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Genetic Tracking of a Rabid Coyote (*Canis latrans*) Detected beyond a Rabies Enzootic Area in West Virginia, USA

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Genetic Tracking of a Rabid Coyote (*Canis latrans*) Detected beyond a Rabies Enzootic Area in West Virginia, USA

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ABSTRACT: Wildlife translocation and cross-species transmission can impede control and elimination of emerging zoonotic diseases. Tracking the geographic origin of both host and virus (i.e., translocation versus local infection) may help determine the most effective response when high-risk cases of emerging pathogens are identified in wildlife. In May 2022, a coyote (*Canis latrans*) infected with the raccoon (*Procyon lotor*) rabies virus variant (RRV) was collected in Lewis County, West Virginia, USA, an area free from RRV. We applied host population genomics and RRV phylogenetic analyses to determine the most likely geographic origin of the rabid coyote. Coyote genomic analyses included animals from multiple eastern states bordering West Virginia, with the probable origin of the rabid coyote being the county of collection. The RRV phylogenetic analyses included cases detected from West Virginia and neighboring states, with most similar RRV sequences collected in a county 80 km to the northeast, within the oral rabies vaccination zone. The combined results suggest that the coyote was infected in an RRV management area and carried the RRV to Lewis County, a pattern consistent with coyote local movement ecology. Distant cross-species transmission and subsequent host movement presents a low risk for onward transmission in raccoon populations. This information helped with emergency response decision-making, thereby saving time and resources.

Key words: Coyote, cross-species transmission, phylogenetics, raccoon rabies virus variant, single-nucleotide polymorphisms, translocation.

The emergence and reemergence of zoonotic pathogens can include spread to new reservoir hosts or geographic areas, which may lead to outbreaks in areas previously free of disease, with potential negative health consequences for humans, domestic animals, and wildlife. Detection of an emerging pathogen in a new geographic location may indicate that local transmission is already established, making it more challenging to contain. When the detection is in a nonreservoir species, there are two possibilities for management to consider: 1) the case has been translocated from an endemic area and was an isolated cross-species transmission (i.e., spillover event) with a low probability of further transmission; and 2) the case is evidence of a local transmission cycle. These two scenarios lead to different management responses. Rapid host and virus genomic characterizations may differentiate between these scenarios and inform management and public health response, thereby saving time, money, and lives.

Determination of the probable geographic origin and location of host infection may help to estimate the risk of onward transmission when an infected animal is found in an area previously known to be free of a specific pathogen. Specifically, this knowledge can inform decisions about the scale of early intervention and management response (Bird and Mazet 2018; Martel et al. 2020). Genomic tools are highly effective at tracking both pathogen and wildlife host movement across landscapes (Barton et al. 2010; Biek and Real 2010; Szanto et al. 2011; Brunker et al. 2020; Gigante et al. 2020). With sufficient sampling, genomic data can be used to estimate the geographic origin

of both the host and the infecting virus, with the potential to distinguish a translocated rabid animal versus local host movement and pathogen spread.

The raccoon rabies virus variant (RRV; family Rhabdoviridae, genus *Lyssavirus*) was first reported in raccoons (*Procyon lotor*) in Florida, US, during the late 1940s; by the late 1970s, the virus had spread to nearby states of the southeastern US (McLean 1971). An unintentional human-mediated translocation of rabid raccoons to the border shared between Virginia and West Virginia, US, resulted in one of the largest epizootics of wildlife rabies in the US (Rupprecht and Smith 1994; Rupprecht et al. 1995; Szanto et al. 2011). The RRV is now the most commonly detected rabies virus in raccoons and other wild carnivores of the eastern US and is one of the predominant exposure risks for domestic animals and humans (Ma et al. 2023). The US Department of Agriculture, Wildlife Services, National Rabies Management Program (NRMP) provides federal leadership and multiagency coordination to prevent the spread of and eventually eliminate RRV through oral rabies vaccination (ORV) combined with enhanced rabies surveillance (ERS; active, targeted surveillance that complements passive public health surveillance; Slate et al. 2009; Elmore et al. 2017).

Long-distance host movements, whether naturally dispersing or human-mediated translocation events, and virus spillover into new reservoir species may threaten rabies control efforts and result in negative health and economic consequences (Rosatte and MacInnes 1989; Russell et al. 2005; Chipman et al. 2008; Slate et al. 2009; Singh et al. 2018; Grome et al. 2022). For example, repeated translocations of RRV into southeastern Canada have diverted resources from RRV elimination programs to containment of the new epizootics; this hinders management program elimination goals (Trewby et al. 2017; Lobo et al. 2018; Nadin-Davis et al. 2020).

In March 2022, a rabid juvenile male coyote (*Canis latrans*) was collected in Lewis County, West Virginia, a region free of RRV (Fig. 1). The coyote, collected through public health surveillance, was diagnosed with RV infection by using the direct fluorescent antibody test (Genevie et al. 2003). The RV was determined to be RRV by antigenic typing at the Centers for Disease Control and Prevention, Atlanta, Georgia, US; this was later confirmed by sequencing (see Supplementary Material). When RRV is found outside the enzootic area, a contingency action is initiated by the NRMP, including ERS and more intensive ORV management. This approach is costly, so an accurate estimation of risk of onward RRV spread can help tailor contingency action response planning for effective control of RRV, while minimizing the resources necessary to reestablish control. We combined host and virus population genetic and phylogenetic methods to assess probable origins of the West Virginia coyote and the infecting RRV to help with the emergency response.

Population genomic analyses were used to determine the origin of the rabid West Virginia coyote. Ear tissue samples from the rabid coyote (ID E22R007878-01) and 54 additional coyotes were combined with coyote genotypes from Heppenheimer et al. (2018). We obtained genotype data from 107,888 single-nucleotide polymorphisms (SNPs) determined to be statistically neutral and unlinked across the genomes of 318 coyotes. We then conducted two unsupervised cluster analyses at different sampling resolutions to provide multiple geographic perspectives, and a supervised population assignment to estimate the probable ancestry of the rabid coyote (for details of the analyses, see Supplementary Material).

Initial clustering revealed that E22R007878-01 had high assignment probability (>88%) to the genetic cluster containing coyotes from Kentucky and West Virginia (Fig. 2; Table 1 and Supplementary Material Table S1). Given that population structure is often hierarchical, we repeated the maximum likelihood cluster analysis at a finer geographic scale to determine whether we could locate a more precise point of origin by using only coyotes from Kentucky and West Virginia and sample E22R007878-01 for K=2–7 genetic partitions. The most likely number of partitions was K=2 and K=3 and the rabid coyote assigned to West Virginia (Supplementary Material Fig. S1A).

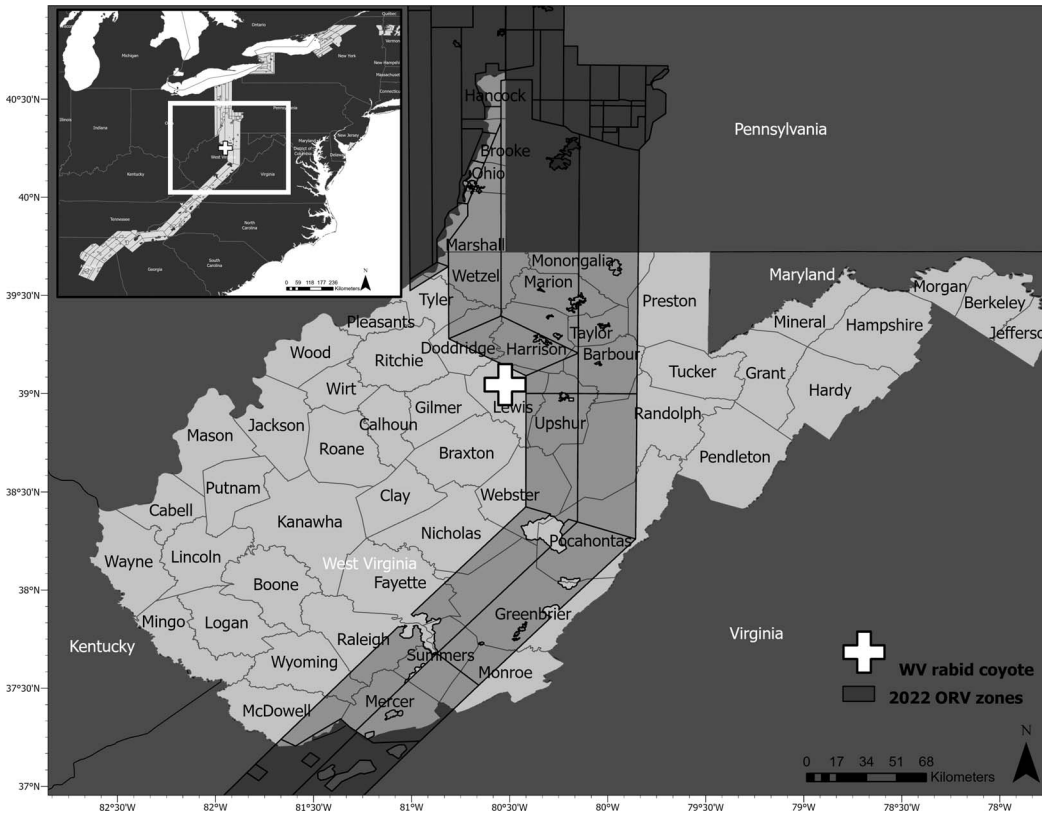

FIGURE 1. Map of West Virginia in the eastern US. The light gray areas are the oral rabies vaccination zones, and the plus sign is the collection location of the rabid coyote (*Canis latrans*; sample E22R007878-01). The inset is to provide perspective on the animal's distance from the oral rabies vaccination zone. East of the vaccination zone is the raccoon rabies virus variant enzootic area.

Although we identified 18,145 alleles private to Kentucky coyotes and 20,019 to West Virginia coyotes, sample E22R007878-01 did not carry any of these private alleles. We then analyzed sample E22R007878-01 with coyotes sampled from West Virginia, considering the hypothesis that the sample would have relatively comparable assignment proportions across all sampled

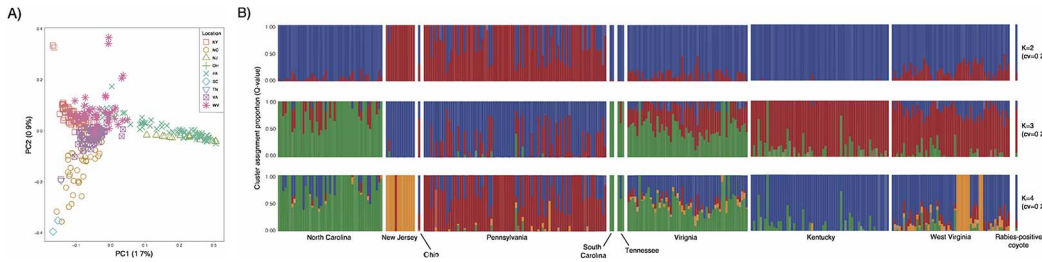

FIGURE 2. Population cluster analysis of 107,888 single-nucleotide polymorphism (SNP) genotypes from eastern US coyotes (*Canis latrans*) by using (A) a principal component analysis (PCA) and (B) the unsupervised maximum likelihood clustering algorithm ADMIXTURE (Alexander et al. 2009) for 2–4 genetic partitions (*K*). The cross-validation (cv) value per *K* is provided. The farthest right sample (single bar) is the rabies-positive coyote (sample E22R007878-01). As *K* increases, West Virginia and Kentucky, USA, form a distinct cluster and the rabid Lewis County, West Virginia, coyote groups with these states.

TABLE 1. ADMIXTURE (Alexander et al. 2009) supervised assignment proportions based on single-nucleotide polymorphisms from a rabid coyote (*Canis latrans*; sample E22R007878-01) collected in West Virginia, USA, to genetic clusters (K=2–4) and associated US states in parentheses.

K and US states included in each training cluster	Assignment proportion E22R007878-01
K=2	
Cluster 1 (Kentucky, North Carolina, South Carolina, Tennessee, Virginia, West Virginia)	0.897
Cluster 2 (New Jersey, Ohio, Pennsylvania)	0.103
K=3	
Cluster 1 (Kentucky, West Virginia)	0.900
Cluster 2 (North Carolina, South Carolina, Tennessee, Virginia)	0.000
Cluster 3 (New Jersey, Ohio, Pennsylvania)	0.100
K=4	
Cluster 1 (Kentucky, West Virginia)	0.885
Cluster 2 (North Carolina, South Carolina, Tennessee, Virigina)	0.000
Cluster 3 (New Jersey)	0.000
Cluster 4 (Ohio, Pennsylvania)	0.115

localities if it did not originate from West Virginia. We found that coyotes sampled in Lewis County clustered with the target sample E22R007878-01 at every genetic partition between K=3 and 10 (Table 2), suggesting that the target sample likely originated in Lewis County.

To identify which sampled coyotes were most closely related to the rabid coyote, we filtered the SNP dataset more stringently to infer interindividual relatedness, producing 1,630 SNPs for 116 coyotes from Kentucky and West Virginia, which included sample E22R007878-01. We obtained 6,670 pairwise relatedness estimates with a mean r=0.01 (SD±0.035). Only eight pairs had r>0.5, with none spanning the boundaries of West Virginia and Kentucky. A focused analysis of sample E22R007878-01 with 53 comparisons with other West Virginia coyotes and 61 comparisons with Kentucky coyotes revealed that mean relatedness to both states was comparable (West Virginia: mean=0.009±0.01, range=0–0.62; Kentucky: mean=0.008±0.01, range=0–0.70; Welch two-sample t-test: t=0.03, df=111.42, P=0.978). Six relatedness values fell within the top 95th percentile of the pairwise relatedness distribution: two were with Kentucky coyotes (r=0.070 in Metcalfe County, 395 miles [636 km] from Lewis County; r=0.042 in Pike County, 214 miles [344 km] away), and four were with West Virginia coyotes (r=0.062 in Wirt County, 64 miles [103 km] away; r=0.049 in Marshall County, 124 miles [200 km] away; r=0.045 in Wetzel County, 69 miles [111 km] away; and r=0.041 in Wirt County, 64 miles [103 km] away). These relatedness levels are probably indicative of proximal relationships (i.e., more than two generations apart).

To determine the possible origin of the RRV, we performed a comparative analysis of RRV sequences from the rabid coyote brain to RRV sequences from West Virginia and neighboring US states: West Virginia [65], Pennsylvania [20], Virginia [14], and Ohio [1] (see Supplementary Material Table S2). Rabies virus from E22R007878-01 had the highest percent nucleotide identity to three RV sequences from Monongalia County, West Virginia, with identical glycoprotein gene sequences and two synonymous nucleotide changes in the nucleoprotein gene (Fig. 3; Supplementary Material Tables S3 and S4). Two additional sequences from Monongalia County had two synonymous nucleoprotein gene changes and either one nonsynonymous or one synonymous glycoprotein gene change.

Phylogenetic analysis of RRV sequences revealed support for three clusters of RRV in West Virginia based on the available sequences

TABLE 2. Coyote (*Canis latrans*) genetic clusters identified by ADMIXTURE (Alexander et al. 2009) in West Virginia, USA. X represents the West Virginia counties that clustered with the rabid coyote (sample E22R007878-01) at $Q>0.90$ for genetic cluster (K)=3–10. In bold is Lewis County, the only county that clustered with the rabid coyote at all values of K.

County	*K*3	*K*4	*K*5	*K*6	*K*7	*K*8	*K*9	*K*10
Barbour					X			
Braxton	X				X			
Doddridge	X	X			X	X		X
Gilmer	X		X	X		X		
Grant	X	X		X			X	
Greenbrier	X							
Hardy				X				
Harrison	X	X		X				
Lewis	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
Monongalia	X						X	
Marion	X		X		X			
Marshall		X						
Mason		X	X					
Mercer	X							
Mineral		X						
Nicholas		X	X	X	X			
Pendleton	X	X	X					X
Pleasants	X			X			X	
Preston			X			X		
Pocahontas	X	X						
Roane	X					X		X
Randolph		X	X					
Upshur					X	X		
Wetzel	X	X					X	
Wirt		X						
Wood								X

(Fig. 4; see Supplementary Material Fig. S2 for maximum likelihood tree). Grant and Monroe counties contained samples that came from multiple clades. The RRV from E22R007878-01 belonged to a large clade of RRV sequences from across Pennsylvania, eastern Ohio, northern West Virginia, southwestern West Virginia, and western Virginia (Fig. 4). Within this large clade, E22R007878-01 belonged to a subclade with high support that included six sequences from Monongalia County.

These host and virus genomic analyses provided high confidence that the rabid coyote originated in Lewis County, the same county where it was collected, and that the origin of RRV infection was most likely from northern West Virginia, possibly in or near Monongalia County, which is an active RRV management area. However, samples were not available from all counties; thus, origin in a nearby county cannot be discounted. It is very unlikely that the coyote was infected with RRV in Kentucky or western West Virginia, because RRV has never been detected in these regions.

The complete explanation of how and where this animal was infected will never be known; however, based on the combined genomic data, we can confidently say it was not a long-distance translocation of RRV (e.g., from Maine or Florida, US) or a migrant animal. Although the rabid coyote showed the strongest genetic association with the county where it was collected, RRV is not enzootic in Lewis County, and the most similar virus was from northern

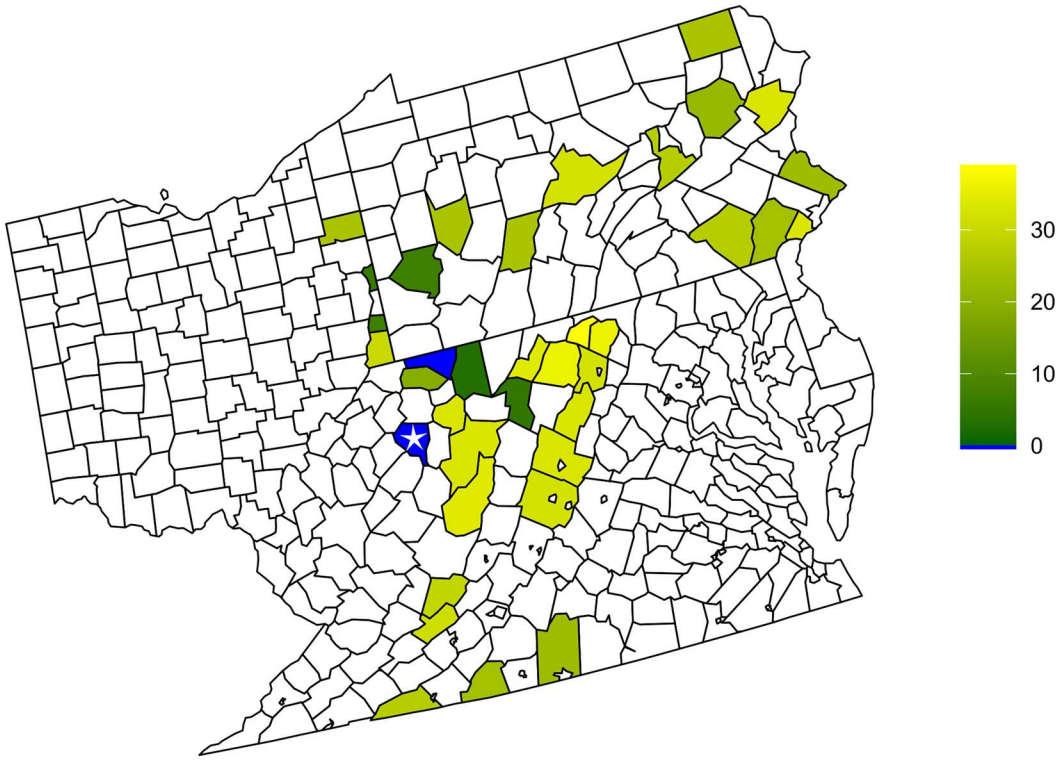

FIGURE 3. Rabies virus sequence similarity to Lewis County, West Virginia, USA, coyote (*Canis latrans*) rabies virus (raccoon, *Procyon lotor*, variant) glycoprotein gene. Color indicates number of nucleotide changes (blue = identical, dark green = one nucleotide change, yellow = most diverged, white = no data). Lewis County is highlighted with a white star, whereas the other blue county is Monongalia County, West Virginia. Nucleotide differences are shown for the most similar sequence from that county when more than one sequences were available (minimum distance).

West Virginia, approximately 80 km away. Studies have suggested that coyotes in the Appalachian plateau, particularly transient individuals, can range >100 km^2, occasionally >500 km^2 (Crawford 1992; Mastro et al. 2019). Given that this individual was a juvenile male, there is a possibility that it did not have an established territory and may have been exhibiting natural dispersal or exploratory movements into areas of northern West Virginia where it was infected with RRV before returning to Lewis County. The incubation time of RV can vary from a few weeks to a few months, depending on host species, infectious dose, and variant (Müller and Freuling 2020); thus, it is possible that the coyote moved across the landscape before displaying clinical signs of rabies virus infection.

Based upon the combined host and viral molecular data from this study, program managers did not implement a full emergency response, which would have included multiple years of intensive ORV management and ERS. Determining that the rabid coyote was a single case of cross-species transmission within a RRV enzootic area, with a low chance of onward local transmission in raccoons, saved both time and resources. Since the detection of the rabid coyote, no additional RRV-infected animals have been found in Lewis County or surrounding counties, despite ERS efforts in the region, thereby supporting the hypothesis of a wandering juvenile coyote.

By combining host and pathogen genomic data, we were able to infer more information about the origin of infection and risk of RRV establishment in a new area than if we had performed only host or viral analysis alone. This approach has potential applications to

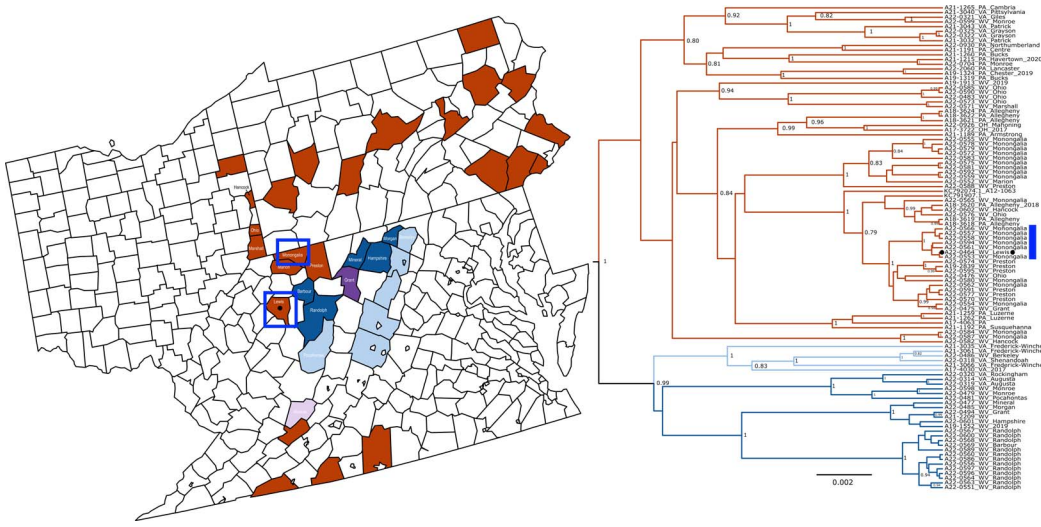

FIGURE 4. Phylogenetic analysis of raccoon (*Procyon lotor*) rabies virus variant glycoprotein gene sequences from West Virginia, Virginia, Pennsylvania, and Ohio, USA. The geographic distributions of major clade are indicated by colored branches on the tree that match colors on the map to the left. Two counties had multiple major clade: Monroe County, West Virginia (pink = light blue + red) and Grant County, West Virginia (purple = dark blue + red). Lewis County is highlighted by the black dot on the phylogeny and map with the blue boxes (map) and bar (phylogeny) representing the clade to which the Lewis County coyote sequence (sample E22R007878-01) belongs. Phylogenetic analysis was performed using BEAST v1.10.4 (Suchard et al. 2018). Posterior support >0.7 is indicated at the branch points. West Virginia counties are labeled on the map; sequences without county information are not included on the map. South central skunk rabies virus variant sequences JQ685938.1 and JQ685968.1 were used to root the tree.

zoonotic pathogens beyond rabies. Nevertheless, the success of our investigation was dependent on the early detection of RRV in a RRV-free county. In this case, a strong, coordinated rabies surveillance system was able to quickly identify this case thorough rapid, routine diagnostic testing, variant typing, and reporting. The resolution of geographic inference is also dependent on sampling breadth for both host species and pathogen genomic analyses, and data must be available rapidly to inform management decisions in a meaningful way. Establishment of geographically curated and genetically diverse genomic databases for both hosts and pathogens, collected through interdisciplinary efforts among geneticists, disease ecologists, and wildlife managers provides a backdrop for linking epizootiology and management of wildlife diseases in real-time during a high-consequence event.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/JWD-D-23-00158.

LITERATURE CITED

Alexander DH, Novembre J, Lange K. 2009. Fast model-based estimation of ancestry in unrelated individuals. *Genome Res* 19:1655–1664.

Barton HD, Gregory AJ, Davis R, Hanlon CA, Wisely SM. 2010. Contrasting landscape epidemiology of two sympatric rabies virus strains. *Mol Ecol* 19:2725–2738.

Biek R, Real LA. 2010. The landscape genetics of infectious disease emergence and spread. *Mol Ecol* 19:3515–3531.

Bird BH, Mazet JAK. 2018. Detection of emerging zoonotic pathogens: An integrated one health approach. *Annu Rev Anim Biosci* 6:121–139.

Brunker K, Jaswant G, Thumbi SM, Lushasi K, Lugelo A, Czupryna AM, Ade F, Wambura G, Chuchu V, et al. 2020. Rapid in-country sequencing of whole virus genomes to inform rabies elimination programmes. *Wellcome Open Res* 5:3.

Chipman R, Slate D, Rupprecht C, Mendoza M. 2008. Downside risk of wildlife translocation. *Dev Biol (Basel)* 131:223–232.

Crawford BA. 1992. *Coyotes in Great Smoky Mountains National Park: Evaluation of methods to monitor relative abundance, movement ecology, and habitat use*. MS Thesis, University of Tennessee, Knoxville, Tennessee, 84 pp.

Elmore SA, Chipman RB, Slate D, Huyvaert KP, VerCauteren KC, Gilbert AT. 2017. Management and modeling approaches for controlling raccoon rabies: The road to elimination. *PLoS Negl Trop Dis* 11:e0005249.

Genevie R, Powell J, Raj P, Rudd R, Rupprecht C, Schnurr D, Smith J, Trimarchi C. 2003. *Protocol for postmortem diagnosis of rabies in animals by direct fluorescent antibody testing: A minimum standard for rabies diagnosis in the United States*. Centers for Disease Control and Prevention. https://www.cdc.gov/rabies/pdf/rabiesdfaspv2.pdf. Accessed August 2023

Gigante CM, Yale G, Condori RE, Costa NC, Long NV, Minh PQ, Chuong VD, Tho ND, Thanh NT, et al. 2020. Portable rabies virus sequencing in canine rabies endemic countries using the Oxford Nanopore MinION. *Viruses* 12:1255.

Grome HN, Yackley J, Goonewardene D, Cushing A, Souza M, Carlson A, Craig L, Cranmore B, Wallace R, et al. 2022. Translocation of an anteater (*Tamandua tetradactyla*) infected with rabies from Virginia to Tennessee resulting in multiple human exposures, 2021. *Morb Mortal Wkly Rep* 71:533–537.

Heppenheimer E, Brzeski KE, Hinton JW, Patterson BR, Rutledge LY, DeCandia AL, Wheeldon T, Fain SR, Hohenlohe PA, et al. 2018. High genomic diversity and candidate genes under selection associated with range expansion in eastern coyote (*Canis latrans*) populations. *Ecol Evol* 8:12641–12655.

Lobo D, DeBenedet C, Fehlner-Gardiner C, Nadin-Davis S, Anderson M, Buchanan T, Middel K, Filejski C, Hopkins J. 2018. Raccoon rabies outbreak in Hamilton, Ontario: A progress report. *Can Commun Dis Rep* 44:116–121.

Ma X, Bonaparte S, Corbett P, Orciari LA, Gigante CM, Kirby JD, Chipman RB, Fehlner-Gardiner C, Thang C, et al. 2023. Rabies surveillance in the United States during 2021. *J Am Vet Med Assoc* 261:1045–1053.

Martel A, Vila-Escale M, Fernández-Giberteau D, Martinez-Silvestre A, Canessa S, Van Praet S, Pannon P, Chiers K, Ferran A, et al. 2020. Integral chain management of wildlife diseases. *Conserv Lett* 13:e12707.

Mastro LL, Morin DJ, Gese EM. 2019. Home range and habitat use of West Virginia *Canis latrans* (coyote). *Northeast Nat* 26:616–628.

McLean RG. 1971. Rabies in raccoons in the southeastern United States. *J Infect Dis* 123:680–681.

Müller T, Freuling CM. 2020. Rabies in terrestrial animals. In *Rabies*, 4th Ed., Fooks AR, Jackson AC, editors. Academic Press, Boston, Massachusetts, pp. 195–230.

Nadin-Davis S, Buchanan T, Nituch L, Fehlner-Gardiner C. 2020. A long-distance translocation initiated an outbreak of raccoon rabies in Hamilton, Ontario, Canada. *PLoS Negl Trop Dis* 14:e0008113.

Rosatte RC, MacInnes CD. 1989. Relocation of city raccoons. *Proc 9th Great Plains Wild Dam Cont Conf*:87–92.

Rupprecht CE, Smith JS. 1994. Raccoon rabies: The re-emergence of an epizootic in a densely populated area. *Semin Virol* 5:155–164.

Rupprecht CE, Smith JS, Fekadu M, Childs JE. 1995. The ascension of wildlife rabies: A cause for public health concern or intervention? *Emerg Infect Dis* 1:107–114.

Russell CA, Smith DL, Childs JE, Real LA. 2005. Predictive spatial dynamics and strategic planning for raccoon rabies emergence in Ohio. *PLoS Biol* 3:e88.

Singh AJ, Chipman RB, de Fijter S, Gary R, Haskell MG, Kirby J, Yu L, Condori RE, Orciari L. 2018. Translocation of a stray cat infected with rabies from North Carolina to a terrestrial rabies-free county in Ohio, 2017. *Morb Mortal Wkly Rep* 67:1174–1177.

Slate D, Algeo TP, Nelson KM, Chipman RB, Donovan D, Blanton JD, Niezgoda M, Rupprecht CE. 2009. Oral rabies vaccination in North America: Opportunities, complexities, and challenges. *PLoS Negl Trop Dis* 3:e549.

Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol* 4:vey016.

Szanto AG, Nadin-Davis SA, Rosatte RC, White BN. 2011. Genetic tracking of the raccoon variant of rabies virus in eastern North America. *Epidemics* 3:76–87.

Trewby H, Nadin-Davis SA, Real LA, Biek R. 2017. Processes underlying rabies virus incursions across US-Canada border as revealed by whole-genome phylogeography. *Emerg Infect Dis* 23:1454–1461.

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SUPPLEMENTARY MATERIALS AND METHODS

RAD sequencing of host genomic DNA

We obtained genomic DNA using the DNeasy Blood and Tissue kit following manufacturer's protocol on a QiaCube extraction robot (Qiagen, Germany). We prepared genomic libraries for the rabid coyote and the 54West Virginia coyotes to conduct RADseq following a modified protocol (Ali et al. 2015). Briefly, we used the *Sbf1* restriction enzyme to digest genomic DNA and ligate a unique 8-bp barcoded biotinylated adapter to the resulting fragments followed by random shearing to 400bp in a Covaris LE220. We then enriched for the adapter ligated fragments using a Dynabeads M-280 streptavidin binding assay followed by a size selection for fragments 300-400bp in size. We purified the enriched library using Agencourt AMPure XP magnetic beads and subsequently prepared them for Illumina NovaSeq 2x150nt sequencing at Princeton University's Lewis Sigler Genomics Institute core facility using the NEBnext Ultra II library prep kit (New England BioLabs, USA).

Bioinformatic processing of host genomic data

We bioinformatically retained sequencing reads that contained the unique barcode and the remnant *SbfI* cut site, which we demultiplexed in *STACKS* v2 using 2bp mismatch for barcode rescue in the *process_radtags* module (Catchen et al. 2013, Rochette et al. 2019). We retained reads with a quality score ≥10 and removed PCR duplicates with the paired-end sequencing filtering option with the *clone_filter* module. We mapped the cleaned reads to the reference dog genome CanFam3.1 assembly (Lindblad-Toh et al. 2005) using *BWA-mem* (Li

2013). We excluded reads with MAPQ<20 and the final alignment was converted to *bam* format in *Samtools* v0.1.18 (Li et al. 2009). We further included RADseq data previously collected from 264 coyotes (VA=54, TN=3, SC=2, PA=82, OH=1, NJ=13, NC=47, and KY=62) that used identical laboratory, library prep method, and sequenced by the same core facility (Table S1).

We discovered SNP loci in in the *STACKS gstacks* module on 318 samples to obtain a catalogue of all polymorphic sites possible (Catchen et al. 2013, Rochette et al. 2019) and increased the minimum significance threshold to require more stringent confidence needed to identify a polymorphic site using the marukilow model (flags –vt-alpha and –gt-alpha, p=0.01). After SNP discovery, we conducted several filtering steps in *VCFtools* v0.1.17 (Danecek et al. 2011) to exclude singleton and private doubleton alleles, removed loci with more than 90% missing data across all samples, and removed individuals with more than 20% missing data. We filtered for a minimum of 3% minor allele frequency (MAF) in *PLINK* v1.90b3i (Chang et al. 2015). For population genetic analyses, we further constructed a "*statistically neutral and unlinked*" dataset of SNPs by excluding sites within 50-SNP windows that exceeded genotype correlations of 0.5 (with the *PLINK* flag --indep-pairwise 50 5 0.5) and deviated from neutrality with the *hwe* flag set to 0.001. We genotyped 555,159 loci across 318 coyotes with an average of 12-fold per-sample sequence coverage (s.d.=7.8, min=3.3, max=74.2). After filtering to remove loci with MAF>3% and >20% missing data, we retained 153,897 SNP loci genotyped for 318 coyotes (Table S1).

Clustering, assignment test, and relatedness of the host to other coyotes

All demographic analyses were conducted on the statistically neutral and unlinked SNP set. We conducted an unsupervised principal component analysis (PCA) in the program *flashPCA* (Abraham and Inouye 2014) and maximum likelihood clustering with the cross-

validation error flag in the program *ADMIXTURE* (Alexander et al. 2009) to assess proportional cluster membership (*Q*) across nine data partitions (*K*=2-10) and the best fit partition. For further population assignment, we used the supervised clustering in *ADMIXTURE* which requires designation of individuals to specified reference populations to estimate cluster assignment probability. We used the *populations* module in *STACKS* to identify alleles private to suspected source coyote populations and tabulate any found in E22R007878-01.

To estimate pairwise relatedness between individual coyotes with the R package *related* (Pew et al. 2015) we further filtered the SNPs to limit genotyping error by increasing the MAF threshold to 20% and excluding SNPs that exceeded a stricter genotype correlation threshold of 0.2 (with the *PLINK* argument --indep-pairwise 50 5 0.2). We used the *coancestry* function, the dyadic likelihood estimator (dvadml=1; Milligan 2003), and permitted inbreeding (allow.inbreeding=TRUE) to estimate relatedness coefficients.

Rabies virus sequencing

We included RRV postmortem samples from the brain tissue of rabid animals collected for routine public health surveillance by health departments of OH, PA, VA, and WV or during routine and emergency response management and activities of the USDA Wildlife Services (Table S2). Total RNA was extracted from brain samples using TRIzol™ reagent and Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA), as described previously (Gigante et al. 2018). Complete RRV nucleoprotein and glycoprotein gene sequencing was performed using the SuperScript IV one-step reverse transcriptase-PCR system (Thermo Fisher, Waltham, MA, USA) following manufacturer's recommendations but in 20 µL reactions with the following primers: **TTTCTGTTGGTGCTGATATTGC**ACGCTTAACAACCAGATCAAAGAA (nucleoprotein gene forward1), TTTCTGTTGGTGCTGATATTGCACGCTTAACAACAAAATCADAGAAG

(nucleoprotein gene forward2), **ACTTGCCTGTCGCTCTATCTTC**AGGAGGRGTGTTAGTTTTTTTC (nucleoprotein gene reverse), **TTTCTGTTGGTGCTGATATTGC**GATGTGAAAAAACTATYAACATCCCTC (glycoprotein gene forward), **ACTTGCCTGTCGCTCTATCTTC**TGTGAKCTATTGCTTRTGTYCTTCA (glycoprotein gene reverse). Separate RT-PCR reactions for each sample were prepared for nucleoprotein and glycoprotein amplification. Nucleoprotein and glycoprotein reactions for the same sample were then combined and barcoded using Takara long amplicon Taq polymerase with GC buffers following the manufacturer's instructions for PCR barcoding for nanopore sequencing (EXP-PBC096 Oxford Nanopore Technologies, Oxford, UK). Sequencing was performed on the MinION using a flongle flow cell version FLG001 after library preparation with the LSK-109 ligation sequencing kit and EXP-PBC096 PCR barcoding kit (Oxford Nanopore Technologies, Oxford, UK).

Rabies virus bioinformatics and analysis

Basecalling was performed using guppy version 4.2.2 (Oxford Nanopore Technologies, Oxford, UK). Reference-based consensus sequences were generated using ivar 0.1 (https://github.com/andersen-lab/ivar) from mapping output of minimap2 v.2.16 (https://github.com/lh3/minimap2) and polished using medaka v1.0.1 (https://github.com/nanoporetech/medaka). Manual indel correction was then performed as described previously for the coding regions of the nucleoprotein and glycoprotein genes (Gigante et al. 2020). The RRV glycoprotein and nucleoprotein gene and amino acid sequences were aligned using MAFFT v.7.450 with the FFT-NS-I x1000 algorithm in Geneious Prime 2019.1.1 (Katoh et al. 2002, Katoh and Standley 2013). A maximum clade credibility tree was estimated

using BEAST 1.10.4 using GTR+ G+I model (determined by AIC in model test in Mega7), and a constant coalescent population (Suchard et al. 2018). Data were split into two partitions (codon sites 1+2, 3) and clock rate was set to 1. Nucleotide or amino acid differences were calculated in Geneious Prime 2019.1.1. Host and virus maps were made in RStudio v.1.3.959 (R version 4.3.0) using the usmap and ggplot2 packages [https://cran.r-project.org/web/packages/usmap/index.html; https://ggplot2.tidyverse.org] (Wickham 2016).

LITERATURE CITED

Abraham G, Inouye M. 2014. Fast principal component analysis of large-scale genome-wide data. *PLoS ONE* 9:e93766.

Alexander DH, Novembre J, Lange K. 2009. Fast model-based estimation of ancestry in unrelated individuals. *Genome Res* 19:1655-1664.

Ali OA, O'Rourke SM, Amish SJ, Meek MH, Luikart G, Jeffres C, Miller MR. 2015. Rad capture (rapture): Flexible and efficient sequence-based genotyping. *Genetics* 202:389-400.

Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA. 2013. Stacks: An analysis tool set for population genomics. *Mol Ecol* 22:3124-3140.

Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. 2015. Second-generation plink: Rising to the challenge of larger and richer datasets. *GigaScience* 4.

Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, et al. 2011. The variant call format and vcftools. *Bioinformatics* 27:2156-2158.

Gigante CM, Dettinger L, Powell JW, Seiders M, Condori REC, Griesser R, Okogi K, Carlos M, Pesko K, Breckenridge M, et al. 2018. Multi-site evaluation of the ln34 pan-lyssavirus real-time rt-PCR assay for post-mortem rabies diagnostics. *PLoS One* 13:e0197074.

Gigante CM, Yale G, Condori RE, Costa NC, Long NV, Minh PQ, Chuong VD, Tho ND, Thanh NT, Thin NX, et al. 2020. Portable rabies virus sequencing in canine rabies endemic countries using the Oxford Nanopore Minion. *Viruses* 12:1255.

Katoh K, Misawa K, Kuma Ki, Miyata T. 2002. Mafft: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 30:3059-3066.

Katoh K, Standley DM. 2013. Mafft multiple sequence alignment software version 7: Improvements in performance and usability. *Mol Biol Evol* 30:772-780.

Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with bwa-mem. *arXiv preprint arXiv:13033997*.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Subgroup GPDP. 2009. The sequence alignment/map format and samtools. *Bioinformatics* 25:2078-2079.

Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas EJ, Zody MC, et al. 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* 438:803-819.

Milligan BG. 2003. Maximum-likelihood estimation of relatedness. *Genetics* 163:1153-1167.

Pew J, Muir PH, Wang J, Frasier TR. 2015. Related: An R package for analysing pairwise relatedness from codominant molecular markers. *Mol Ecol Resour* 15:557-561.

Rochette NC, Rivera-Colón AG, Catchen JM. 2019. Stacks 2: Analytical methods for paired-end sequencing improve radseq-based population genomics. *Mol Ecol* 28:4737-4754.

Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. 2018. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol* 4: vey016.

Wickham H. 2016. *Ggplot2: Elegant graphics for data analysis.* Springer-Verlag, New York.

Supplementary Tables

See separate spreadsheet file for Tables S1-S4:

Table S1. Sample ID, state, county, NCBI sequence read archive accession number (SRA ID) and additional metadata for coyote (*Canis latrans*) tissue samples used for DNA extraction to determine the origin of a rabid coyote. Also included are the mean sequencing depth, proportion of missing data, PCA coordinates, and ADMIXTURE assignment probabilities.

Table S2. Details for rabies virus samples sequenced and analyzed to determine the origin of a rabid coyote (*Canis latrans*) captured in West Virginia, USA.

Table S3. Rabies virus glycoprotein genetic distances. Number of nucleotide and amino acid differences for rabies virus sequences from USA states West Virginia, Virginia, Pennsylvania and Ohio relative to the Lewis County, West Virginia rabid coyote (*Canis latrans*).

Table S4. Nucleoprotein genetic distances. Number of nucleotide and amino acid differences for rabies virus sequences from USA states West Virginia, Virginia, Pennsylvania and Ohio relative to the Lewis County, West Virginia rabid coyote (*Canis latrans*).

Supplementary Figures

Figure S1. Population cluster analysis of 107,888 single nucleotide polymorphism (SNP) genotypes from West Virginia and Kentucky, US, coyotes using **A)** the unsupervised maximum-likelihood algorithm ADMIXTURE for 2-7 genetic partitions (K), with K=5-7 not shown. The cross-validation (cv) value per K is provided. The farthest right sample (single bar) is the rabies-positive coyote (sample E22R007878-01). The values of K with the highest likelihood were K = 2 and K = 3 which grouped the Lewis County rabid coyote with West Virginia coyotes. **B)** To evaluate any possible geographic substructure, we mapped assignment proportions for K=7 per geographic region for Kentucky and West Virginia. The pie charts on the map represent the assignment proportion of coyote genotypes from the area with the "E22R" indicative of the rabies-positive coyote which matched coyotes from both Kentucky and West Virginia.

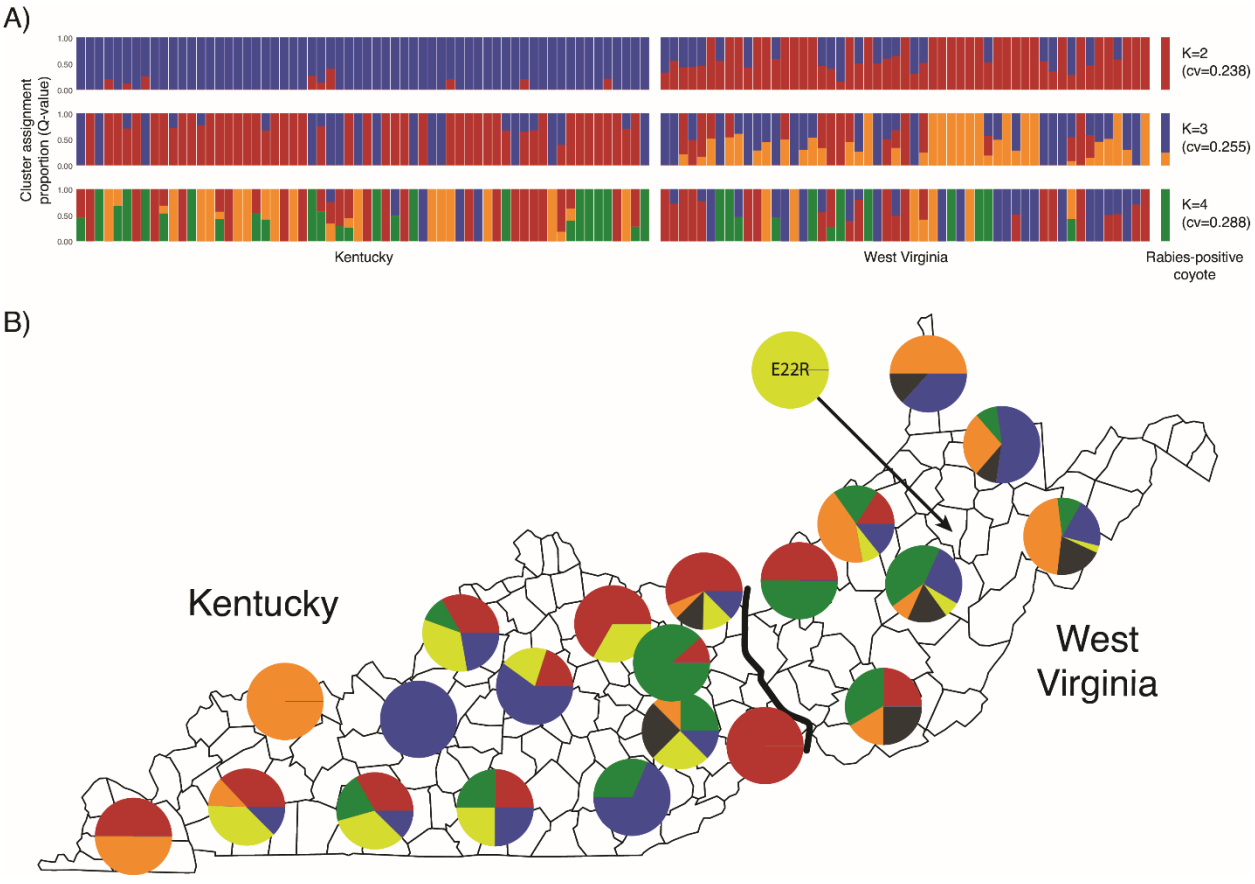

Figure S2. Mid-point rooted Maximum likelihood phylogenetic tree of raccoon rabies virus variant glycoprotein sequences for comparison to Bayesian trees. The tree was generated using raxml-ng and the same alignment as the BEAST trees. We used two partitions (codon sites 1+2, 3) and GTR+G+I model. Support values are based on 1000 bootstraps. There are many more polytomies than the BEAST trees, but the rabid Lewis County coyote (denoted by •) is in a clade with Monogalia County sequences.