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Genetic diversity of feline cowpox virus, Germany 2000 – 2008

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Abstract

Recent cowpox virus (CPXV) infections of humans in Europe transmitted from cats and pet rats have risen public awareness of this rare zoonosis. Based on serosurveys wild rodents are regarded as primary reservoir hosts. Cats can become infected while hunting and could therefore serve as a sentinel for CPXV strains circulating in wild rodents. In a retrospective study we analysed formalin-fixed paraffin-embedded skin samples from cats with a histologically proven CPXV infection. Specimens had been collected in different parts of Germany during 2000 to 2008. Following DNA extraction part of the hemagglutinin gene was amplified and sequenced from samples. A phylogenetic analysis was inferred resulting in a total of 21 different CPXV genetic variants. The geographic distribution was imposed on a map.

Keywords

Orthopoxvirus, cowpox virus, cats, Germany, hemagglutinin

1 Introduction

2 For many years cowpox virus (CPXV), a member of the genus *Orthopoxvirus* (OPV), family
3 *Poxviridae*, was associated with localized lesions in cattle. However, during the last 3 decades
4 CPXV has not been isolated from cattle anymore. Instead, domestic cats and zoo animals are
5 currently those animal species most frequently diagnosed with CPXV (Bennett et al., 2008). Wild
6 rodents seem to be the primary reservoir host, shown by serological transmission studies (Hazel
7 et al., 2000). Cats, zoo animals and humans are accidental hosts with humans getting infected by
8 close contact to infected cats or pet rats (Schulze et al, 2006; Campe et al., 2009; Ninove et al.,
9 2009). The fact that most feline CPXV infections occur in the late summer (Pfeffer et al., 2002) –
10 where rodent populations usually are at their peak level – underlines the hypothesis that domestic
11 cats can be regarded as a sentinel for CPXV prevalence among rodents. In general, CPXV
12 infections predominantly manifest as skin lesions which progress from papule, vesicle, pustule,
13 ulcer to scarring. In cats, CPXV infections are often misdiagnosed clinically as pyoderma,
14 eosinophilic granuloma or neoplasm. In most cases these clinical differential diagnoses can be
15 ruled out by histopathological examination and a CPXV infection can be confirmed. Sequence
16 data from sporadic CPXV infections or small outbreaks has been published on a case-by-case-
17 basis in the last years (Schulze et al, 2006; Campe et al., 2009; Ninove et al., 2009), some
18 indicate possible chains of infection. However, systematic data on the genetic diversity of CPXV
19 does not exist. Recent rat-to-human infections in Germany and France (Campe et al., 2009;
20 Ninove et al., 2009) or cat-to-human infections in Italy (Carletti et al., 2009) have re-raised the
21 question for virus population dynamics and genetic diversity of CPXV in Europe and suggest
22 improved surveillance efforts. To fulfil this need we conducted a retrospective study on
23 formalin-fixed paraffin-embedded tissues from histopathologically confirmed feline cowpox
24 infections aiming to describe the genetic diversity of CPXV in Germany.

1 **Material and Methods**

2 **Sample Collection.** Routine tissue specimens received for histopathological examination had
 3 been processed in an automatic tissue processor, embedded in paraffin blocks, sectioned at 5–6
 4 μm on a standard rotary microtome, and mounted on glass microscope slides for automated
 5 staining with hematoxylin–eosin. Samples with the characteristic histopathologic features of a
 6 necrotizing and eosinophilic dermatitis and folliculitis with large intracytoplasmic eosinophilic
 7 A–type inclusion (ATI) bodies were considered CPXV positive. Scanning the report database
 8 (January 2000 to January 2008) for the keywords “pox”, “orthopox” and “cat” resulted in 73
 9 cases. Corresponding formalin–fixed paraffin–embedded (FFPE) blocks were retrieved and
 10 stored individually in a plastic box. For every sample the following data from each report were
 11 included in a software database (BioNumerics 5.1, Applied Maths NV, Sint–Martens–Latem,
 12 Belgium): case number (year/individual code), species, preliminary diagnosis, kind of material,
 13 place of origin, postal code, country of origin and month of transmittal. Using the postal code and
 14 place of origin coordinates were generated for display in Google Maps (Google Maps, 2009).

15 **DNA extraction.** To reduce contamination, all initial sample handling and preparation was done
 16 in a class 2 biosafety cabinet with single–use scalpels (Aesculap AG, Tuttlingen, Germany).
 17 Approximately 3 x 1 x 3 mm tissue sections were removed and transferred into sterile, DNase–
 18 free Eppendorf safe–lock Biopur 1.5 mL test tubes (Eppendorf AG, Hamburg, Germany). The
 19 DNA elution was performed with the QIAamp® DNA FFPE Tissue Kit (QIAGEN, Hilden,
 20 Germany) according to the manufacturer's instructions. The final elution step was done in 60 μL
 21 of ATE buffer.

22 **Orthopox virus–specific PCR.** A PCR yielding an approximately 270 bp amplicon within the
 23 OPV hemagglutinin (HA) gene was performed on a Perkin Elmer GenAmp® PCR System 2400
 24 (Applied Biosystems, Singapore, Singapore) with the following DNA Master mix: 6 μL of
 25 LightCycler® FastStart DNA Master SYBR Green I (Roche Applied Science, Mannheim,

Germany), 3 µL each of primer HA fw (5'-TAC TTT TGT TAC TAA TAT CAT TAG-3') and HA rev (5'-AGC AGT CAA TGA TTT AAT TGT-3') both at 10 µM (TIB MOLBIOL, Berlin, Germany), 7.2 µL MgCl₂ (final concentration of 4,8 mM MgCl₂) and 25.8 µL PCR-grade water. For amplification 5 µL of template DNA were added. PCR-grade water served as no-template-control (NTC) and vaccinia virus (VACV) strain Lister-Elstree (ATCC VR-1549, LGC Standards GmbH, Wesel, Germany) served as positive control.

The PCR was performed in a touch-down format with a pre-incubation of five minutes at 94°C, its first ten cycles starting at 94°C for one minute, then reducing the annealing temperature from 65°C to 55°C in one-degree steps for one minute and an extension phase for one minute at 72°C. Further amplification was done by 30 cycles each consisting of one minute at 94°C, 55°C and 72°C. After ten minutes at 72°C samples were held at 4°C. 4 µl of the amplicon were electrophorized in a 2 % agarose gel.

Sequencing and analysis. HA-positive samples were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The final elution step was done with 50 µl molecular biology grade water (5 Prime GmbH, Hamburg, Germany) and 3 µl were electrophorized in a 2 % agarose gel to determine the DNA concentration by using a 50 – 2000 bp AmpliSize Molecular Ruler (BioRad, Munich, Germany). DNA was adjusted to give a total yield of about 10 µg/ml and was shipped for commercial sequencing (Eurofins MWG GmbH, Ebersberg, Germany). Contigs were build using BioNumerics 5.1, sequences were aligned using ClustalW in MEGA4 (Tamura et al., 2007). The final phylogenetic analysis was also done using MEGA4, the diagram in Figure 5 was created using Base-by-Base (Brodie et al., 2004).

Nucleotide sequence accession numbers. 72 sequences representing part of the HA gene were deposited into GenBank and assigned the numbers FJ944556 to FJ944627.

Results

Samples and DNA extraction. The report search yielded 73 hits for the keywords “pox” or “orthopox” of feline origin. 72 samples were sent in from small animal practices in Germany, one from Luxemburg. In 52 cases preliminary diagnoses were conveyed by the sender, in nine cases an orthopox virus infection was suspected. The other preliminary diagnoses were: neoplasm (n = 15), eosinophilic granuloma (14), bacterial infection (9), mycosis (3), autoimmune disease (1) and herpes virus infection (1). The places of origin were distributed all over Germany with a focus on the South and West of Germany (Figure 3).

We were able to amplify part of the HA gene from 72 out of the 73 samples. Despite repeated attempts, one sample did not show an amplicon.

The seasonal distribution of the accumulated case numbers over the period from January 2000 to January 2008 is shown in Figure 1. Almost 72 % (n = 52) of the cases were found in the months August to November with a peak in September (n = 18). Sample numbers ranged from 2 (in 2006) up to 17 (in 2007) with a median of 9 samples per year.

Phylogeny and geographical distribution. After sequencing and eliminating primer-specific sequences the adjusted lengths of the resulting sequences varied from 214 to 229 base pairs (exact sizes were either 214, 217, 223 or 229 bp). All sequences were located within the hemagglutinin gene's reading frame, no frameshifts were observed. BLAST search identified all 72 study sequences as CPXV with query coverages ranging from 98 to 100 % and strain identities from 97 to 100 %. A total of 21 different genetic variants were found, ten of these have not been described in GenBank so far. The specifications of these genetic variants are outlined in Table 1 and Figure 5.

The phylogenetic tree resulting from a Neighbor-Joining analysis with 500 bootstraps is shown in Figure 2. Based on the branching, two major groups A and B with up to three subgroups were

defined. To correlate the phylogenetic analysis with the place of origin a map overlay was designed in Google Maps that shows the different subgroups and origin of the samples (Kayser et al., 2009).

Discussion

Zoonotic diseases originating from wild animal reservoirs such as tularemia, hantavirus or monkeypox virus infections are of growing interest to physicians worldwide. The reservoir host of CPXV has long been unknown, but it is now widely accepted that bank voles (*Clethrionomys glareolus*) and wood mice (*Microtus agrestis*) are primary reservoir hosts (Chantrey et al., 1999; Hazel et al., 2000). A good correlation between population density and seroconversion rates (up to 60 % against OPV) with yearly peak infection levels in the rodent population has been observed in late summer and autumn. A link between feline CPXV infections acquired from hunting CPXV-infected wild rodents was shown by a serologic study in Germany demonstrating a seroconversion rate of 2 % in 2.173 cats investigated (Czerny et al., 1996). The prevalence of CPXV in local rodent populations is certainly the cause for sporadic CPXV infections in cats which subsequently can infect humans. In this study the peak month with regard to time of infection is September, which confirms results from a previous study in cats (Pfeffer et al., 2002) and correlates well with data from rodent population studies (Chantrey et al., 1999; Hazel et al., 2000). The winter 2006/2007 was extremely mild in Germany, which – together with other climatic factors - led to a significant rise in vole populations and subsequent hantavirus infections among humans in 2007 (Piechotowski et al., 2008). And indeed, the highest number of CPXV infections in cats (n = 17) was observed in 2007 (Figure 1).

Since CPXV has never been isolated from rodent species in Western Europe, the use of molecular techniques for analysing feline CPXV could shed light on a potential genetic diversity of CPXV in the primary rodent host. In the past, open reading frame (ORF) sequences of the

1 hemagglutinin (HA) protein have proven very useful to allocate a given isolate to a distinct OPV
2 species, thereby confirming the current taxonomy of OPV species which is based on phenotypic
3 markers. In this study we attempted to sequence the entire ORF of the HA gene from FFPE
4 specimens. However, pretests in our lab failed to amplify the entire ORF (which accounts for 900
5 to 951 bp, depending on the strain investigated) in the majority of the 73 FFPE blocks (data not
6 shown). The success rate of this PCR approach largely depends on the fixative itself, the age of
7 the FFPE block and the duration of fixation, since all these factors are likely to promote DNA
8 degradation and making amplification sometimes impossible (Johansen et al., 2004). We
9 therefore chose a second PCR assay amplifying a much shorter sequence within the HA gene
10 (about 270 base pairs). The segment amplified lies on the upstream end of the HA gene (starting
11 at nucleotide 41 of the CPXV type strain Brighton Red). This rather short sequence of the HA
12 gene allowed identification of all 72 amplified samples as CPXV using BLAST search (BLAST,
13 2009). CPXV always was the top hit, in four cases (CPXV 2007/33255 (FJ944556), CPXV
14 2005/27380 (FJ944603), CPXV 2006/28727 (FJ944615), CPXV 2004/28150 (FJ944626)) a
15 sequence conserved in both, vaccinia and cowpox virus was found.

16 ATIs are regarded as a pathognomonic marker for an infection with CPXV. This was confirmed
17 to nearly 100 % in this study by PCR findings. A re-examination of one hematoxylin and eosin
18 (HE)-stained tissue section that did not yield a CPXV amplicon revealed a pattern characteristic
19 of a viral infection. However, inclusions were not clearly located intracytoplasmic. A herpes
20 virus could therefore be the causative agent in this case. The high correlation between PCR and
21 light microscopy supports the key role and the high sensitivity and specificity of histopathology.
22 Only 17 % of the preliminary diagnoses were correctly made by the sender of the sample. CPXV
23 in cats seems to be “underdiagnosed” by the general practitioner since the reasons for submission
24 of skin specimens to the pathologist were mostly other dermatologic diseases and not a suspected
25 CPXV infection.

1 The phylogenetic tree in Figure 2 reveals two major groups (A and B), group B could be further
 2 divided into three subgroups based on the clustering. Group A specimens have been sent in from
 3 the northern part of Germany, whereas group B specimens have a higher incidence in the south.
 4 Both groups overlap to some extent in central Germany.

5 It is remarkable that subgroup B.2 (red markers) shows close clustering in and around Berlin
 6 (including the strain Biber V940/97, described by Hentschke et al., 1999). Strain GRI-90 from
 7 Moscow is loosely related to this small cluster. Unfortunately the study lacks further data from
 8 Mecklenburg–Western Pomerania (the federal state north of Berlin) and in general from the area
 9 of the former German Democratic Republic to draw conclusions on the appearance of further
 10 subgroup B.2 isolates. It would also be valuable to add data from Poland and the Czech Republic
 11 to the comparison to see if the two–group–distribution extends further to the East of Europe.

12 Group B.1 is very small and to some extent heterogeneous, in our opinion conclusions should not
 13 yet be drawn out of its existence. In this case again, data from Austria and Italy could add
 14 valuable information to the map. The reference strains all fit well into the clustering, they were
 15 added to illustrate the potential of combined phylogenetic analyses and the use of geographical
 16 information systems.

17 It could be argued that the sequence variation described in this study is merely a clonal selection
 18 in the infected cat of highly different CPXVs circulating in the reservoir host. However, OPVs
 19 are genetically remarkably stable in vivo and in vitro. This was shown in a recent study of human
 20 monkeypox in the Democratic Republic of Congo. Monkeypox–like CPXV has a broad host
 21 range and is maintained in a rodent reservoir, however, the HA gene sequence of 50 human
 22 monkeypox virus isolates was 100 % identical (Rimoin et al., 2007) which argues against a
 23 variation in the reservoir host.

24 Isolates of CPXV do differ in restriction enzyme pattern and generally greater differences occur
 25 between geographically distinct isolates. Based on bioinformatic analyses of the genomes

sequenced so far it has been proposed that the two CPXV reference strains Brighton (UK) and Grishak (Russia) are quite divergent and should be reclassified as separate OPV species (Gubser et al., 2004).

Regarding the apparent variation among isolates in central Europe which was also confirmed in this study, CPXV might have originated from central Europe. A geographically and host-related independent evolution might explain the genetic divergence seen today. Isolation of new or a more detailed genomic characterization of existing strains might increase the number of genetic variants considerably. However, due to the shortness of the amplicon the resulting phylogenetic tree in Figure 2 should be interpreted with caution: while the full HA gene has proven to be well-suited for phylogenetic analyses, this is the first attempt to perform an analysis on such a short segment. It might also be that an even larger genetic variation would have appeared if the full length HA gene would have been amplified, but it is also possible that the segment under investigation shows an unusually high grade of base exchanges. A certain degree of blur in the phylogenetic tree must be attributed to the short amplicon.

A large database is a prerequisite for an adequate prediction of risk to humans, but the proper understanding of animal surveillance data is the key challenge for linking veterinary research to human risk prediction (Scotch et al., 2009). The data generated in this study using cats as a sentinel for CPXV infections might add to the understanding of CPXV distribution in Germany and show the potential of integrating geographical information with sequence data in outbreak investigations and risk prediction. Due to its self-limiting course in humans and its low impact on public health, CPXV infections are not a notifiable disease in Germany, surrogate parameters as presented in this study seem to be the only practical approach to human risk prediction.

Conclusion

1 CPXV infection – although relatively rare in animals and uncommon among humans – has
2 gained some recognition through micro-outbreaks in recent years. The fact that immunity to
3 smallpox infection (with its cross-reactivity to CPXV) in the world population is constantly
4 decreasing due to cessation of mandatory smallpox vaccination nearly 30 years ago, puts more
5 persons to the risk of zoonotic CPXV infections. Since CPXV is not a notifiable disease in
6 Germany and distinct virological diagnosis relies on specialized labs, it is often not included in
7 the differential diagnoses of most general practitioners (veterinary or human). A higher grade of
8 public awareness and a linkage between human and veterinary health authorities through
9 databases will reduce the individual risk of infection. This study can serve as part of this effort by
10 providing data on the geographical distribution of CPXV in Germany.

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3 study.

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References

- Begon, M., Telfer, S., Smith, M. J., Burthe, S., Paterson, S., Lambin, X., 2009. Seasonal host dynamics drive the timing of recurrent epidemics in a wildlife population. *Proc. R. Soc. B* 276, 1603–1610.
- Bennett, M., Smith, G. L., Baxby, D., 2008. Cowpox virus. In: Mahy, B. W. J., van Regenmortel, M. (Eds.), *Encyclopedia of Virology*, Academic Press, London, pp. 574–580.
- BLAST, Basic Local Alignment Search Tool, 2009. <http://blast.ncbi.nlm.nih.gov/Blast.cgi>
- Brodie, R., Smith, A. J., Roper R. L., Tcherepanov, V., Upton, C., 2004. Base-By-Base: Single nucleotide-level analysis of whole viral genome alignments, *BMC Bioinformatics* 5, 96.
- Burthe, S., Telfer, S., Lambin, X., Bennett, M., Carslake, D., Smith, A., Begon, M., 2006. Cowpox virus infection in natural field vole *Microtus agrestis* populations: delayed density dependence and individual risk. *J. Anim. Ecol.* 75, 1416–1425.
- Campe, H., Zimmermann, P., Glos, K., Bayer, M., Bergemann, H., Dreweck, C., Graf, P., Weber, B. K., Meyer, H., Büttner, M., Busch, U., Sing, A., 2009. Cowpox virus transmission from pet rats to humans, Germany. *Emerg. Inf. Dis.* 15, 777–780.
- Carletti, F., Bordi, L., Castilletti, C., Di Caro, A., Falasca, L., Gioia, C., Ippolito, G., Zaniratti, S., Beltrame, A., Viale, P., Capobianchi, M. R., 2009. Cat-to-human orthopoxvirus transmission, northeastern Italy. *Emerg. Inf. Dis.* 15, 499–500.
- Chantrey, J., Meyer, H., Baxby, D., Begon, M., Bown, K. J., Hazel, S. M., Jones, T., Montgomery, W. I., Bennett, M., 1999. Cowpox: reservoir hosts and geographic range. *Epidemiol. Infect.* 122, 455–460.
- Czerny, C. P., Wagner, K., Gessler, K., Mayr, A., Kaaden, O. R., 1996. A monoclonal blocking-ELISA for detection of orthopoxvirus antibodies in feline sera. *Vet. Microbiol.* 52, 185–200.
- Eis-Hübinger, A. M., Gerritzen, A., Schneeweis, K. E., Pfeiff, B., Pullmann, H., Mayr, A., Czerny, C. P., 1990. Fatal cowpox-like virus infection transmitted by cat. *Lancet* 336, 880.

- 1 Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap.
- 2 Evolution 39, 783–791.
- 3 Google Maps, 2009. <http://maps.google.com/>
- 4 Gubser, C., Hué, S., Kellam, P., Smith, G. L., 2004. Poxvirus genomes: a phylogenetic analysis.
- 5 J. Gen. Virol. 85, 105–117.
- 6 Hazel, S. M., Bennett, M., Chantrey, J., Bown, K., Cavanagh, R., Jones, T. R., Baxby, D., Begon,
- 7 M., 2000. A longitudinal study of an endemic disease in its wildlife reservoir: cowpox and wild
- 8 rodents. Epidemiol. Infect. 124, 551–562.
- 9 Hentschke, J., Meyer, H., Wittstadt, U., Burkhardt, S., Ochs, A., Aue, A., 1999. An outbreak of
- 10 cowpox in beavers (*Castor fiber canadensis*) and bearcats (*Ailurus fulgens*) in Berlin zoo.
- 11 Tierärztl. Umsch. 54, 311–316 (Article in German).
- 12 Johansen, I. S., Thomsen, V. Ø., Forsgren, A., Hansen, B. F., Lundgren, B., 2004. Detection of
- 13 Mycobacterium tuberculosis complex in formalin–fixed, paraffin–embedded tissue specimens
- 14 with necrotizing granulomatous inflammation by strand displacement amplification. J. Mol.
- 15 Diagn. 6, 231–236.
- 16 Kaysser, P., v. Bomhard, W., Dobrzykowski, L., Meyer, H., 2009.
- 17 [http://maps.google.com/maps/ms?ie=UTF&msa=0&msid=117602373455318976828.00046872c](http://maps.google.com/maps/ms?ie=UTF&msa=0&msid=117602373455318976828.00046872c56e10a195632)
- 18 [56e10a195632](http://maps.google.com/maps/ms?ie=UTF&msa=0&msid=117602373455318976828.00046872c56e10a195632)
- 19 Ninove, L., Domart, Y., Vervel, C., Voinot, C., Salez, N., Raoult, D., Meyer, H., Capek, I.,
- 20 Zandotti, C., Charrel, R. N., 2009. Cowpox virus transmission from pet rats to humans, France.
- 21 Emerg. Inf. Dis. 15, 781–784.
- 22 Pfeffer, M., Pfleghaar, S., von Bomhard, D., Kaaden, O. R., Meyer, H., 2002. Retrospective
- 23 investigation of feline cowpox in Germany. Vet. Rec. 150, 50–51.

- 1 Piechotowski, I., Brockmann, S., Schwarz, C., Winter, C., Ranft, U., Pfaff, G., 2008. Emergence
2 of hantavirus in South Germany: rodents, climate and human infections. *Parasitol. Res.* 103, 131–
3 137.
- 4 Rimoin, A. W., Kisalu, N., Kebela-Ilunga, B., Mukaba, T., Wright, L. L., Formenty, P., Wolfe,
5 N. D., Shongo, R. L., Tshioko, F., Okitolonda, E., Muyembe, J. J., Ryder, R. W., Meyer, H.,
6 2007. Endemic human monkeypox, Democratic Republic of Congo, 2001–2004. *Emerg. Inf. Dis.*
7 13, 934–937.
- 8 Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing
9 phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.
- 10 Schulze, C., Alex, M., Schirrmeier, H., Hlinak, A., Engelhardt, A., Koschinski, B., Beyreiß, B.,
11 Hoffmann, M., Czerny, C. P., 2007. Generalized fatal cowpox virus infection in a cat with
12 transmission to a human contact case. *Zoonoses Public Health* 54, 31–37.
- 13 Scotch, M., Odofin, L., Rabinowitz, P., 2009. Linkages between animal and human health
14 sentinel data. *BMC Vet. Res.* 5, 15.
- 15 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics
16 Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.
- 17 Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using
18 the Neighbor-Joining method. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11030–11035.

1 **Tables**

- 2 Table 1. Features of 21 cowpox virus genetic variants detected in the study.

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Figure captions

Figure 1. Seasonal distribution of cowpox virus (CPXV) infections in cats (black: accumulated numbers from January 2000 to January 2008, n = 72; grey: case numbers from 2007, n = 17).

Figure 2. Evolutionary relationships of 80 CPXV sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0,17798791 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 214 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). For reference eight additional CPXV strains were included (by accession number, sequences were truncated for facilitated alignment using the primer pair applied for amplification described above): AF482758 (Brighton Red), AY298785 (Callithrix jacchus), AY902287 (Nancy), AY902260 (Biber V940/97), AY944028 (CPV91ger3), EF612709 (ITA 1 PG), FJ654467 (Shiva), X94355 (GRI-90). Data is presented using virus acronym, sequence identifier, GenBank accession number (study samples) or virus acronym, city of origin, strain, GenBank accession number (reference strains).

Figure 3. Geographical distribution of 77 feline specimens which proved CPXV positive. Group markers (corresponding to the groups in Figure 2) use the following colour scheme: A –

1 turquoise, B.1 – green, B.2 – red, B.3 – blue. Reference strains in Great Britain, Italy and Russia
2 are not shown.

3
4 Figure 4. Paw pad of a twelve year old female cat: necrotizing dermatitis with abundant brightly
5 eosinophilic intracytoplasmic inclusion bodies. Hematoxylin–eosin stain (400 X, bar = 200 μ m).

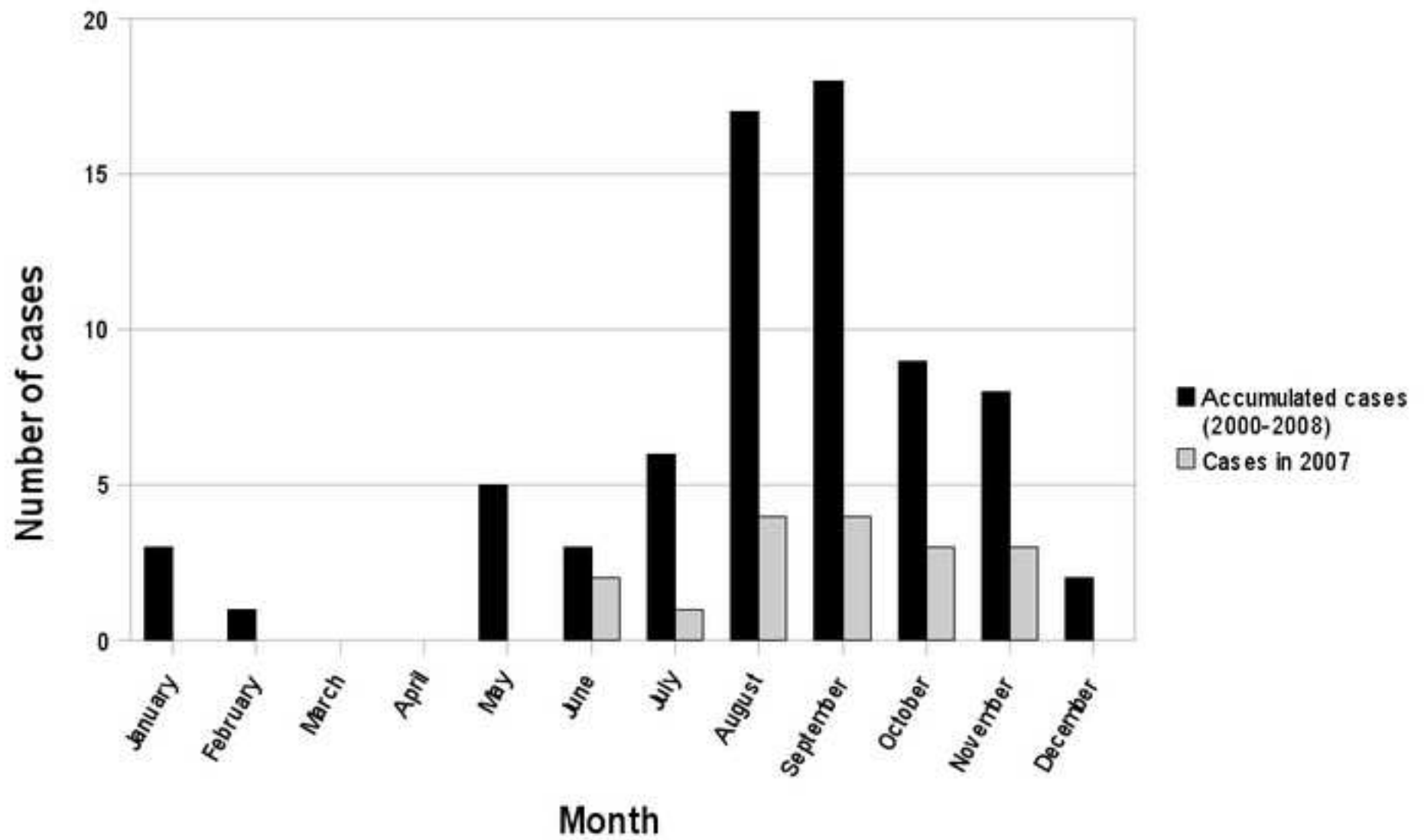
6
7 Figure 5. Graphical overview of 21 genetic variants of cowpox virus detected in the study.

1 **Conflict of interest statement**

- 2 The authors declare that they have no conflict of interest.

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Figure 1



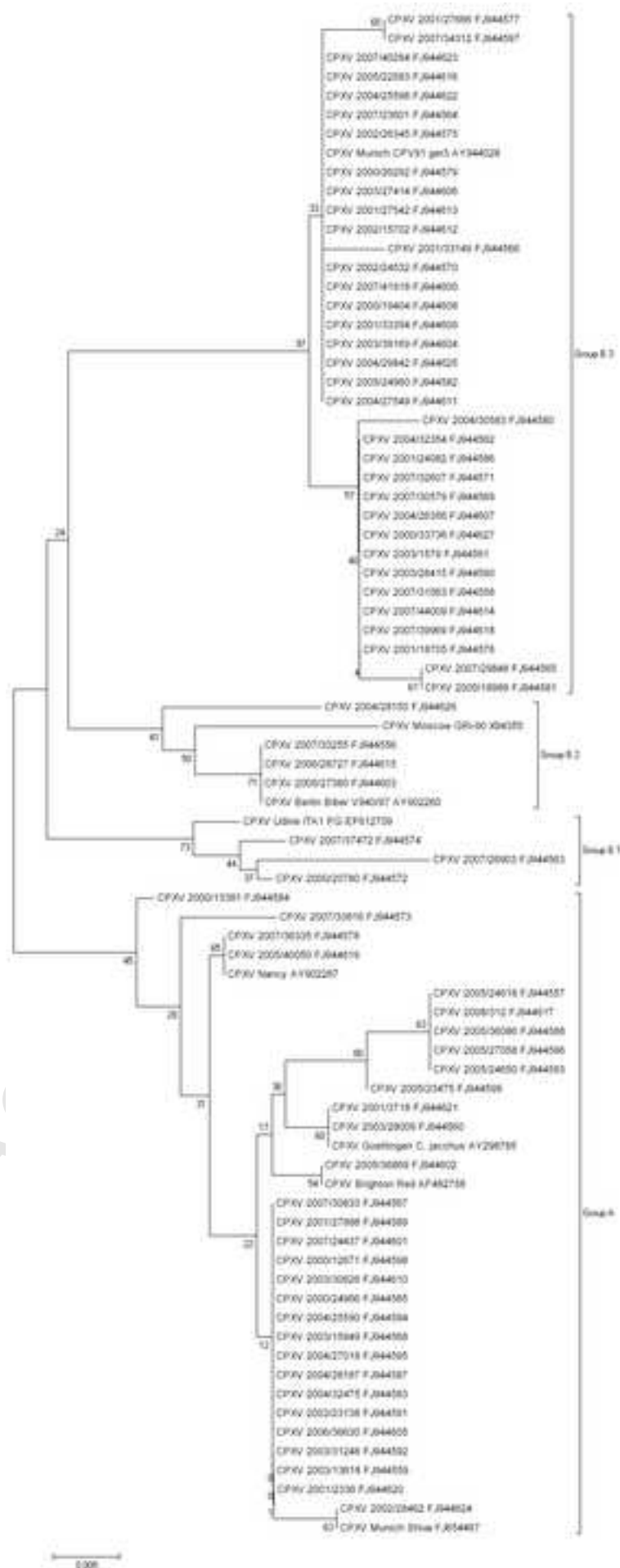




Figure 4

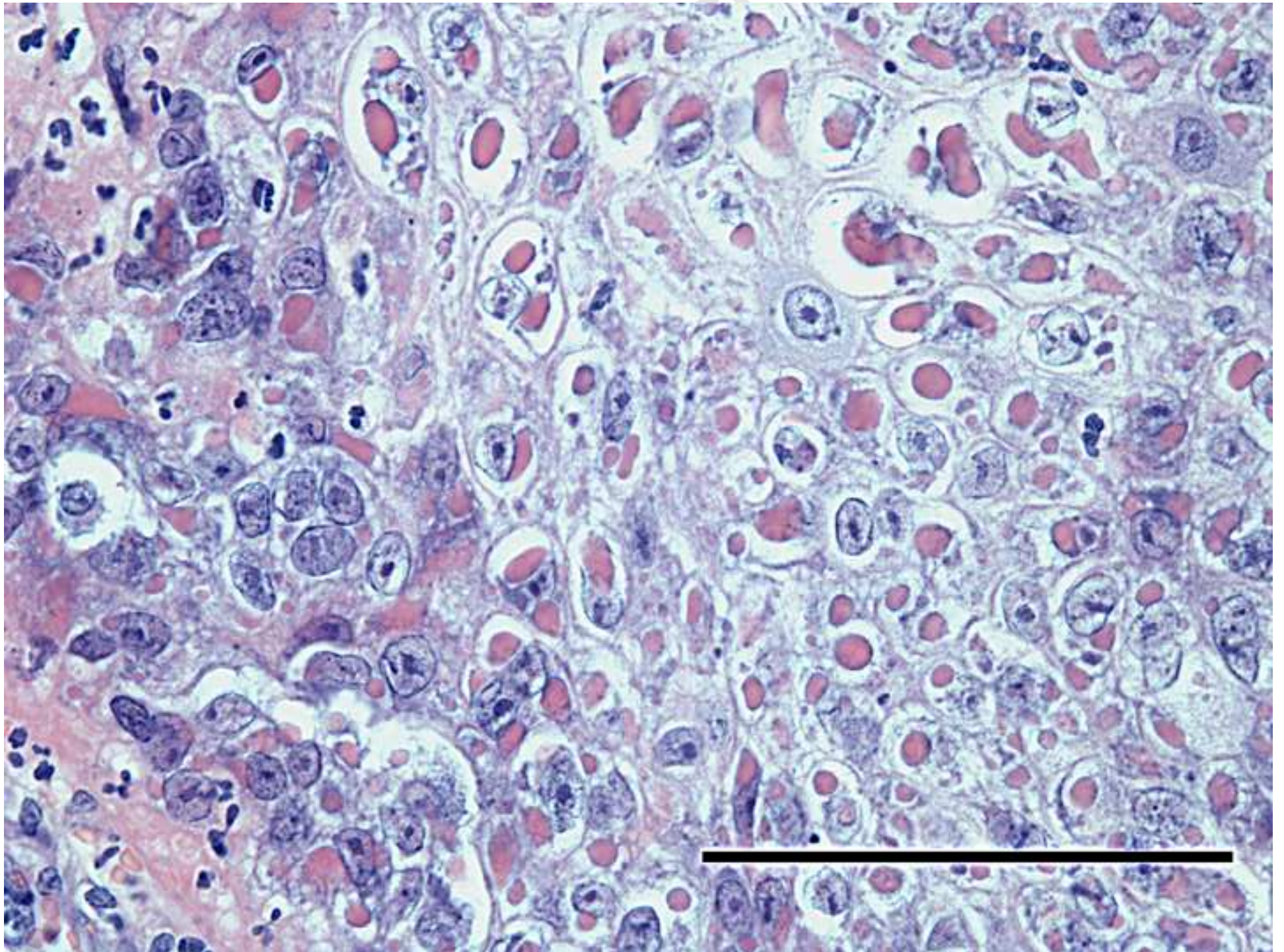
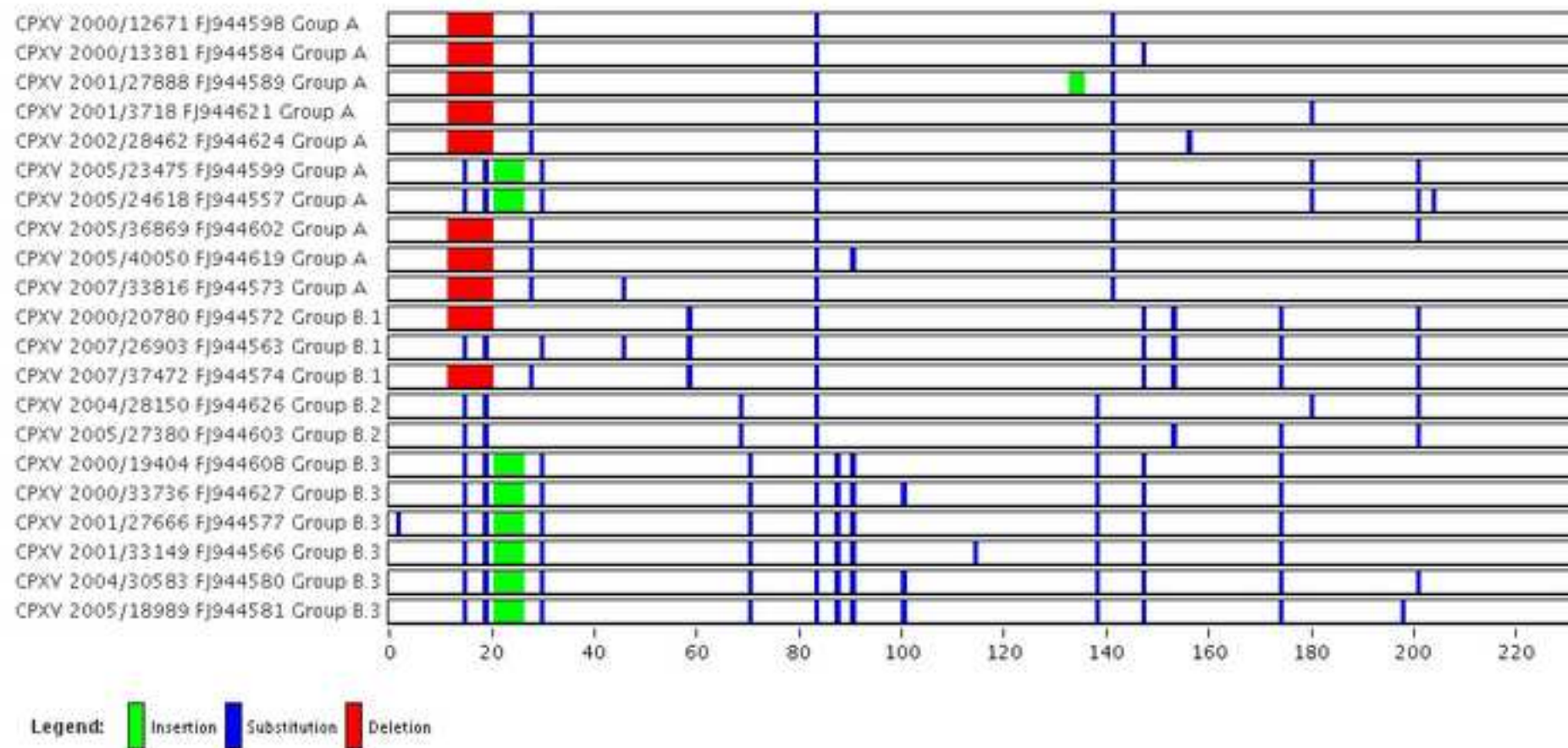


Figure 5

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Sequence number	Accession number	Sequence previously described	Group according to Figure 2
CPXV 2000/12671	FJ944598	yes	A
CPXV 2000/13381	FJ944584	no	A
CPXV 2001/27888	FJ944589	no	A
CPXV 2001/3718	FJ944621	yes	A
CPXV 2002/28462	FJ944624	yes	A
CPXV 2005/23475	FJ944599	yes	A
CPXV 2005/24618	FJ944557	yes	A
CPXV 2005/36869	FJ944602	yes	A
CPXV 2005/40050	FJ944619	yes	A
CPXV 2007/33816	FJ944573	no	A
CPXV 2000/20780	FJ944572	no	B.1
CPXV 2007/26903	FJ944563	no	B.1
CPXV 2007/37472	FJ944574	no	B.1
CPXV 2004/28150	FJ944626	no	B.2
CPXV 2005/27380	FJ944603	yes	B.2
CPXV 2000/19404	FJ944608	yes	B.3
CPXV 2000/33736	FJ944627	yes	B.3
CPXV 2001/27666	FJ944577	yes	B.3
CPXV 2001/33149	FJ944566	no	B.3
CPXV 2004/30583	FJ944580	no	B.3
CPXV 2005/18989	FJ944581	no	B.3

* Identical to strain Brighton Red AF482758.