry with the result that there have been no isolations of IPNV in freshwater sites since then. There still appears to be some level of horizontal transmission between sea sites which has been identified as an important factor in the spread of IPN disease through modelling studies carried out in both Scotland [21] and Ireland [26]. The Irish model suggested that while it was possible to eradicate IPNV in freshwater sites it would be more difficult for the marine sites and this has proven to be the case. Although there have only been a handful of IPN cases reported in recent years, the more virulent form of the virus in Ireland is now the dominating form and thus presents a challenge for controlling the disease in Ireland.

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**Conflict of interest** The authors declare that they have no conflict of interest

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310 Legends to the Figures.

311

312 **Fig 1** A condensed phylogenetic tree showing relationships between Irish infectious
313 pancreatic necrosis virus (IPNV) isolates from 1993 to 2013, based on a 529 bp sequence of
314 the VP2 gene segment. The tree was constructed using the neighbour-joining method and
315 1000 bootstrap replicates were performed for each analysis to assess the likelihood of the tree
316 construction. Only values >70 are indicated.

317

318 **Fig 2** A condensed phylogenetic tree showing relationships between infectious pancreatic
319 necrosis virus (IPNV) genogroup 5 isolates from different geographical locations, based on a
320 529 bp sequence of the VP2 gene. The tree was constructed using the neighbour-joining
321 method and 1000 bootstrap replicates were performed for each analysis to assess the
322 likelihood of the tree construction. Only values > 70 are indicated.

323

324

325 **Table 1** Infectious pancreatic necrosis virus isolates from Ireland (between 1993 and 2013, sites referred as A
326 through Q) and selected isolates from different geographic regions used as a comparison in the present study.

| # | Isolate | Host | Origin | Site/Reference | Accession no. |
|---|---|---|---|---|---|
| 1 | F484-1993 | Rainbow trout | FW | A | JX898743 |
| 2 | F561-1993 | Atlantic salmon | FW | B | KJ801308 |
| 3 | F699-1994 | Atlantic salmon | FW (smolt) | C | JX898745 |
| 4 | F811-1994 | Arctic charr | FW | C | JX880106 |
| 5 | F893-1995 | Atlantic salmon | SW | D | JX898739 |
| 6 | F1050-1996 | Atlantic salmon | SW | E | KJ811971 |
| 7 | F1619-1998 | Atlantic salmon | FW (smolt) | F | JX880107 |
| 8 | F1703-1999 | Atlantic salmon | SW | G | JX880108 |
| 9 | F1902-2000 | Atlantic salmon | SW | H | JX898740 |
| 10 | F2017-2000 | Rainbow trout | FW | I | JX898744 |
| 11 | F3108-2006 | Atlantic salmon | SW | H | JF430402 |
| 12 | F3202-2006 | Atlantic salmon | FW (fry) | J | JF430403 |
| 13 | F59-2009 | Atlantic salmon | SW | K | KJ811983 |
| 14 | F11-2010 | Atlantic salmon | SW | H | KJ811980 |
| 15 | F81-2010 | Atlantic salmon | SW (brood) | L | KJ811992 |
| 16 | F200-2010 | Atlantic salmon | FW (brood) | B | JX898741 |
| 17 | F6-2011 | Atlantic salmon | FW (wild) | M | JX898742 |
| 18 | F11-2011 | Atlantic salmon | SW | N | JX898734 |
| 19 | F37-2011 | Atlantic salmon | SW | H | KJ811986 |
| 20 | F46-2011 | Atlantic salmon | SW | O | JX898737 |
| 21 | F50-2011 | Atlantic salmon | SW | P | JX898735 |
| 22 | F51-2011 | Atlantic salmon | SW | N | JX898738 |
| 23 | F57-2011 | Atlantic salmon | SW | N | JX898736 |
| 24 | F37-2012 | Atlantic salmon | SW | Q | KJ811985 |
| 25 | F46-2012 | Atlantic salmon | SW | Q | KJ811987 |
| 26 | F106-2013 | Atlantic salmon | FW (brood) | B | KJ801312 |
| 27 | IRIPN | Rainbow trout | Iran | [8] | KC489465 |
| 28 | 1146 | Rainbow trout | Spain | [7] | AJ489222 |
| 29 | Sole ABV | Senegal sole | Spain | [12] | EF493156 |
| 30 | Sp | Rainbow trout | Denmark | [5] | AF342728 |
| 31 | Fr21 | Rainbow trout | France | [15] | L40582 |
| 32 | Fr10 | Rainbow trout | France | [5] | AY026482 |
| 33 | Sp975-99 | Atlantic salmon | Scotland | [1] | AJ829474 |
| 34 | Sp757-02 | Atlantic salmon | Scotland | [1] | AJ877116 |
| 35 | Sp432-00 | Atlantic salmon | Scotland | [1] | AJ811908 |
| 36 | N1 | Atlantic salmon | Norway | [14] | D00701 |
| 37 | NVI-010 | Atlantic salmon | Norway | [28] | AY379744 |
| 38 | NVI-013 | Atlantic salmon | Norway | [28] | AY379738 |
| 39 | NVI-015 | Atlantic salmon | Norway | [28] | AY379740 |
| 40 | NVI-016 | Atlantic salmon | Norway | [28] | AY379742 |
| 41 | NVI-023 | Atlantic salmon | Norway | [28] | AY379737 |
| 42 | FS19 | Atlantic salmon | Norway | [17] | HQ864643 |
| 43 | C-1.3 | Atlantic salmon | Chile | [22] | HQ457169 |
| 44 | C-2.6 | Atlantic salmon | Chile | [22] | HQ457172 |
| 45 | C-3.3 | Atlantic salmon | Chile | [22] | HQ457175 |
| 46 | C10 | Atlantic salmon | Chile | [22] | HQ457178 |
| 47 | VCh3351 | Atlantic salmon | Chile | [6] | JN642220 |
| 48 | OV2 | Oyster | England | [5] | AY026484 |
| 49 | NZ6 | Chinook salmon | N. Zealand | [9] | EU869270 |
| 50 | NZ10 | Turbot | N. Zealand | [9] | EU869271 |
| 51 | TAB98 | Atlantic salmon | Australia | [9] | EU672429 |
| 52 | TAB02 | Atlantic salmon | Australia | [9] | EU869272 |
| 53 | 578 | Turbot | Spain | [7] | AJ489228 |
| 54 | Te virus 2 | Tellina *sp*. | England | [5] | AF342731 |

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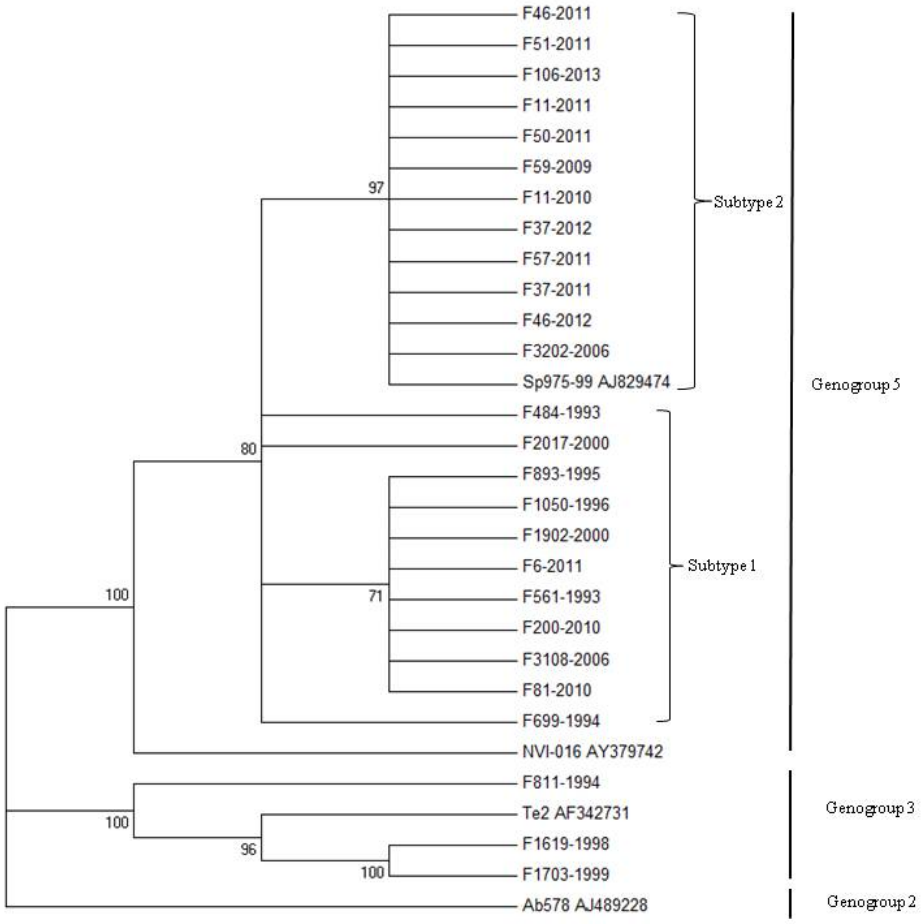
329 **Table 2** Amino acid variations in the VP2 region of the Irish infectious pancreatic necrosis virus isolates
330 compared with previously published isolates (isolate details are shown in Table 1).

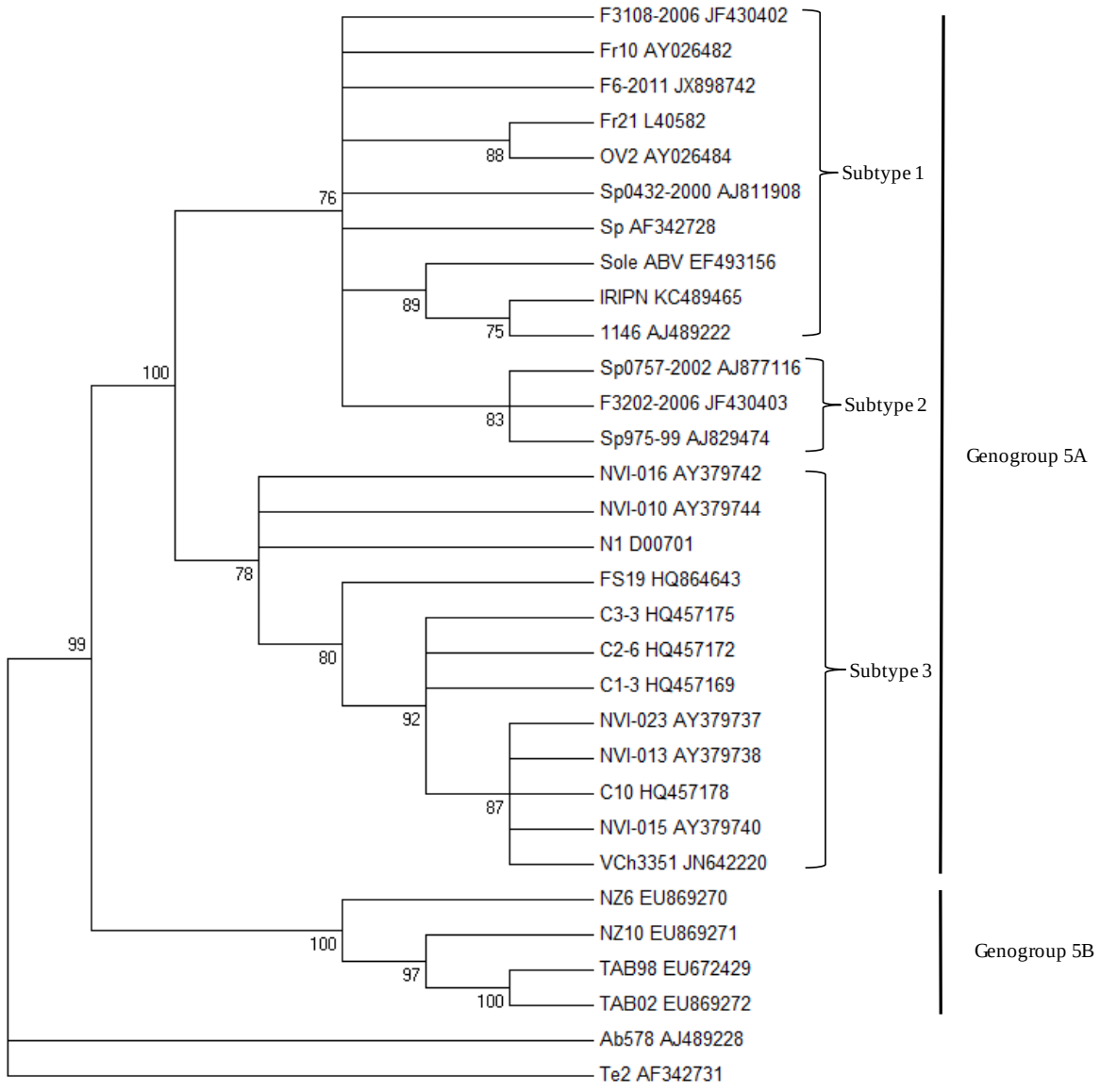
| No. | Isolate | 217 | 221 | 247 | 252 | 314 |
|---|---|---|---|---|---|---|
| 1 | F484-1993 | P | T | A | D | I |
| 2 | F561-1993 | P | T | A | N | I |
| 3 | F699-1994 | P | T | A | N | I |
| 4 | F811-1994 | P | T | P | A | V |
| 5 | F893-1995 | P | T | A | N | I |
| 6 | F1050-1996 | P | T | A | N | I |
| 7 | F1619-1998 | P | T | P | A | V |
| 8 | F1703-1999 | P | T | P | A | V |
| 9 | F1902-2000 | P | T | A | N | I |
| 10 | F2017-2000 | P | T | A | N | I |
| 11 | F3108-2006 | P | T | A | N | I |
| 12 | F3202-2006 | P | T | A | V | V |
| 13 | F59-2009 | P | A | A | V | V |
| 14 | F11-2010 | P | A | A | V | V |
| 15 | F81-2010 | P | T | A | N | I |
| 16 | F200-2010 | P | T | A | N | I |
| 17 | F6-2011 | P | T | A | N | I |
| 18 | F11-2011 | P | A | A | V | V |
| 19 | F37-2011 | P | A | A | V | V |
| 20 | F46-2011 | P | A | A | V | V |
| 21 | F50-2011 | P | A | A | V | V |
| 22 | F51-2011 | P | A | A | V | V |
| 23 | F57-2011 | P | A | A | V | V |
| 24 | F37-2012 | P | T | A | V | V |
| 25 | F46-2012 | P | A | A | V | V |
| 26 | F106-2013 | P | A | A | V | V |
| 27 | IRIPN | P | T | A | D | I |
| 28 | 1146 | P | T | A | N | I |
| 29 | Sole ABV | P | T | A | N | I |
| 30 | Sp | P | T | A | N | I |
| 31 | Fr21 | P | T | A | N | I |
| 32 | Fr10 | P | T | A | E | I |
| 33 | Sp 975-99 | P | A | A | I | X |
| 34 | Sp757-02 | P | A | A | V | I |
| 35 | Sp432-00 | P | T | A | N | I |
| 36 | N1 | P | T | A | N | I |
| 37 | NVI-010 | P | T | A | N | I |
| 38 | NVI-013 | T | A | T | V | I |
| 39 | NVI-015 | T | A | T | V | I |
| 40 | NVI-016 | P | T | A | N | I |
| 41 | NVI-023 | T | A | T | V | I |
| 42 | FS19 | P | A | A | V | V |
| 43 | C-1.3 | P | A | A | V | I |
| 44 | C-2.6 | P | A | A | V | I |
| 45 | C-3.3 | P | A | A | V | I |
| 46 | C10 | T | A | T | V | I |
| 47 | VCh3351 | T | A | T | V | I |
| 48 | OV2 | P | T | V | N | I |
| 49 | NZ6 | P | T | A | T | I |
| 50 | NZ10 | P | T | A | T | I |
| 51 | TAB98 | P | A | A | T | I |
| 52 | TAB02 | P | A | A | T | I |
| 53 | Ab AJ489228 | P | T | A | A | V |
| 54 | Te AF342731 | P | T | P | A | V |

331 P, proline, A, alanine, T, threonine, V, valine, N, asparagine, I, isoleucine, D, aspartic acid, E, glutamic acid.

FIGURE 1


F46-2011
F51-2011
F106-2013
F11-2011
F50-2011
F59-2009
F11-2010
F37-2012
F57-2011
F37-2011
F46-2012
F3202-2006
Sp975-99 AJ829474
F484-1993
F2017-2000
F893-1995
F1050-1996
F1902-2000
F6-2011
F561-1993
F200-2010
F3108-2006
F81-2010
F699-1994
NVI-016 AY379742
F811-1994
Te2 AF342731
F1619-1998
F1703-1999
Ab578 AJ489228
97
80
100
71
100
96
100
Subtype 2
Subtype 1
Genogroup 5
Genogroup 3
Genogroup 2

336 FIGURE 2

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