

Mystery of fatal ‘Staggering disease’ unravelled: Novel rustrela virus causes severe encephalomyelitis in domestic cats

Kaspar Matiasek¹, Florian Pfaff², Herbert Weissenböck³, Claudia Wylezich², Jolanta Kolodziejek⁴, Sofia Tengstrand⁵, Frauke Ecke⁶, Sina Nippert⁷, Philip Starcky², Benedikt Litz², Jasmin Nessler⁸, Peter Wohlsein⁹, Christina Baumbach¹⁰, Lars Mundhenk¹¹, Andrea Aebischer¹², Sven Reiche¹², Pia Weidinger⁴, Karin M. Olofsson¹³, Cecilia Rohdin^{14,15}, Christiane Weissenbacher-Lang³, Julia Matt³, Marco Rosati¹, Thomas Flegel¹⁶, Birger Hörnfeldt⁶, Dirk Höper², Rainer G. Ulrich^{7,17}, Norbert Nowotny^{4,18}, Martin Beer², Cecilia Ley^{5,13}, Dennis Rubbenstroth^{2,*}

¹Section of Clinical & Comparative Neuropathology, Institute of Veterinary Pathology, Centre for Clinical Veterinary Medicine, Ludwig-Maximilians-Universität München, Munich, Germany

²Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

³Institute of Pathology, University of Veterinary Medicine Vienna, Vienna, Austria

⁴Institute of Virology, University of Veterinary Medicine Vienna, Vienna, Austria

⁵Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

⁶Department of Wildlife, Fish and Environmental Studies, Swedish University of Agricultural Sciences (SLU), Umeå, Sweden

⁷Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

⁸Department of Small Animal Medicine and Surgery, University of Veterinary Medicine Hannover, Hannover, Germany

⁹Department of Pathology, University of Veterinary Medicine Hannover, Hannover, Germany

¹⁰State Office for Agriculture, Food Safety and Fisheries, Rostock, Germany

¹¹Institute of Veterinary Pathology, Freie Universität Berlin, Berlin, Germany

¹²Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

¹³Department of Pathology and Wildlife Diseases, National Veterinary Institute (SVA), Uppsala, Sweden

¹⁴Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

¹⁵Anicura, Albano Small Animal Hospital, Danderyd, Sweden

¹⁶Department of Small Animal Medicine, Leipzig University, Leipzig, Germany

¹⁷German Centre for Infection Research (DZIF), Partner site Hamburg-Lübeck-Borstel-Riems, Greifswald-Insel Riems, Germany

¹⁸College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates.

***Corresponding author:**

Dennis Rubbenstroth, Dennis.Rubbenstroth@fli.de, Tel.: +49-38351-7-1521

39 **ABSTRACT**

40 'Staggering disease' is a neurological disorder considered a threat to European domestic cats (*Felis*
41 *catus*) for almost five decades. However, its aetiology has remained obscure. Rustrela virus (RusV),
42 a relative of rubella virus, has recently been shown to be associated with encephalitis in a broad
43 range of mammalian hosts. Here, we report the detection of RusV RNA and antigen by metagenomic
44 sequencing, RT-qPCR, *in-situ* hybridization and immunohistochemistry in brain tissues of 28 out of
45 29 cats with non-suppurative meningoencephalomyelitis and 'staggering disease'-like neurological
46 disorder from Sweden, Austria, and Germany, but not in non-affected control cats. Screening of
47 possible reservoir hosts in Sweden revealed RusV infection in wood mice (*Apodemus sylvaticus*).
48 Our work strongly indicates RusV as the long-sought cause of feline 'staggering disease'. Given its
49 broad host spectrum and considerable geographic range, RusV may be the aetiological agent of
50 neuropathologies in further mammals, possibly even including humans.

51

52 INTRODUCTION

53 Throughout mammalian species, inflammatory disorders of the central nervous system (CNS) are
54 associated with substantial suffering, mortality and long-term neurological deficits.
55 Aetiopathogenically, they can be broadly categorised into infectious and immune-mediated
56 disorders¹. All too often, however, the cause of an encephalitis remains unknown and leaves
57 clinicians, patients and owners of affected pets with considerable uncertainty about its origin,
58 treatment options and, hence, prognosis. The latter holds true particularly for the large
59 histopathologically convergent panel of non-suppurative, lymphohistiocytic encephalitides. A
60 considerable proportion of these cases remains unsolved using conventional diagnostic methods,
61 such as immunohistochemistry (IHC), *in-situ* hybridization (ISH), and polymerase chain reaction
62 (PCR) techniques for regional neurotropic pathogens^{2, 3, 4, 5, 6}.

63 One of those controversial encephalitides of possibly infectious origin is the so-called ‘staggering
64 disease’ of domestic cats (*Felis catus*). It has been described first in the 1970s in the Swedish Lake
65 Mälaren region between Stockholm and Uppsala⁷, which remains a hotspot of ‘staggering disease’
66 to the present. Neurologic disorders possessing striking similarity with this disease entity have been
67 described also in domestic cats in other European countries, particularly in Austria^{6, 8, 9, 10, 11}, and
68 even in other felids^{12, 13}.

69 The most prototypic clinical sign is hind leg ataxia with a generally increased muscle tone resulting
70 in a staggering gait. In addition, a broad range of other neurologic signs may occur, including the
71 inability to retract the claws, hyperaesthesia and occasionally tremors and seizures. Behavioural
72 alterations may range from enhanced vocalization, depression and becoming more affectionate to
73 rarely aggression^{7, 8, 14, 15}. The disease progression usually lasts a few days to a few weeks, but may
74 also continue for more than a year, and it generally results in euthanasia— for animal welfare
75 reasons. The histopathology of ‘staggering disease’ is characterized by a non-suppurative,
76 predominantly lymphohistiocytic meningoencephalomyelitis with angiocentric immune cell invasion
77 and perivascular cuffing predominantly in the grey matter of the CNS^{7, 8, 14, 15, 16}.

78 While the microscopic pattern has suggested a viral origin of ‘staggering disease’, its aetiological
79 agent has remained undisclosed for almost five decades. Borna disease virus 1 (BoDV-1; species
80 *Orthobornavirus bornaense*; family *Bornaviridae*), which causes neurologic disorders in various
81 mammals including humans¹⁷, has for a long time spearheaded the panel of aetiological
82 candidates^{16, 18, 19, 20, 21, 22, 23}. However, results suggesting natural BoDV-1 infections in cats with
83 ‘staggering disease’ in Sweden remained inconclusive and were later refuted on the grounds of
84 new standards^{17, 24, 25}.

85 Fortunately, advances in clinical metagenomics over the last years have provided us with promising
86 tools for the detection of new or unexpected pathogens involved in hitherto unexplained
87 encephalitides^{26, 27, 28, 29, 30, 31}. By application of an established metagenomic workflow³², we now

88 detected rustrela virus (RusV; *Rubivirus strelense*; *Matonaviridae*) sequences in brains of cats with
89 'staggering disease'-like neurological disorder. RusV is a recently discovered relative of rubella virus
90 (RuV; *Rubivirus rubellae*)³², the causative agent of rubella in humans^{30, 33}. It was first identified in
91 the brains of various mammals in a zoo close to the Baltic Sea in Northern Germany^{30, 34}. These
92 animals had suffered from neurologic disorders and lymphohistiocytic encephalitis^{30, 31, 34}. Yellow-
93 necked field mice (*Apodemus flavicollis*) without apparent encephalitis were considered as possible
94 reservoir hosts of the virus in that area^{30, 34}.

95 Here, we now report the presence of RusV in the brains of cats with non-suppurative
96 lymphohistiocytic meningoencephalomyelitis and 'staggering disease'-like disorders from Sweden,
97 Austria, and Germany, by metagenomic analysis and further independent methods, including
98 reverse transcription real-time PCR (RT-qPCR), ISH and IHC. In contrast, RusV was not detected in
99 the brains of control cats without neurologic disease or with encephalopathies of other causes from
100 the same or nearby regions. Thus, our results indicate that RusV is indeed the causative agent of
101 long-known 'staggering disease' in domestic cats.

102

103 RESULTS

104

105 Failure to detect BoDV-1 infection in cats with 'staggering disease'

106 In an attempt to investigate the aetiology of 'staggering disease', frozen or formalin-fixed paraffin-
107 embedded (FFPE) brain samples from 29 cats with non-suppurative encephalitis and/or the clinical
108 presentation of 'staggering disease' from Sweden (n=15), Austria (n=9), and Germany (n=5) were
109 examined for the presence of bornaviruses by RT-qPCR assays detecting the RNA of BoDV-1 and
110 other orthobornaviruses²⁷, (**Supplemental Table S1**), or by IHC using a monoclonal antibody
111 targeting the BoDV-1 nucleoprotein (**Supplemental Figure S1**). Neither bornavirus RNA nor antigen
112 could be detected.

113

114 RusV sequences identified in cats with 'staggering disease' by metagenomic analysis

115 Selected samples were subsequently analysed using a generic metagenomic sequencing
116 workflow³². In an initial analysis using blastx, sequence reads with the highest identity to RusV were
117 identified in 14 out of 15 tested samples from these three countries (**Table 1**). Additional high
118 throughput sequencing (HTS), assisted by target enrichment using the panRubi myBaits set v2), a
119 newly developed set v3 achieved complete RusV genome sequences for three cats from Sweden
120 (animals SWE_13, SWE_14 and SWE_15) and one cat from Northeastern Germany (GER_04), as
121 well as a complete and an almost genome sequences for two cats from Austria (AUT_02 and
122 AUT_06, respectively). The newly identified RusV sequences clearly clustered with other RusV
123 sequences when compared to related matonaviruses (**Figure 1a**), based on amino acid (aa)
124 sequences of the structural polyprotein (p110/sPP). The genome nucleotide (nt) sequences from
125 Austria and Sweden formed separate phylogenetic lineages in comparison to the sequences from
126 Germany (**Figure 1b**). While sequence GER_04 possessed at least 92.1% nt sequence identity with
127 the previously published German RusV sequences, the minimum nt identities of the Swedish and
128 Austrian sequences to the German sequences were only 76.7%, or 76.0%, respectively, but 80.7%
129 to each other (**Supplemental Figure S2**). The genome organization of the newly discovered RusV
130 sequences (**Figure 1c**) was consistent with those of previously published RusV genomes^{30, 34}. Using
131 a sliding window analysis, we identified a highly conserved region at the 5' terminus of the RusV
132 genomes (approximate positions 1 to 300). Regions of particularly high variability covered the
133 intergenic region between the p200 and p110 open reading frames (ORF) as well as a stretch of
134 the p150-encoding sequence around nt positions 2,100-2,600 (**Figure 1c**).

135

136 **Detection of RusV RNA using a broadly reactive panRusV RT-qPCR assay**

137 Since the initially published RT-qPCR assay RusV Mix-1³⁰ was unable to detect RusV RNA in samples
138 from Sweden and Austria (data not shown), we designed a new set of primers and probe targeting
139 the highly conserved region at the 5' end of the genome (**Supplemental Figure S3; Supplemental**
140 **Table S1**). This newly established panRusV assay readily detected RusV RNA in the brains of all 15
141 Swedish cats with 'staggering disease', eight out of nine Austrian cats^{8, 11}, and three out of five cats
142 from Germany (**Table 1**). Results were moderately to strongly positive for frozen tissue (cycle of
143 quantification [Cq] values 20 to 32), and rather weakly positive for animals where only FFPE
144 material was available (Cq 27 to 36; **Supplemental Table S2; Supplemental Figure S4a**). In contrast,
145 RusV RNA was not detected in frozen brain samples from 21 control cats without encephalitis
146 originating from Sweden, Austria, and Germany, or in eight cats from Germany suffering from other
147 types of encephalitis (**Table 1**).

148

149 **Detection of RusV RNA and antigen in neural tissue by ISH and IHC**

150 To confirm and further characterize RusV infection in the cats, we employed viral RNA detection by
151 ISH and antigen detection by IHC (**Figure 2**). An RNAscope ISH probe was designed to target the
152 highly conserved stretch at the 5' terminus of the RusV genome (**Supplemental Figure S3**). Specific
153 ISH staining was observed for 22 out of 26 tested cats from all three countries (**Table 1; Figures 2a**
154 **to d**). Two animals revealed inconclusive results and two were ISH-negative (**Supplemental Table**
155 **S2**).

156 In addition, we performed IHC using a newly generated mouse monoclonal antibody targeting the
157 RusV capsid protein. Specific immunopositivity mirroring the ISH pattern (**Figures 2e to h**) was seen
158 in 27 out of 29 analysed cats with 'staggering disease', but not in any brain from 18 tested control
159 cats (**Table 1**). IHC identified RusV antigen in two cases that had been negative by RT-qPCR from
160 FFPE brain tissue (AUT_03 and GER_02), whereas one RT-qPCR-positive individual (AUT_05)
161 remained negative by IHC (**Supplemental Table S2**).

162 By both, ISH and IHC, a specific granular chromogen staining was observed predominantly in
163 perikarya of pyramidal neurons of cerebral cortices, namely of isocortex (**Figures 2c, g**) and
164 hippocampus proper (**Figure 2f**), granule cells of dentate gyrus (**Figure 2b**), Purkinje cells of the
165 cerebellum (**Figures 2a, e**), multipolar neurons of brain stem and cerebellar roof, and in ventral
166 horn neurons of the spinal cord (**Figures 2d, h**). On occasion, cytoplasmic immunoreactivity was
167 also noted in individual interposed neuroglial and microglial cells. In addition, some small dot-like
168 reactions were spotted in a scattered pattern amongst the neuropil and white matter. Notably, viral
169 RNA and protein often did not colocalize with inflammatory lesions (**Figure 2f**).

170

171 **Demographic data, clinical disease and histopathology of RusV-infected cats**

172 Among the 29 cats in this study that met the criteria of 'staggering disease', 28 cats were identified
173 as RusV-positive by at least one of the employed methods (**Table 1; Supplemental Table S2**).
174 Twenty-two (78.6%) of them were neutered or intact males (**Supplemental Table S3**), which is
175 consistent with previous studies on 'staggering disease'^{14, 15, 35}. All affected animals were adults,
176 with a median age of 3.1 years (range 1.5 to 12.3; **Supplemental Figure S5a; Supplemental Table**
177 **S3**), and all had outdoor access (where reported) (**Supplemental Table S3**). The onset of disease
178 had occurred more often in winter and spring (December to May: 18 cases) as compared to summer
179 and autumn (June to November: 8 cases; **Supplemental Figure S5b; Supplemental Table S3**).

180 Typically observed clinical signs included gradually deteriorating gait abnormalities, with abnormal
181 posture, stiff gait, ataxia, hind limb-predominant weakness progressing to non-ambulatory
182 tetraparesis and proprioceptive deficits. In addition, fever, behavioural changes such as abnormal
183 vocalization or affectionate behaviour, hypersalivation, depression, hyperaesthesia in dorsal back
184 and lumbar/tail region, reduced spinal reflexes and postural reactions, affection of cranial nerves
185 and opisthotonus were reported in some of the cases. In one animal, generalized seizures were
186 specifically recorded (**Supplemental Table S4**)^{6, 8}. Duration from the reported disease onset to
187 euthanasia ranged from one week to more than one year, with most of the cats being euthanized
188 within less than two months (median two weeks) (**Supplemental Figure S5c; Supplemental Table**
189 **S3**).

190 In congruence with previous reports on 'staggering disease'^{8, 11, 15}, histopathological examination
191 of brain and spinal cord revealed widespread, polio-predominant angiocentric lymphocytic and/or
192 lymphohistiocytic infiltrates throughout the cases (**Figures 3 and 4; Supplemental Table S4**).
193 Occasionally, they were accompanied by oligofocal astrogliosis and microglial proliferates, a few
194 degenerating neurons and neuronophagic nodules (**Figure 4**). Inflammation was most pronounced
195 in the brain stem (**Figures 3a, 4b, c**), cerebral cortices (**Figures 3b, c**), and all levels of the spinal
196 cord, while – independent of the ISH and IHC signal (**Figures 2a, e**) – they were less evident in the
197 cerebellum (**Figure 3a**). Apart from the parenchyma, lymphohistiocytic infiltrates and fewer plasma
198 cells were present also in the leptomeninges (**Figure 3a**). Potentially viral inclusion bodies were not
199 observed.

200

201 **Detection of RusV RNA in rodents from Southern Sweden**

202 We furthermore screened brain samples from 116 rodents that had been collected between 1995
203 and 2019 during monitoring studies near Grimsö in Örebro county (**Supplemental Figure S6**), which
204 is situated approximately 80 km Southwest of the origin of the closest RusV-infected cat detected
205 in this study (**Figure 5**). PanRusV RT-qPCR detected RusV RNA in eight out of 106 (7.5%) wood mice
206 (synonym 'long-tailed field mice'; *Apodemus sylvaticus*) with Cq values ranging from 20 to 35

207 **(Supplemental Figure S4b)**. In contrast, we did not detect RusV RNA in ten yellow-necked field mice
208 from the same location. The positive individuals were collected in the years 1996 (n=2), 1997
209 (n=3), 2005 (n=2), and 2011 (n=1). All positive animals had been trapped during fall season, which
210 is consistent with the considerably higher number of wood mice trapped during fall (n=94) as
211 compared to spring (n=12; **Supplemental Figure S6**).

212 None of the positive animals showed inflammatory lesions in their brain tissues (data not shown).
213 Sample quality allowed for ISH analysis of brain tissue for only four RusV-positive individuals. All of
214 them exhibited specific staining, whereas one RT-qPCR-negative wood mouse did not when tested
215 in parallel (**Supplemental Figure S7**).

216

217 **Phylogenetic analysis and spatial distribution of RusV sequences from cats and wood mice**

218 To allow for a detailed phylogenetic analysis, we aimed at generating RusV sequence information
219 for all positive cats and wood mice. However, whole RusV genome sequencing by HTS is
220 sophisticated and laborious³⁴. Particularly for those individuals with only FFPE material available,
221 the generated sequences were highly fragmented. Thus, we designed primers specifically targeting
222 a stretch of 409 nt within the highly conserved region at the 5' end of the genome to be applied for
223 conventional RT-PCR and subsequent Sanger sequencing (**Supplemental Table S1; Supplemental**
224 **Figure S3**). Using this approach, sufficient sequence information was generated for 23 RusV-
225 positive cats and all eight RusV-positive wood mice. Phylogenetic analysis of these sequences
226 together with all previously published RusV sequences revealed three clearly distinguishable clades
227 for sequences originating from Sweden, Austria, or Northern Germany, with the Swedish and
228 Austrian clades being more closely related to each other than to the Northern German clade (**Figure**
229 **5a**). The Swedish clade revealed three distinguishable subclades. One subclade harboured
230 sequences from ten cats from an area of about 9,000 km² around the city Uppsala. A second
231 subclade included three RusV sequences from cats from the same region and all sequences from
232 wood mice from Grimsö. The third subclade was constituted by only a single sequence from a cat
233 from Stockholm (**Figure 5**). The sequences of both cats from Northeastern Germany belonged to
234 the previously published Northern German clade (**Figures 5a, b**)^{30, 34}. Surprisingly, sequence
235 fragments available for cat GER_01, which originated from Hannover in Central Germany, were
236 more closely related to sequences of the Austrian clade than to the Northern German clade (**Figures**
237 **5a, b**).

238

239 DISCUSSION

240 For almost five decades, ‘staggering disease’ in domestic cats had been suspected as a cohesive
241 entity with a uniform, presumably viral, but still unknown aetiology^{7, 8, 11, 14, 15}. While BoDV-1 had
242 been discussed as a candidate for causing ‘staggering disease’^{16, 19, 21, 22, 23}, proof of natural
243 infections complying with current diagnostic standards could not be presented^{17, 24, 25}. Here we
244 used robust diagnostic approaches that had been demonstrated to successfully detect a broad
245 range of orthobornaviruses, including cases of BoDV-1-induced encephalitis in humans and
246 domestic mammals^{27, 36, 37, 38}. Nevertheless, we were not able to detect bornavirus RNA in any of
247 the 29 tested cats from three different countries with clinicopathological features consistent with
248 ‘staggering disease’. Thus, our results clearly refute the hypothesis of BoDV-1 being the causative
249 agent of ‘staggering disease’.

250 Instead, we were able to unequivocally confirm RusV infection in almost all of these cats. We
251 consistently detected RusV RNA and antigen by employing independent diagnostic assays,
252 including RT-qPCR, genome sequencing, ISH and IHC in the majority of these individuals. Only minor
253 inconsistencies between results of the assays occurred, presumably due to genetic variability of
254 the involved RusV variants, quality of the available material and differential sensitivities of the
255 employed assays that may have led to false negative results of single tests, particularly for
256 individuals for which only archived FFPE material was available. Experimental RusV infection of
257 cats, to reproduce the disease and thereby fulfil Henle-Koch’s postulates, has not been performed
258 so far due to the lack of a virus isolate. However, we demonstrate a clear association between
259 infection and disease, with almost all animals of the ‘staggering disease’ group being RusV-positive,
260 whereas the virus was not detected in any control cat without neurologic disease or with other types
261 of encephalitis. Furthermore, clinical course and histopathologic lesions observed for cats with
262 ‘staggering disease’ in this and previous studies^{7, 8, 14, 15} closely resembled those described for
263 other RusV-infected mammals in Germany^{30, 34}. Thus, RusV can be considered as the causative
264 agent of ‘staggering disease’ with high certainty.

265 We detected RusV infection of cats in the Lake Mälaren region in Sweden and Northeast of Vienna
266 in Austria, two traditional hotspots of ‘staggering disease’^{7, 8, 11, 15, 35}, as well as in Northern
267 Germany, where RusV had been initially discovered^{30, 34}, but ‘staggering disease’ had not yet been
268 reported. Phylogenetic analyses revealed the RusV sequences from the three regions to belong to
269 separate genetic clusters, with the Swedish and Austrian sequences being more closely related to
270 each other than to those from Northern Germany. The considerable genetic variability with only
271 75% minimal nt sequence identities among the different lineages posed a major challenge for the
272 generation of broadly reactive diagnostic tools. However, a particularly conserved sequence stretch
273 at the 5’ terminus of the genome allowed for the design and application of versatile primers and

274 probes for PCR and ISH assays. Furthermore, a monoclonal mouse antibody targeting the RusV
275 capsid protein proved suitable for the detection of all three major RusV lineages.

276 While yellow-necked field mice are considered as putative reservoir hosts of RusV in Northern
277 Germany^{30, 34}, we surprisingly detected RusV in Sweden only in the closely related wood mice but
278 not in yellow-necked field mice from the same area in Örebro county. Since the majority of tested
279 individuals from this location were wood mice, it remains to be elucidated, whether this discrepancy
280 is mainly a result of different species compositions of the analysed sample collections or whether
281 it represents a diverging adaptation of RusV variants to alternative reservoir hosts.

282 The route of RusV transmission within its presumed reservoir as well as from there to other hosts
283 remains unknown. The tissue tropism in zoo animals and yellow-necked field mice in Germany was
284 described as restricted almost exclusively to the CNS, with occasional detection of RusV RNA in
285 peripheral nerve fibres. Viral shedding has not been described so far^{30, 34}. In the future, detailed
286 data on tissue distribution needs to be obtained also for RusV-infected cats and wood mice, but
287 this was beyond the scope of this study. Furthermore, the possibility of RusV shedding by infected
288 cats remains to be elucidated. However, the apparently spatially restricted occurrence of the
289 phylogenetic clusters argues in favour of a continuous viral spread only within a locally bound
290 reservoir, including small mammals, whereas more mobile hosts, including domestic animals that
291 may be transported over long distances, serve predominantly as erroneous dead-end hosts. Similar
292 patterns have been evidenced for rodent and shrew reservoir-bound viruses such as BoDV-1, rat
293 hepatitis E virus or Puumala orthohantavirus^{25, 39, 40, 41}. The sporadic occurrence of ‘staggering
294 disease’ in domestic cat populations, the apparent lack of outbreak series within cat holdings, as
295 well as the almost exclusive restriction to cats with outdoor access, often originating from rural
296 areas, further support this assumption^{8, 14, 15, 35}.

297 Previous studies had also suggested a seasonal occurrence of ‘staggering disease’ with more cases
298 in winter and spring than summer and autumn¹⁵. Although higher case numbers and a more
299 systematic sampling scheme are required for solid statistical evaluation, the same tendency was
300 observed also in our study. This seasonal pattern may be attributable to fluctuating reservoir
301 populations. During the small mammal monitoring in Grimsö, Sweden, numbers of *Apodemus* spp.
302 trapped in fall were much higher than in spring. In addition, movement of small rodents towards
303 and into human dwellings during winter is frequently reported and has been discussed to be
304 associated with transmission of zoonotic pathogens such as Puumala orthohantavirus to
305 humans⁴². Increased exposure to *Apodemus* spp. during fall and winter might also facilitate RusV
306 transmission to cats. However, since the incubation period of RusV-induced disease is unknown,
307 time points of infection cannot be reliably estimated so far. Changes of reservoir populations may
308 also explain long-term temporal patterns of ‘staggering disease’ occurrence. While cases have been
309 continuously observed in the Swedish Lake Mälaren region from at least the 1970s until today^{7, 15}.

310 ^{22, 23, 35}, ‘staggering disease’ in the districts north-east of Vienna has been diagnosed mainly during
311 the early 1990s^{8, 11}, but appears to have ceased thereafter.

312 In summary, we provide convincing evidence of an association of RusV infection with ‘staggering
313 disease’ in cats, strongly supporting a causative role. Our results demonstrate a much broader
314 genetic diversity and spatial distribution of RusV than initially appreciated, and we identified the
315 wood mouse as an additional potential reservoir host. The availability of broadly reactive diagnostic
316 tools may lead to the detection of RusV in encephalitic cats also in regions where ‘staggering
317 disease’ has not been evident before. Furthermore, given the broad range of affected zoo animals^{30,}
318 ³⁴, RusV may be found to be responsible also for additional neurologic disorders in other
319 mammalian species, possibly even including humans. Thus, future research should include the
320 investigation of a possible zoonotic potential of RusV.

321

322 MATERIAL AND METHODS

323 Samples and data collection

324 Fresh-frozen or formalin-fixed paraffin-embedded (FFPE) brain and/or spinal cord samples from 29
325 cats fulfilling the inclusion criteria for this study (lymphohistiocytic meningoencephalomyelitis,
326 meningoencephalitis, encephalomyelitis or encephalitis of unknown cause, and clinical signs
327 suggestive of 'staggering disease') were provided by different laboratories from Sweden, Austria,
328 and Germany (**Table 1; Supplemental Tables S2, S3, and S4**). The samples dated back to 1991 to
329 1993 (Austria) or 2017 to 2022 (Germany and Sweden). Some of these cases were published
330 previously^{6, 8, 11}. In addition, frozen brain samples from 21 cats originating from Sweden, Austria,
331 and Germany without encephalitis were included as controls. An additional control group was
332 composed of eight cats from Germany that had suffered from encephalitis of other types or causes,
333 such as CNS manifestation of feline coronavirus (FCoV)-associated feline infectious peritonitis (FIP),
334 vasculitic disorders and immune-mediated limbic encephalitis (**Table 1**)⁶. Metadata were provided
335 by the submitters and/or extracted from the previous publications, including course and duration
336 of disease, age, sex, origin, and outdoor access of the cats (**Supplemental Table S3**), as well as
337 clinical signs (**Supplemental Table S4**).

338 Furthermore, the study includes archived frozen brain samples from yellow-necked field mice (*A.*
339 *flavicollis*; n=10) and wood mice (*A. sylvaticus*; n=106) that had been collected near Grimsö, Örebro
340 county, Sweden, as part of the Swedish Environmental Monitoring Program of Small Rodents at the
341 Grimsö Wildlife Research Station⁴³. Trapping was approved by the Swedish Environmental
342 Protection Agency (latest permission: NV-412-4009-10) and the Animal Ethics Committee in Umeå
343 (latest permissions: Dnr A 61-11), and all applicable institutional and national guidelines for the
344 use of animals were followed. Species identities were confirmed by cytochrome b gene sequencing
345 as described previously⁴⁴.

346

347 RNA extraction

348 Fresh-frozen samples were mechanically disrupted in 1 ml TRIzol Reagent (Life Technologies,
349 Darmstadt, Germany) by using the TissueLyser II (Qiagen, Hilden, Germany) according to the
350 manufacturers' instructions. After the addition of 200 µl chloroform and a centrifugation step
351 (14,000 × g, 10 min, 4 °C), the aqueous phase was collected and added to 250 µl isopropanol.
352 Total RNA was extracted using the silica bead-based NucleoMagVet kit (Macherey & Nagel, Düren,
353 Germany) with the KingFisher™ Flex Purification System (Thermo Fisher Scientific, Waltham, MA,
354 USA) according to the manufacturers' instructions. *In vitro*-transcribed RNA of the enhanced green
355 fluorescent protein (eGFP) gene was added during the extraction procedure as described by
356 Hoffmann *et al.*⁴⁵.

357 RNA extraction from FFPE tissue was performed with a combination of truXTRAC FFPE total NA Kit
358 (Covaris, Brighton, UK) and the Agencourt RNAdvance Tissue Kit (Beckman Coulter, Krefeld,
359 Germany). FFPE sections were loaded into a microTUBE-130 Screw-Cap (Covaris) together with
360 110 μ l Tissue Lysis Buffer and 10 μ l proteinase K solution (both Covaris). The lysate was processed
361 with a M220 Focused ultrasonicator (Covaris) according to the manufacturer's recommendations
362 for acoustic pellet resuspension. The tube was subsequently incubated at 56 °C in a thermal shaker
363 at 300 rpm overnight (no longer than 18 hours). Subsequently, the sample tube was cooled to room
364 temperature and centrifuged at 5,000 \times g for 15 min using a microTUBE-130 centrifuge adapter.
365 A volume of 100 μ l supernatant was transferred into a clean 1.5 ml reaction tube without
366 transferring any wax or paraffin. After another centrifugation (5 min at 20,000 \times g), 85 μ l of the
367 lower phase with the RNA-containing tissue pellet was transferred into a clean 1.5 ml reaction tube.
368 It was incubated at 80 °C for 20 min and then cooled to room temperature, before 175 μ l B1 buffer
369 (Covaris) were added, mixed, and briefly centrifuged. Thereafter, 250 μ l of 65% isopropanol were
370 added, mixed, and briefly centrifuged. Subsequently, the preparations were further processed with
371 the Agencourt RNAdvance Tissue Kit (Beckman Coulter) with the KingFisher™ Flex Purification
372 System (Thermo Fisher Scientific) according to the manufacturer's instructions.

373

374 **Metagenomic analysis and complete genome sequencing by high throughput sequencing (HTS)**

375 Total RNA was sequenced using a universal metagenomics sequencing workflow³². In brief, total
376 RNA was extracted from fresh-frozen tissue samples using a cryoPREP impactor (Covaris) along
377 with the Agencourt RNAdvance Tissue Kit (Beckman Coulter) and a KingFisher™ Flex Purification
378 System (Thermo Fisher Scientific). Then, 350 ng RNA per sample were reverse-transcribed into
379 cDNA using the SuperScript IV First-Strand cDNA Synthesis System (Thermo Fisher Scientific) and
380 the NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs,
381 Ipswich, MA, USA). Subsequently, cDNA was processed to generate barcoded sequencing libraries
382 as described in detail elsewhere³². The cDNA was fragmented to 200 base pairs (bp) length (for
383 FFPE material) or 500 bp length (for fresh-frozen material) using an M220 Focused ultrasonicator
384 (Covaris). Subsequent library preparation was performed as described previously, with the following
385 modification for FFPE material during size exclusion: small fragments were retained and purified
386 twice with 1.2 \times Ampure XP Beads (Beckman Coulter). Libraries were quantified with the QIAseq
387 Library Quant Assay Kit (Qiagen) and sequenced on an Ion Torrent S5XL instrument using Ion 530
388 chips and chemistry for 400 bp reads, or Ion 540 chips and chemistry for 200 bp reads (Thermo
389 Fisher Scientific) for fresh-frozen or FFPE material, respectively. In addition to the original
390 sequencing libraries, 7 μ l of the libraries were used to apply a capture enrichment with the panRubi
391 v2 myBaits panel as described elsewhere³⁴. For samples with expected major sequence divergence
392 (>20%) from the initially available RusV sequences from Northern Germany that were used for
393 designing the panRubi v2 myBaits panel, a hybridization temperature of 61 °C was used for 24-26

394 hours. In addition, a new panRubi myBaits panel was designed (v3) adding preliminary genome
395 information from samples of Sweden and Austria to the v2 panel. The panRubi v3 myBaits panel
396 consists of 19,982 baits (60-nt oligonucleotides arranged every 20 nt, 3x tiling; GC content of
397 67.3%) and was collapsed at 98% sequence identity. This panel was applied with a hybridization
398 temperature of 64 °C.

399 For selected RusV-positive samples, we additionally applied a depletion protocol in order to
400 decrease the amount of host-derived ribosomal RNA (rRNA) within the total RNA and thereby
401 increase the virus-to-background ratio. In detail, we used a Pan-Mammal riboPOOL reaction kit
402 (siTOOLs Biotech, Planegg, Germany) for 0.2 and 1 µg total RNA following the manufacturer's
403 instructions. The rRNA-depleted RNA was then used for strand-specific library preparation with the
404 Collibri Stranded RNA Library Prep Kit (Thermo Fisher Scientific). The libraries were checked for
405 sufficient quality and quantity using the 4150 TapeStation System (Agilent Technologies, Santa
406 Clara, CA, USA) with the High Sensitivity D1000 ScreenTape and reagents (Agilent Technologies) as
407 well as a Qubit Fluorometer (Thermo Fisher Scientific) along with the dsDNA HS Assay Kit (Thermo
408 Fisher Scientific). Pooled libraries were sequenced using a NovaSeq 6000 (Illumina, San Diego, CA,
409 USA) running in 100 bp mode.

410

411 ***De novo* assembly and sequence annotation of HTS-derived sequences**

412 The raw sequences from Ion Torrent and Illumina systems were processed as described
413 previously³⁴. Briefly, the platform-specific adapters were initially removed from the reads and the
414 sequences were trimmed according to their quality using either 454 Sequencing Systems Software
415 (version 3.0) or Trim Galore (version 0.6.6)⁴⁶ with automated adapter selection, for Ion Torrent and
416 Illumina reads, respectively. Subsequently, the reads were filtered according to their average G+C
417 content using PRINSEQ-lite (version 0.20.4)⁴⁷ with a G+C threshold of ≥60 mol%. The trimmed and
418 filtered reads were used for *de novo* assembly using SPAdes genome assembler (version 3.15.2)⁴⁸
419 running in single cell mode (--sc) and Ion Torrent mode (--iontorrent) as required. Subsequently, all
420 contigs were mapped back to an appropriate RusV reference sequence using Geneious generic
421 mapper with medium sensitivity (Geneious Prime 2021.0.1; Biomatters, Auckland, New Zealand),
422 and a consensus sequence was generated. The final sequence was annotated according to an
423 appropriate RusV reference genome using ORF detection as provided by Geneious Prime 2021.0.1.

424

425 **Bornavirus and RusV RT-qPCRs and design of adapted broad range RusV-specific primers and 426 probes**

427 Two RT-qPCR assays were applied for the detection of either a broad range of orthobornaviruses
428 (panBorna v7.2; **Supplemental Table S1**) or specifically BoDV-1 (BoDV-1 Mix-1; **Supplemental Table**

429 **S1)** following previously published procedures^{27, 38}. Initial screening for RusV-specific RNA was
430 performed using a TaqMan-based RT-qPCR assay (RusV Mix-1; **Supplemental Table S1**) targeting
431 the initially discovered RusV sequences from a zoo in Northern Germany as described by Bennett
432 *et al.*³⁰. The exogenously supplemented eGFP RNA was amplified as RNA extraction control as
433 described previously⁴⁵.

434 To establish a new RT-qPCR assay for the detection of a broader range of RusV sequences, all
435 available sequences from Northern Germany and Sweden were aligned and a set of primers and
436 probe (panRusV-2; **Supplemental Table S1; Supplemental Figure S3**) was designed to target a
437 highly conserved region at the 5' terminus of the genome. RT-qPCR was performed with AgPath-ID
438 One-Step RT-PCR reagents (Thermo Fisher Scientific), panRusV-2 primers (final concentration:
439 0.8 μ M each) and probe (0.4 μ M), eGFP primers (0.2 μ M) and probe (0.15 μ M), and 2.5 μ l extracted
440 RNA in a total volume of 12.5 μ l. The reaction was performed with the following cycler setup: 45 °C
441 for 10 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec.
442 A standard preparation of a RusV-positive donkey brain³⁰ served as positive control and was used
443 for the calibration of Cq values in each RT-qPCR analysis.

444

445 **Determination of partial p150-encoding RusV sequences by Sanger sequencing**

446 Highly conserved primer binding sites in the same alignment as described above were also
447 identified for the amplification of 449 nt at the 5' end of the p150-encoding sequence by
448 conventional RT-PCR (**Supplemental Table S1; Supplemental Figure S3**). RNA extracted from frozen
449 brain samples from all cats and rodents with positive panRusV RT-qPCR results was analysed using
450 the following One-Step RT-PCR conditions: 2.5 μ l RNA were amplified in a total volume of 25 μ l
451 using the SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Thermo
452 Fisher Scientific) and 0.4 μ M each of primers RusV_80+ and RusV_528- (**Supplemental Table S1;**
453 **Supplemental Figure S3**). The cycler setup consisted of 50 °C for 30 min, 94 °C for 2 min, followed
454 by 40 cycles of 94 °C for 30 sec, 63 °C for 30 sec, and 68 °C for 25 sec, and a final elongation step
455 at 68 °C for 5 min. Following separation and visualization by gel electrophoresis, amplification
456 products were purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, Freiburg, Germany)
457 and Sanger sequencing service was provided by Microsynth SeqLab (Balgach, Switzerland).
458 Amplicons were sequenced in both directions and consensus sequences of 409 bp lengths were
459 generated after *de novo* assembly of quality- and primer-trimmed raw sequences in Geneious Prime
460 2021.0.1.

461

462 **Phylogeny and geographic mappings**

463 Phylogenetic analysis of RusV sequences generated in this study was performed together with
464 representative sequences of all currently known matonaviruses^{30, 49}, as well as all publicly available
465 RusV sequences from the INSDC database^{30, 34}. For the phylogeny within the known matonaviruses,
466 the aa sequences of the sPP were aligned using MUSCLE (version 3.8.425)⁵⁰ with a maximum of
467 100 iterations. A maximum likelihood (ML) phylogenetic tree was then calculated using IQ-TREE2
468 (version 2.2.0)⁵¹ running in automatic model selection mode (FLU+F+I+G4) and applying 100,000
469 ultra-fast bootstrap replicates⁵². For phylogenetic analysis of RusV nt sequences, the complete or
470 nearly complete RusV sequences were aligned using MAFFT (version 7.450)⁵³. A ML tree was then
471 calculated as described above (model: TIM3+F+I). The alignment was further used for sequence
472 comparison with a sliding window approach that calculated the pairwise distances (Jukes Cantor
473 1969 model) within a window of 200 nt every 50 nt. A phylogenetic tree of partial p150 protein-
474 coding sequences of 409 nt length was built as described above (model: TN+F+G4).

475

476 **Histopathological examination**

477 Brain and spinal cord samples of cats were harvested on *post mortem* examination via extensive
478 craniectomy-laminectomy. For histology, brain and spinal cord tissues from cats as well as brain
479 tissue from all eight RusV-positive wood mice were fixed in 10% neutral-buffered formalin. Fixed
480 neural tissues were routinely sampled, processed in an automatic tissue processor, embedded in
481 paraffin wax, sectioned at 2–4 µm, and stained with histological standard stains including
482 haematoxylin-eosin (H.E.), Nissl and Luxol-Fast-blue stains.

483 Slices were microscopically examined for the presence of non-suppurative, lymphohistiocytic
484 encephalitis, meningoencephalitis and/or meningoencephalomyelitis. Inflammation was graded
485 mild to severe based on the extent of inflammatory cell infiltrates. Mild encephalitis comprised few
486 perivascular infiltrates, most of which showed one to two layers of cells and were not necessarily
487 present in all investigated locations. One or two larger infiltrates in a single location were allowed
488 to occur in this category. Moderate encephalitis comprised several infiltrates per location, showing
489 three to five layers of cells, allowing single locations with larger or smaller infiltrates. Severe
490 encephalitis comprised many perivascular infiltrates, most of which showed several layers of cells
491 (>5) in the majority of investigated locations.

492

493 **Detection of RusV-specific RNA by *in-situ* hybridization (ISH)**

494 A custom-designed RNAscope probe was provided by Advanced Cell Diagnostics (Newark, NJ, USA)
495 based on the consensus sequence of the available RusV sequences from Sweden, targeting the
496 highly conserved region at the 5' end of the RusV genome. A probe targeting the messenger RNA

497 (mRNA) of the ubiquitous, widely expressed housekeeping gene peptidyl-prolyl-isomerase-B (*Felis*
498 *catus*-PPIB; cat. no. 455011) was used as positive control, while a probe targeting bacterial
499 dihydropicolinate reductase (DapB; cat. no. 310043) was used as a negative control probe. Viral
500 nucleic acid was determined using ISH with the RNAscope® 2.5 High Definition RED assay
501 (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's instructions.
502 Briefly, brain slides were deparaffinized and pre-treated with 1× Target Retrieval solution and
503 RNAscope® Protease Plus solution prior to hybridization with the target probe. Subsequently, the
504 tissue was treated with a series of pre-amplifiers and amplifiers followed by the application of a
505 chromogenic substrate. The samples were counterstained with Hematoxylin Gill No. 2 (Merck,
506 Darmstadt, Germany).

507 Brain sections of a RusV-positive capybara³⁰ served as positive control and showed positive
508 reactivity with the specific RusV RNAscope probe. A brain sample from a RusV-negative control cat
509 incubated with the RusV RNAscope probe and a brain sample from a RusV-positive cat incubated
510 with an irrelevant RNAscope probe (*Mycoplasma hyopneumoniae*) served as negative controls and
511 yielded no reactivity (data not shown). The scoring of the signals was performed as described in
512 **Supplemental Table S5**.

513

514 **Detection of RusV and BoDV-1 antigen by immunohistochemistry (IHC) staining**

515 Brain slides were evaluated for expression of RusV capsid protein using a mouse monoclonal
516 primary antibody (2H11B1; see below). The slides were deparaffinised and underwent antigen
517 retrieval in the microwave (750 W, 20 min) being immersed in 10 mM citrate buffer (pH 6.0) before
518 incubation with the primary antibody (dilution 1:100) at 4 °C for 18 hours. Successful labelling was
519 demonstrated using ImmPRESS® polymer anti-mouse IgG (LINARIS Biologische Produkte,
520 Dossenheim, Germany) coupled to peroxidase and a diaminobenzidine tetrahydrochloride staining
521 kit (ImmPACT DAB substrate HRP; BIOZOL Diagnostica, Eching, Germany) according to the
522 manufacturers' instructions. After peroxidase reaction, sections were counterstained with
523 hematoxylin. Sections of a RusV-positive capybara brain³⁰ served as virus-positive tissue control,
524 whereas brain sections of cats from the control groups that had been tested negative for RusV by
525 RT-qPCR served as negative tissue control (**Supplemental Figure S8**). Specificity of the anti-mouse
526 IgG polymere was evaluated by two sections each of capybara brain and of PCR-confirmed RusV
527 positive cat SWE_07, in which 2H11B1 antibody was replaced by horse serum and by anti-FCoV
528 mouse monoclonal (FIPV 3-70, LINARIS Biologische Produkte, Dossenheim, Germany;
529 **Supplemental Figure S8**). The scoring of the signals was performed as described in **Supplemental**
530 **Table S5**.

531 Cat brain slides were furthermore assessed for the expression of BoDV-1 nucleoprotein using
532 murine monoclonal antibody Bo18⁵⁴ with the ABC detection kit (biotinylated goat anti-mouse IgG;

533 BIOZOL Diagnostica) and diaminobenzidine tetrahydrochloride (ImmPACT DAB substrate HRP;
534 BIOZOL Diagnostica). Brain slides of a horse confirmed as BoDV-1-infected by RT-qPCR served as
535 positive control. Replacement of Bo18 antibody by an irrelevant mouse monoclonal antibody (FIPV
536 3-70) was used as negative reagent control on BoDV-1-positive horse tissue and RusV-positive
537 feline brain SWE_07.

538

539 **Recombinant protein production and generation of a monoclonal anti-RusV capsid protein antibody**

540 A synthetic DNA string fragment encoding aa 128 to 308 of the RusV capsid protein, based on the
541 sequence from an infected donkey from Northern Germany (accession number MN552442.2), was
542 ordered from GeneArt synthesis (Thermo Fisher Scientific) and inserted into the pEXPR103
543 expression vector (IBA Lifesciences, Göttingen, Germany) in-frame with a Strep-tag-coding
544 sequence at the 3' end. The protein with a C-terminal Strep-tag was expressed in Expi293 cells
545 (Thermo Fisher Scientific) and subsequently purified using Strep-Tactin XT Superflow high capacity
546 resin (IBA Lifesciences) following the manufacturer's instructions.

547 For monoclonal antibody generation, two female BALB/c mice were immunized intraperitoneally
548 with 20 µg of purified capsid protein in compliance with the national and European legislation, with
549 approval by the competent authority of the Federal State of Mecklenburg-Western Pomerania,
550 Germany (reference number: 7221.3-2-042/17). The immunization, as well as the generation of
551 hybridoma cells were performed as described previously⁵⁵.

552

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561 immunohistochemistry.

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566 the Swedish Environmental Protection Agency through the Swedish Wildlife Management Fund
567 (grant no. 2020-00093).

568

569 **DATA AVAILABILITY**

570 The novel RusV sequences have been made publicly available in the INSDC database under the
571 accession numbers ON641041 to ON641071.

572

573 **AUTHOR CONTRIBUTIONS**

574 K.M., M.B., C.L., and D.R. initiated the study. K.M., H.W., S.T., and C.L. evaluated the histopathology.
575 F.P., C.W., and B.L. performed metagenomics sequencing. J.K., P.S., P.We. and D.R. established
576 and performed PCR assays and Sanger sequencing. F.P. and D.R. performed phylogenetic analyses.
577 H.W., C.W.L., and J.M. established and performed RNAscope *in-situ* hybridization. K.M. and M.R.
578 established and performed immunohistochemistry. A.A. and S.R. generated the RusV-specific
579 monoclonal antibody. K.M., H.W., S.T., F.E., S.N., J.N., P.Wo., L.M., C.B., K.M.O., C.R., T.F., B.H., and
580 C.L. provided samples from cats or rodents together with clinical and pathological diagnosis and
581 metadata. K.M., F.P., H.W., C.W., and C.L. wrote parts of the manuscript. D.H., R.G.U, N.N., and M.B.
582 supervised parts of the study. D.R. conceptualized the study and wrote and finalized the
583 manuscript. The manuscript was critically reviewed through the contributions of all authors.

584

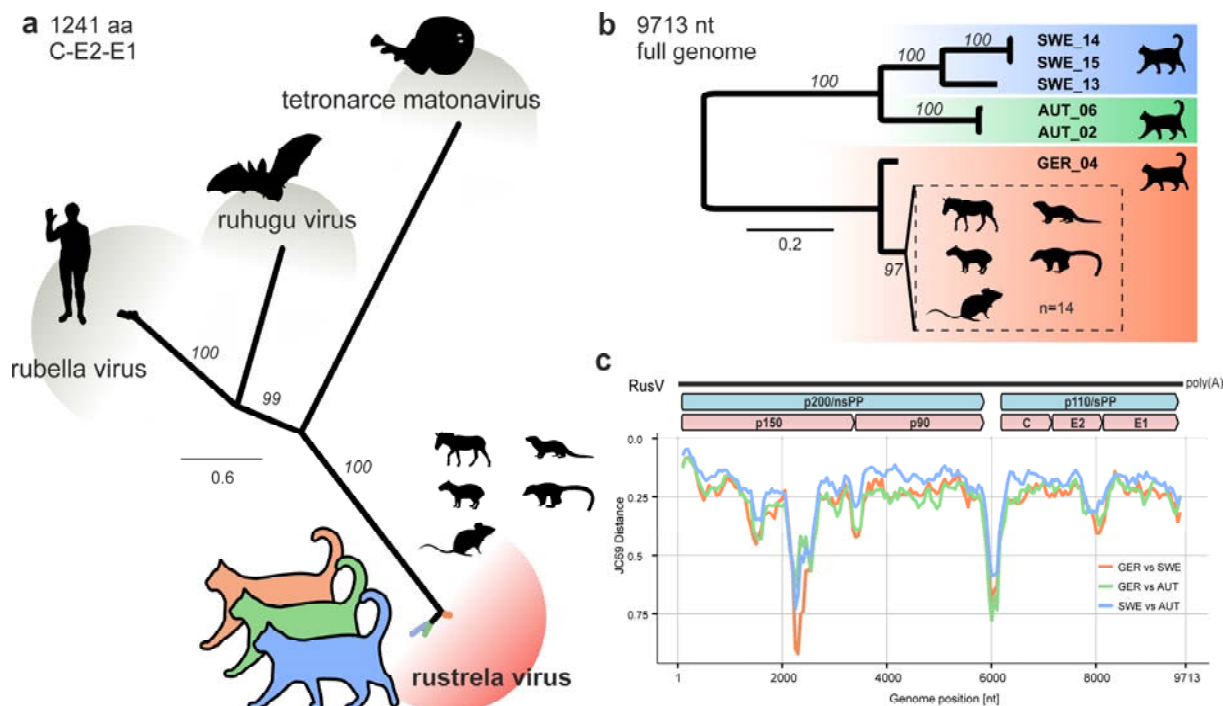
585 **COMPETING INTERESTS STATEMENT**

586 The authors declare to have no competing interests.

587

588 FIGURES

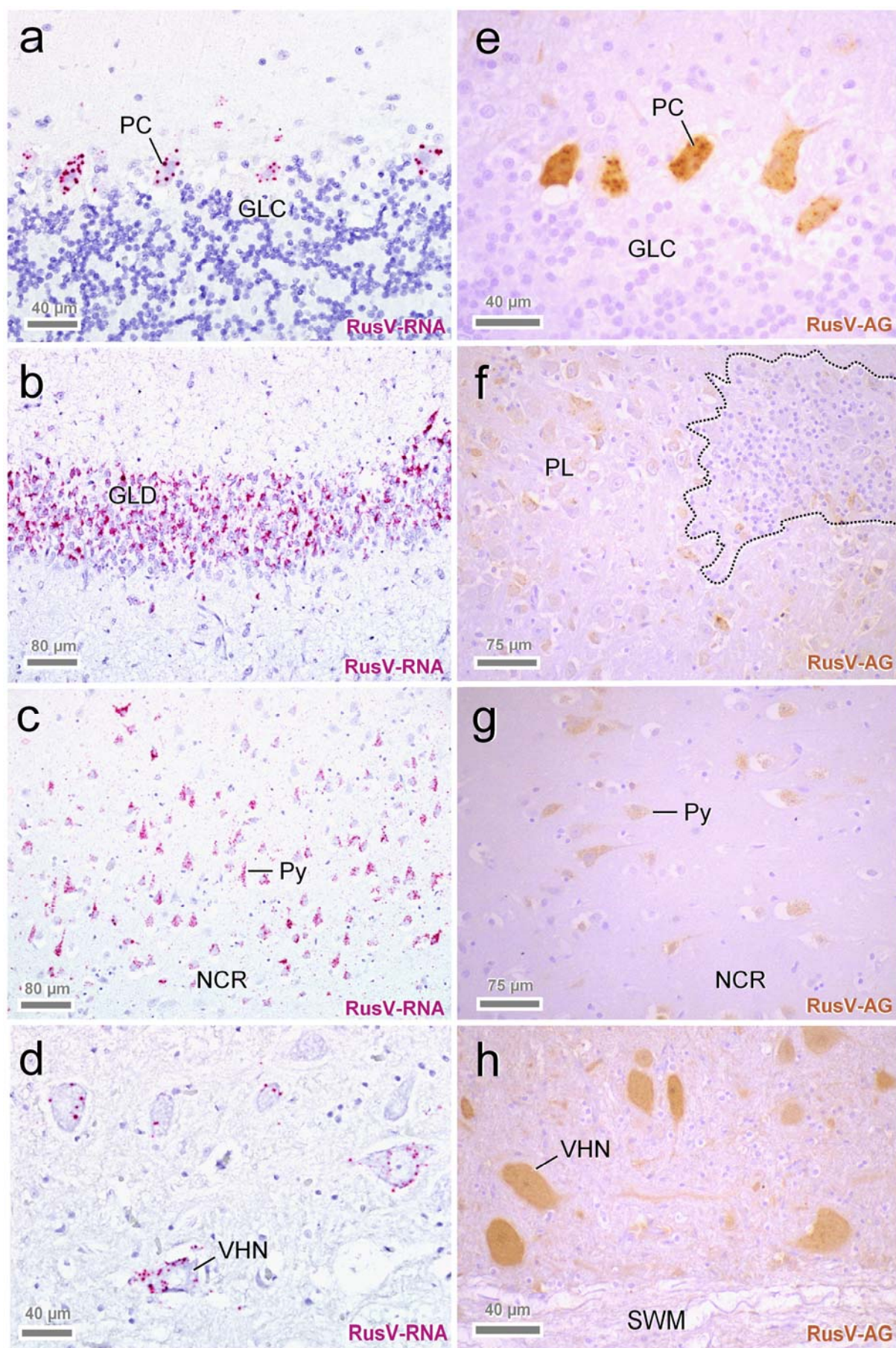
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591 **Figure 1. Sequence comparison of complete rustrela virus (RusV) genome sequences from cats**
 592 **from Sweden, Austria, and Germany. (a)** The amino acid sequences of the structural polyprotein
 593 (p110/sPP) of all known matonaviruses were aligned and a maximum-likelihood (ML) phylogenetic
 594 tree was calculated (IQ-TREE2 version 2.2.0; FLU+F+I+G4; 100,000 ultrafast bootstraps).
 595 Bootstrap support values are shown in italics. **(b)** ML tree of complete or nearly complete RusV
 596 genome sequences from cats with ‘staggering disease’ and all publicly available RusV sequences
 597 (IQ-TREE2 version 2.2.0; TIM3+F+I; 100,000 ultrafast bootstraps). Sequences from Sweden,
 598 Austria, and Germany are highlighted in blue, green, and orange, respectively. Sequences from a
 599 previously identified German RusV cluster from zoo animals with encephalitis and apparently
 600 healthy yellow-necked field mice (*Apodemus flavicollis*)^{30, 31, 34} are presented in a dashed box.
 601 Bootstrap support values are shown at the nodes. **(c)** The genetic variability of RusV lineages from
 602 Sweden, Austria, and Germany is presented as mean pairwise JC69 distance using a sliding window
 603 analysis (window: 200 nt; step size: 50 nt). The genomic organization of RusV is shown, highlighting
 604 the non-structural (p200/nsPP) and structural (p110/sPP) polyprotein open reading frames, as well
 605 as the mature cleavage products protease (p150), RNA-directed RNA polymerase (p90), capsid
 606 protein (C), and glycoproteins E2 and E1.

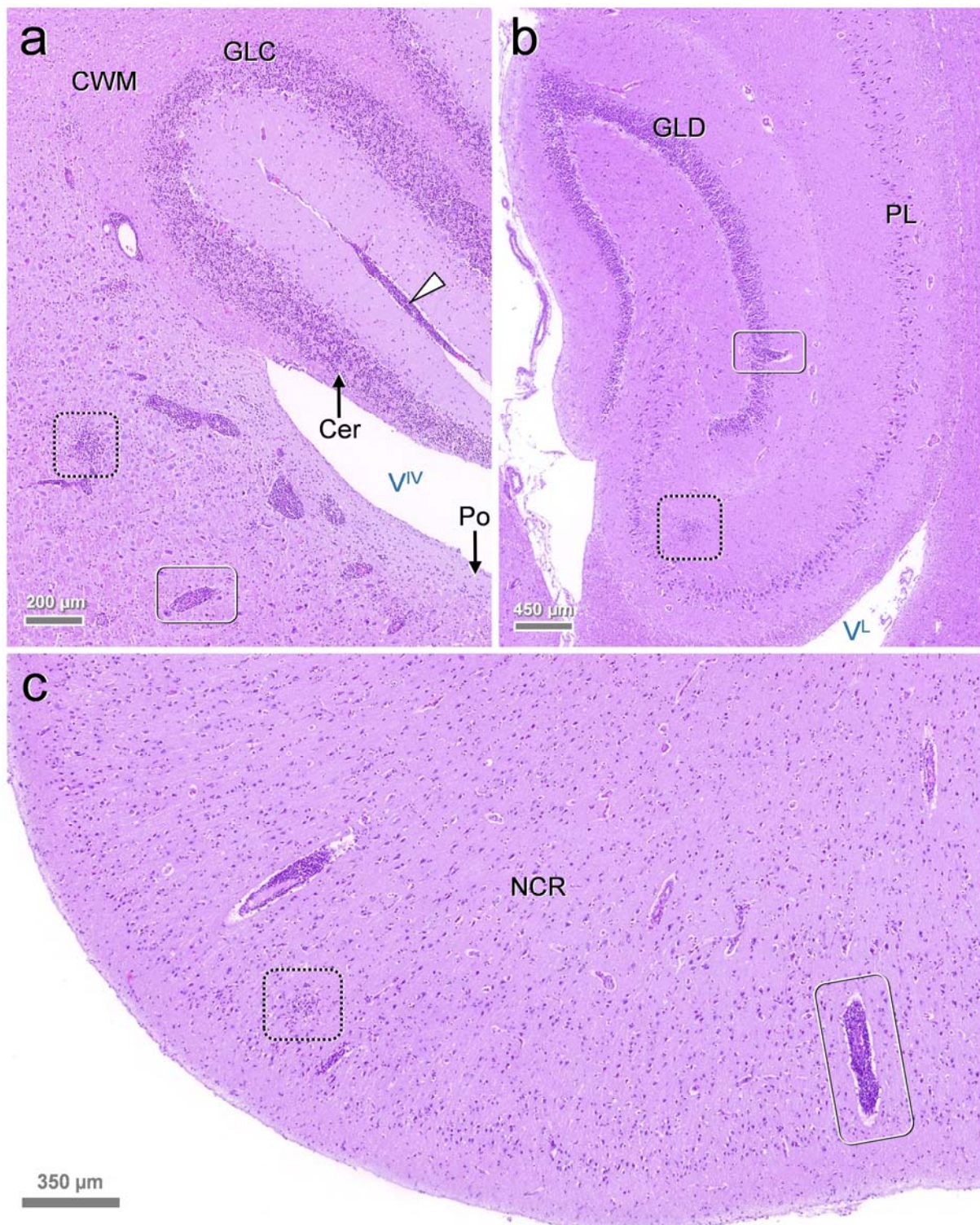
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609 **Figure 2. Detection of rustrela virus (RusV) RNA by RNAscope *in-situ* hybridization (a-d) and RusV**
610 **antigen by immunohistochemistry (e-h) in the central nervous system of encephalitic cats.** Both
611 virus RNA and capsid protein were located mainly in the cytoplasm of different nerve cell
612 populations. Typical are spherical reaction products, which may coalesce to more extensive and/or
613 diffuse staining. Neurons with the highest viral load were particularly Purkinje cells (**a, e: PC**),
614 granule cells of dentate gyrus (**b: GLD**), pyramidal cells within hippocampal pyramidal cell layer (**f:**
615 **PL**), and neocortex (**c, g: Py**). Also, numerous RusV positive cells are seen in lower brainstem and
616 spinal ventral horn neurons (**d, h: VHN**). Note that expression of virus RNA and antigen is far more
617 widespread than inflammatory changes (**f: dashed line**) and mostly affects neurons without
618 cytopathic effects.

619 GLC: granule cell layer of cerebellar cortex; GLD: granule cell layer of dentate gyrus; NCR:
620 neocortical ribbon; PC: Purkinje cell; PL: pyramidal cell layer; Py: pyramidal cell; SWM: spinal white
621 matter; VHN: ventral horn neuron. Sources: (**a**) cat SWE_03; (**b**) SWE_11; (**c**) SWE_05; (**d, f**) AUT_09;
622 (**e**) AUT_04; (**g**) SWE_08; (**h**) AUT_08.

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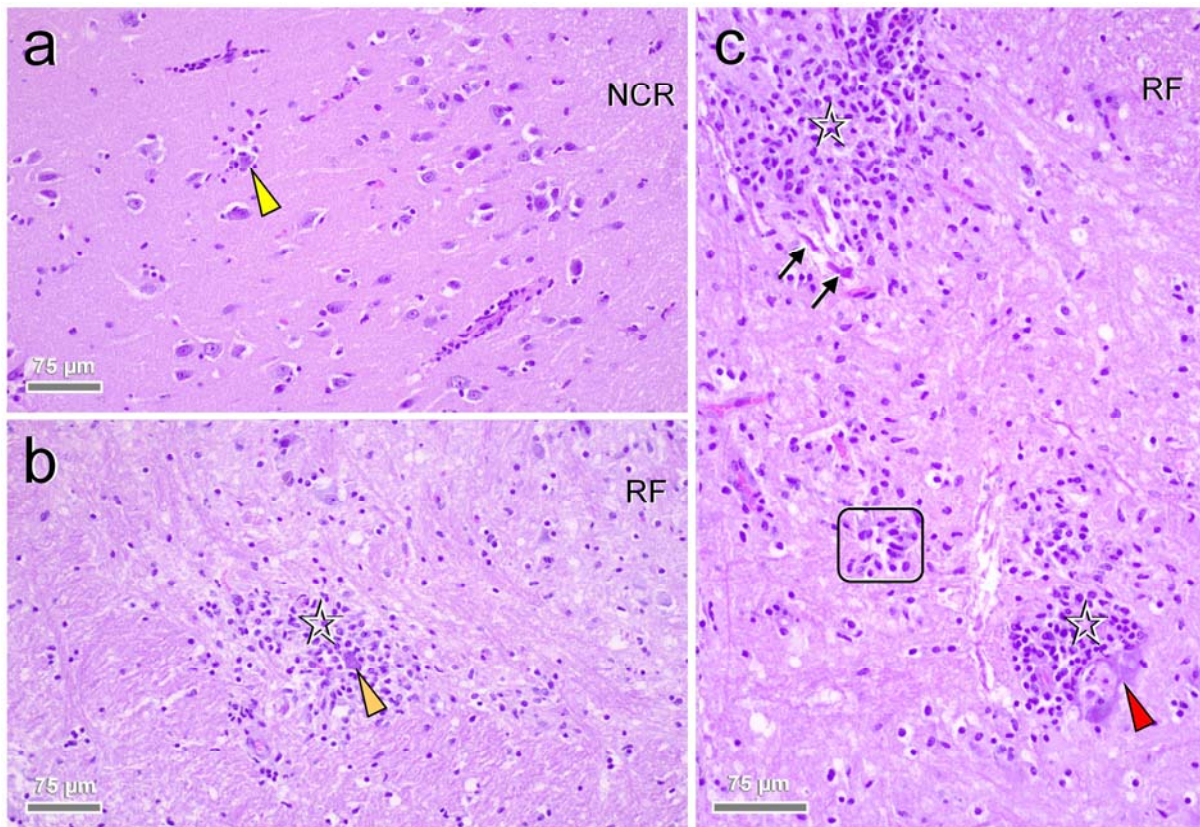
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625 **Figure 3. Encephalitic pattern in rustrela virus (RusV)-infected cats.** Histology typically features
626 polio-predominant, perivascular lymphohistiocytic cuffs (**a-c**: solid boxes) and angiocentric
627 infiltrates (**a-c**: dashed boxes). They are most prominent in brainstem (**a**: Po), hippocampus
628 formation (**b**) and neocortex (**c**). Leptomeningeal infiltrates (**a**: white arrowhead) also occur in areas
629 with sparse parenchymal invasion such as the cerebellum (**a**: Cer).

630 Stain: hematoxylin eosin (H.E.). Anatomical landmarks: Cer: cerebellum; CWM: cerebellar white
631 matter; GLC: granule cell layer of cerebellar cortex; GLD: granule cell layer of dentate gyrus; NCR:

632 neocortical ribbon; PL: pyramidal cell layer; Po: pons; V^{IV}: fourth ventricle; V^L: lateral ventricle.
633 Sources: (a) cat SWE_04; (b, c) SWE_07.

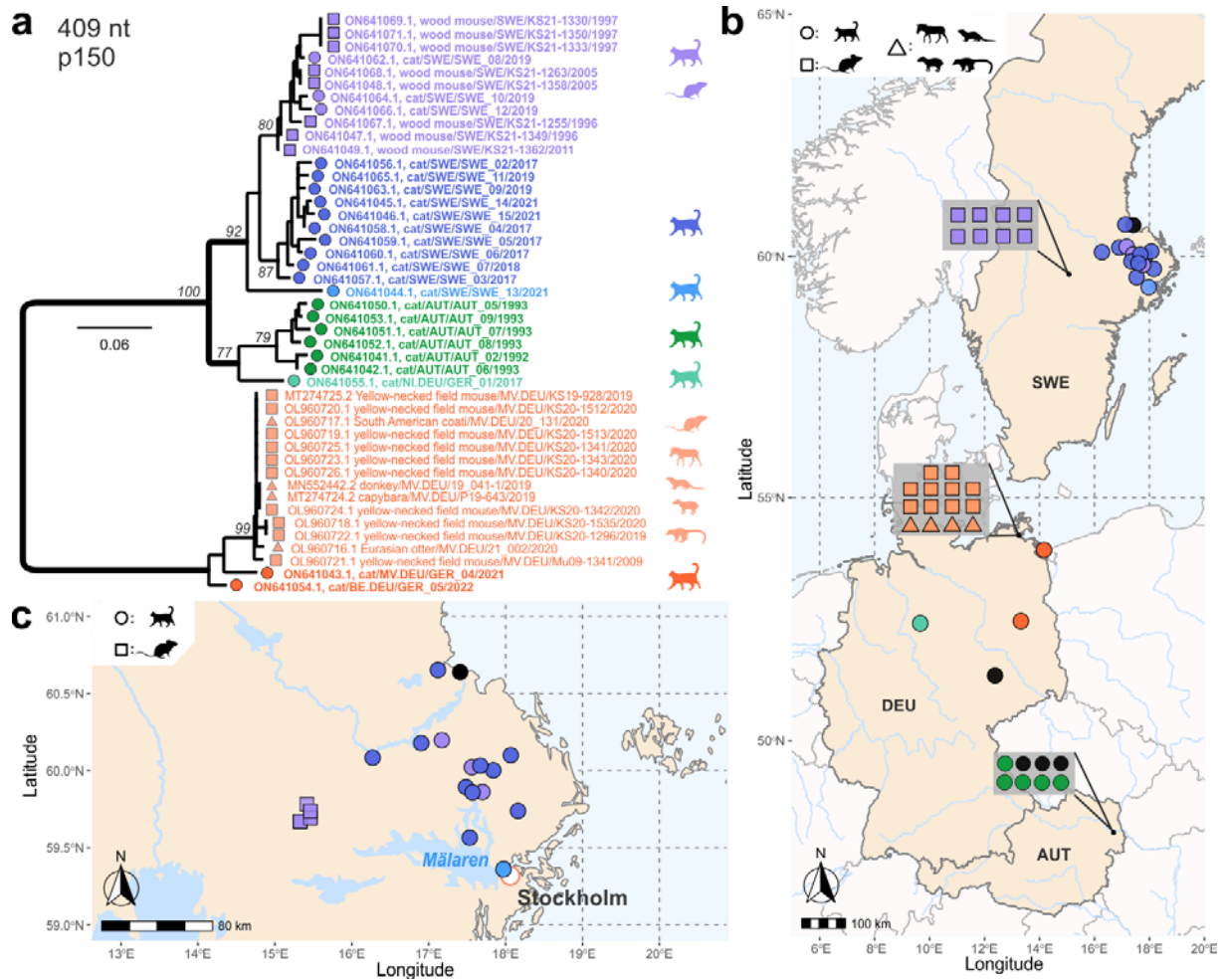
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640 **Figure 4. Close-up pathology and cellular damage of rustrela virus (RusV) infection within the brain.**
641 Infected brains show neurons (a-c: arrowheads) with (c: red arrowhead) and without (a, b: yellow
642 and orange arrowheads) degenerative features, early (a, b: yellow and orange arrowheads) and
643 advanced (c: red arrowhead) neuronophagia suggestive of a neuronotropic pathogen. Focal
644 dropout of neurons goes with formation of microglial stars (c: frame). Inflammatory infiltrates (b, c:
645 asterisks) mingle with focal glial proliferates. Dystrophic axons (c: black arrows) are occasionally
646 present within the perilesional area.

647 Stain: hematoxylin eosin (H.E.). Anatomical landmarks: NCR: neocortical ribbon; RF: reticular
648 formation. Sources: (a) cat SWE_06; (b, c) SWE_04.

649



650

651 **Figure 5. Phylogenetic analysis and spatial distribution of rustrela virus (RusV) infections in Europe.**

652 (a) Maximum likelihood (ML) phylogenetic tree of partial RusV sequences (409 nucleotides,

653 representing genome positions 100 to 508 of donkey-derived RusV reference genome

654 MN52442.2; IQ-TREE version 2.2.0; TN+F+G4; 100,000 ultrafast bootstraps). Only bootstrap

655 values ≥ 70 at major branches are shown in the phylogenetic tree. RusV sequence names are shown

656 in the format “host/ISO 1366 code of location (federal state.country)/animal ID/year”. (b, c)

657 Mapping of the geographic origin of RusV-positive animals in Europe (b) and in the Lake Mälaren

658 region in Sweden (c). Colours represent the phylogenetic clades of the sequences (a). RusV-positive

659 cats that failed to deliver sequences are depicted in black. The respective host animals are shown

660 as circles (cats), squares (*Apodemus* spp.), and triangles (zoo animals). Symbols in grey boxes

661 represent individuals from the same or very close locations.

662 AUT = Austria, DEU/GER = Germany, SWE = Sweden; BE = Berlin, MV = Mecklenburg-Western

663 Pomerania, NI = Lower Saxony.

664

665 **TABLES**

666

667 **Table 1. Rustrela virus (RusV) detection in brain samples from cats with or without signs of**
 668 **'staggering disease'.**

Group Country	Years	n	RusV detection (positive / total animals)				
			HTS	RT-qPCR	ISH	IHC	total ^a
Cats matching the criteria of 'staggering disease'							
Sweden	2017-2021	15	9/9	15/15	14/14	15/15	15/15
Austria	1991-1993	9	3/4	8/9	7/9	8/9	9/9
Germany	2017-2022	5	2/2	3/5	1/3	4/5	4/5
Cats with other types of encephalitis							
Germany	2017-2020	8	0/3	0/8	n.d.	0/8	0/8
Cats without encephalitis							
Sweden	2021-2022	7	n.d.	0/7	n.d.	0/1	0/7
Austria	2021	5	n.d.	0/5	n.d.	n.d.	0/5
Germany	2018-2020	9	n.d.	0/9	n.d.	0/9	0/9

669 HTS: high throughput sequencing followed by metagenomic analysis; ISH: *in-situ* hybridization using
 670 RNAscope; IHC: immunohistochemistry; n.d.: not determined

671 a Cats were considered RusV-positive if RusV RNA or antigen was detected by at least one of the
 672 applied methods.

673

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