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Two novel poxviruses with unusual genome rearrangements: NY_014 and Murmansk

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Abstract The genome sequence and annotation of two novel poxviruses, NY_014 and Murmansk, are presented. Despite being isolated on different continents and from different hosts, the viruses are relatively similar, albeit distinct species. The closest known relative of the novel viruses is Yoka poxvirus. Five novel genes were found in these genomes, two of which were MHC class I homologs. Although the core of these genomes was well conserved, the terminal regions showed significant variability with large deletions and surprising evidence of recombination with orthopoxviruses.

Keywords Poxvirus · Orthopoxvirus · Yoka poxvirus · Zoonotic · Murmansk · Virus virulence · Complete genome

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Introduction

Poxviruses are large, complex viruses with linear, double-stranded DNA genomes that replicate entirely in the cytoplasm and infect insects (subfamily *Entomopoxvirinae*) and vertebrates (subfamily *Chordopoxvirinae*). Chordopoxviruses have been placed into 10 genera, i.e., Avi-, Capri-, Cervid-, Crocodylid-, Lepori-, Mollusci-, Ortho-, Para-, Sui-, and Yatapoxvirus; however, there are a number of fully sequenced viruses that are currently not assigned to a genus and are likely to require the designation of several new genera. These include salmon gill poxvirus (SGPV) isolated from salmon [1], Yoka poxvirus [2], Cotia virus and Embu virus [3] isolated from mosquitos, and Pteropoxvirus isolated from the Australian little flying fox (a bat) [4]. When further reports of novel poxviruses with only part of their genomes sequenced are taken into account, it is apparent that the numbers of *Chordopoxvirus* species and genera have taken a recent leap. Included in this group are a novel poxvirus isolated in big brown bats [5] that has been proposed to be designated as a new genus *Chiropoxvirus*, a poxvirus isolated from humans in the country of Georgia that has been classified as a novel *Orthopoxvirus* species [6] and two novel poxviruses from grey kangaroos (Dr. M. O'Dea, personal communication).

All of the Chordopoxviruses share a common core of about 80 genes that regulate and perform transcription, replication, and virus assembly functions [7]. However, the viral genomes are substantially varied in size, encoding between 129 and 328 genes. Thus, it is clear that there is an abundance of viral genes that are specific to various subsets of the Chordopoxviruses. Many of these genes are of "unknown function," but are suspected to encode some type of host range or virulence factor because of their non-essential nature.

Poxviruses first assigned to a genus of the subfamily *Chordopoxvirinae* can be considered "low hanging fruit" discovered by virtue of their abundance and, or ability to cause observable effects in humans and animals. In contrast, those that have been discovered more recently have been isolated from relatively poorly surveyed hosts (fish, bats, mosquitos, birds, and aquatic mammals) or rare infections of humans.

Here, we report the complete genome sequences of two novel poxvirus species: one, NY_014, from an immune-suppressed patient living in the US [8] and the second, Murmansk from a root vole (*Microtus oeconomus*) isolated in Russia [9]. Phylogenetic analysis reveals that these two genomes are remarkably similar considering where they were isolated, and indicates that they should be placed in a new genus together with Yoka poxvirus.

Materials and methods

Viral strains

The isolation of NY_014 from a renal transplant patient with a progressive panniculitis/blistering rash in Upstate New York, USA and DNA isolation has been described previously [8]. Although not substantiated, this infection may have been acquired from a feral cat.

The Murmansk virus was isolated as follows [9]: during the period of June–July 1985, 225 rodents of different species were captured in the forest-tundra of Kolsky Peninsula, Russia, (latitude N 65°, longitude E 38°). Pooled organs (brain, liver, and spleen) from each species were analyzed for viral activity by intracerebral inoculation of suckling baby mice. From one such pool of root vole (*Microtus oeconomus*) organs, the LEIV-11411 Mur-Lovozero (Murmansk) viral strain was isolated. Electron microscopic study suggested that it belonged to the *Poxviridae* family. It had a low hemagglutinin activity, and formed hemorrhagic plaques on chorioallantoic membranes and plaques on Vero cell monolayers.

DNA extraction and PCR

To extract DNA, the Murmansk strain was propagated on Vero cells, and after 3 days cells were harvested and freeze-thawed three times. Low-speed centrifugation removed cellular debris and DNA was extracted from the supernatant using the Magna Pure apparatus (Roche, Germany). NY_014 was propagated on BSC40 cells and DNA was extracted as described by [8]. Use of a pan-orthopoxvirus real-time PCR assay [10] produced no signal with both viruses, whereas the pan-poxvirus PCR [11] method produced a specific amplicon. Sequencing of the amplicons resulted in unique sequences with no identical counterparts in the GenBank database.

Genome sequencing and de novo assembly

The purified Murmansk viral DNA was sequenced using the Illumina HiSeq 2500 platform (Illumina, Inc, San Diego, CA). The de novo assembly of the viral genome was performed using CLC genomic workbench software (CLC bio, Aarhus, Denmark) with an average coverage of over 1000×. The NY_014 assembly has an average coverage of 500×. Briefly, raw reads were imported into CLC and quality was assessed to remove duplicate reads, low-quality reads (quality score >0.01), and reads with >2 ambiguous nucleotides. After de novo assembly at the default setting, output contigs were screened against poxviruses (Taxid:10240) using BLAST. Four contigs were generated from the Murmansk DNA and three contigs were generated from NY_014. The order and orientation of the contigs were determined based on the BLAST results, and the gaps between the contigs were filled by Sanger sequencing using designed specific primers based on the assembled contigs. The gaps in the de novo assembling were due to tandem repeats and the inverted terminal repeats sequences (ITRs) at both the ends of poxvirus genome. The ITR regions usually form separate contigs and have elevated level of raw reads coverage relative to other contigs. ITR contigs were manually added to both the ends of the assembled genomes, and PCR and sequencing were used to confirm the assembly of the ITR ends.

Genome and phylogenetic analysis

The two genomes were annotated using the Genome Annotation Transfer Utility (GATU) [12] with Yoka poxvirus (YKV) and Cowpox virus strain Brighton Red (CPXV-BR) as the reference genomes. This program first transfers known gene positions to the target genomes and then identifies possible novel ORFs for further evaluation by the annotator. All predicted ORFs were searched against the NCBI nr database using BLAST programs [13]. Multiple sequence alignments (MSA) were performed with MAFFT [14] and ClustalO [15] in Base-By-Base (BBB). Phylogenetic analysis were performed using both maximum likelihood and neighbor-joining methods with MEGA v7 [16] and 500 bootstrap replicates; these methods generated similar trees. Dotplots were calculated and visualized using JDotter [17]. BBB, JDotter, and GATU are available at the Viral Bioinformatics Resource Centre (virology.uvic.ca).

Viruses and sequence accession numbers used for analysis

The following viruses were also used in the phylogenetic analysis [species/strain name (abbreviation; GenBank accession number)]: the Orthopox species, Camelpox virus CMS (CMLV; AY009089.1), Cowpox virus Brighton Red (CPXV; NC_003663.2), Ectromelia virus Moscow (ECTV; AF012825.2), Monkeypox virus Zaire-96-I-16 (MPXV; NC_003310.1), Vaccinia virus Western Reserve (VACV; NC_006998.1), Variola virus United Kingdom 1946 Harvey (VARV; DQ441444.1), Taterapox virus (TATV; NC_008291.1), Raccoonpox virus Herman (RCNV; NC_027213.1), Skunkpox virus WA (SKPV, NC_031038.1), and Volepox virus CA (VPXV, NC_031033.1); the Avipoxvirus species, Canarypox virus (CNPV; NC_005309.1), Fowlpox virus Iowa (FWPV; NC_002188.1), Penguinpox virus (PEPV; NC_024446.1), Pigeonpox virus (PIPV; NC_024447.1), and Turkeypox virus (TKPV; NC_028238.1); the Molluscipoxvirus species, Molluscum Contagiosum virus (MOCV; NC_001731.1); the Leporipoxvirus species, Myxoma virus Lausanne (MYXV; NC_001132.2) and Rabbit Fibroma virus (RFV; NC_001266.1); the Suipoxvirus species, Swinepox virus (SWPV; NC_003389.1); the Capripoxvirus species, Lumpy Skin Disease virus Neethling 2490 (LSDV; NC_003027.1), Sheeppox virus TU-V02127 (SPPV; NC_004002.1), and Goatpox virus Pellor (GTPV; NC_004003.1); the Cervidpoxvirus species, Deerpox virus W-1170-84 (DPV; NC_006967.1); the Yatapoxvirus species, Yaba-like disease virus (YLDV; NC_002642.1), Yaba monkey tumor virus (YMTV; NC_005179.1), and Tanapox virus (TANV; NC_009888.1); the Crocodylidpoxvirus species, Nile crocodilepox virus (CRV; NC_008030.1); the Parapox virus species, Bovine Papular Stomatitis virus AR02 (BPSV; NC_005337.1), Orf virus OV-SA00 (ORFV; NC_005336.1), Pseudocowpox virus VR634 (PCPV; NC_013804.1), and the unassigned viruses Cotia virus SPAn232 (COTV; NC_016924.1), Squirrel poxvirus (SQPV; NC_022563.1), and Yoka poxvirus (YKV; NC_015960.1). Accession numbers for genomic and MHC class I-like sequences used are indicated in their respective figures. The genome sequences of NY_014 and Murmansk poxviruses have been deposited in GenBank with accession numbers MF001305 and MF001304, respectively.

Results

NY_014 and Murmansk genome characteristics

The genome sequences of NY_014 and Murmansk were 200,223 bp (ITR = 1646 bp; A + T = 70.5%) and 204,055 bp (ITR = 4182 bp; A + T = 70.2%), respectively. There were only limited tandem repeat sequences in the ITRs of both NY_014 and Murmansk. 197 and 206 ORFs were annotated in the NY_014 and Murmansk genomes, respectively. We took a conservative approach to the process, annotating (1) ORFs matching previously characterized poxvirus genes, (2) unique ORFs larger than 65 codons, and (3) ORFs matching more than 50% of a characterized poxvirus gene with promoter region and initiating Met codon intact. The goal was to limit annotations to those ORFs with a high likelihood of encoding a polypeptide with some biological function. The NY_014 and Murmansk genome annotations are presented in Table 1. The lists of genes in Table 1 confirm an overall co-linear arrangement of the two new genomes with the Yoka poxvirus genome throughout the central core. The NY_014 and Murmansk genomes share 187 genes, which have an average aa identity of 94.3%; for the core region, this average increases to 98.1%. Although the core regions of these genomes have high similarity, towards the right end of the central region of the genome, Murmansk has two genes (Murmansk-156 and -157) that are absent from NY_014. These two genes, which are also absent from Yoka poxvirus, are orthologs of orthopoxvirus genes that encode a semaphorin-like protein (CPXV-BR-176) and a chemokine binding protein (CPXV-BR-178) with 50 and 34% aa identity, respectively. Table 1 also highlights the considerable variation at the left and right termini between these genomes and Yoka poxvirus, which is in part because the Yoka poxvirus genome is approximately 25 kbp shorter than the NY_014 and Murmansk genomes. To identify those genes without orthologs in Yoka poxvirus, we searched the NCBI non-redundant (nr) database using BLASTP [13]. The ITRs are unremarkable, although they are different sizes in the two viruses with one and four genes duplicated for NY_014 and Murmansk genomes, respectively. Such differences are commonly seen among the poxviruses and it is unclear whether having two copies of particular genes makes much difference to the biology of the viruses.

NY_014 and Murmansk phylogenetic analysis

Considering the locations from which these two viruses were isolated (New York, USA, and Murmansk, close to the Russia/Sweden border), we were somewhat surprised by their high level of nucleotide similarity. Alignment of the central core genes revealed that NY_014 and Murmansk viruses are approximately 98% identical (nucleotide); thus, they are each other's closest relative. The next closest known relative is Yoka poxvirus, which shares approximately 85% nucleotide identity with each of these two novel viruses. These relationships suggest that these three viruses should be placed in a common genus, proposed to ICTV as *Centapoxvirus*. In order to increase the reliability of the phylogenetic analysis, alignments were

Table 1 Annotation of Murmansk and NY_014 genomes. Yoka poxvirus was used as the primary reference genome

NY_014	Murmansk	Reference	NY_014 AA Size	Murmansk AA Size	Product
NY_014-001	Mur-001	CPXV-AUS-209	166	166	TNF-alpha receptor (CrmE)
NY_014-002		RCNV-Herman-003	593		Ankyrin
NY_014-003		RCNV-Herman-005	172		Hypothetical protein
NY_014-004		RCNV-Herman-006f/007f	210		Putative TLR signaling inhibitor, alpha-amanitin sensitivity
NY_014-005		RCNV-Herman-011	779		Ankyrin
NY_014-006		RCNV-Herman-012	434		Ankyrin
	Mur-002	CPXV221		317	TNF receptor (CrmD)
	Mur-003	CPXV220		563	Ankyrin
	Mur-004	CPXV009		152	Hypothetical protein
	Mur-005	CPXV010		206	Putative TLR signaling inhibitor, alpha-amanitin sensitivity
	Mur-006	CPXV218[a]		174	Hypothetical protein
NY_014-007	Mur-007	CPXV219	1875	1875	Surface glycoprotein
NY_014-008	Mur-008	CPXV191	180	183	TNF receptor (CrmC)
NY_014-009	Mur-009	CPXV220	571	569	Ankyrin
	Mur-010	CPXV218[a]		170	Hypothetical protein
NY_014-010	Mur-011	CPXV218[a]	187	187	Hypothetical protein
NY_014-011	Mur-012	CPXV003[a]	280	281	Chemokine binding protein
NY_014-012	Mur-013	CPXV218[a]	174	175	Hypothetical protein
NY_014-013	Mur-014	YKV008	246	249	Zinc Finger-Like Protein
NY_014-014	Mur-015	CPXV024	101	101	Soluble IL-18 Binding Protein (Bsh-D7L)
	Mur-016	CPXV024[a]		119	Soluble IL-18 Binding Protein (Bsh-D7L)
	Mur-017	CPXV008[a]		653	Ankyrin
NY_014-015	Mur-018	CPXV193[a]	303	308	BTB Kelch-domain containing protein
NY_014-016	Mur-019	CPXV018[a]	173	172	Hypothetical protein
	Mur-020	CPXV020[a]		149	Hypothetical protein (Bang-D3L)
NY_014-017	Mur-021	YKV009	116	119	Secreted EGF-like Protein
NY_014-018	Mur-022	YKV010c	320	320	IL-1 Receptor antagonist
NY_014-019	Mur-023	CPXV034	257	257	Complement binding (secreted)
NY_014-020	Mur-024	YKV011c	516	517	POZ/BTB Kelch-domain protein (Cop-C2L)
NY_014-021	Mur-025	YKV012c	205	205	Putative TLR signaling inhibitor (Cop-C1L)
NY_014-022	Mur-026	YKV013c	119	119	Anti-apoptotic Bcl-2-like protein (Cop-N1L)
NY_014-023	Mur-027	YKV014c	222	222	Putative TLR signaling inhibitor, alpha-amanitin sensitivity
NY_014-024	Mur-028	YKV015c	464	464	Ankyrin (Cop-M1L)
NY_014-025	Mur-029	CPXV041	276	275	Ankyrin/NFkB inhibitor
NY_014-026	Mur-030	YKV016c	84	84	IFN resistance, PKR/eIF-alpha inhibitor (Cop-K3L)
NY_014-027	Mur-031	CPXV044	420	419	Nicking-Joining Enzyme
NY_014-028	Mur-032	CPXV045	279	279	Putative monoglyceride lipase
NY_014-029	Mur-033		123	124	Hypothetical protein (NY_014-029)

Table 1 continued

NY_014	Murmansk	Reference	NY_014 AA Size	Murmansk AA Size	Product
NY_014-030	Mur-034	YKV019c	165	164	Caspase-9 (apoptosis) inhibitor (mitochondrial-associated)
NY_014-031	Mur-035	CPXV050	472	472	Kelch-like protein
NY_014-032	Mur-036	YKV020c	320	320	Ribonucleotide Reductase small subunit
NY_014-033	Mur-037		321	320	MHC class I protein
NY_014-034	Mur-038		336	334	MHC class I protein
NY_014-035	Mur-039	CPXV052	327	304	36 kDa major membrane protein
NY_014-036	Mur-040	YKV022c	66	71	Hypothetical protein
NY_014-037	Mur-041	CPXV054	82	83	Hypothetical protein
NY_014-038	Mur-042	YKV023c	64	64	Hypothetical protein
NY_014-039	Mur-043	YKV024c	212	212	S–S bond formation pathway protein substrate (Cop-F9L)
NY_014-040	Mur-044	YKV025c	440	440	Essential Ser/Thr kinase Morph (Cop-F10L)
NY_014-041	Mur-045	YKV026c	349	351	RhoA signaling inhibitor, virus release protein (Cop-F11L)
NY_014-042	Mur-046	YKV027c	644	644	Exclusive to IEV (Cop-F12L)
NY_014-043	Mur-047	YKV029c	373	373	Major IEV antigen (Cop-F13L)
NY_014-044	Mur-048		172	176	Hypothetical protein (NY_014-044)
NY_014-045	Mur-049	YKV031c	49	49	IMV protein (YMTV-28.5L)
NY_014-046	Mur-050	YKV032c	148	148	Unknown Conserved (Cop-F15L)
NY_014-047	Mur-051	YKV033c	221	221	Non-functional Serine Recombinase (Cop-F16L)
NY_014-048	Mur-052	YKV034	100	100	DNA-binding phosphoprotein (VP11; Cop-F17R)
NY_014-049	Mur-053	YKV035c	472	472	Poly (A) polymerase catalytic subunit (VP55)
NY_014-050	Mur-054	YKV036c	736	736	IEV morphogenesis (Cop-E2L)
NY_014-051	Mur-055	YKV037c	184	185	dsRNA-binding, IFN resistance/ PKR inhibitor (Z-DNA binding)
NY_014-052	Mur-056	YKV038c	261	260	RNA polymerase (RPO30)
NY_014-053	Mur-057	YKV039	371	368	Virosome component
NY_014-054	Mur-058	YKV040	566	566	Virion protein (Cop-E6R)
NY_014-055	Mur-059	YKV041	269	269	ER-localized membrane protein, virion core protein (Cop-E8R)
NY_014-056	Mur-060	YKV043c	1007	1007	DNA polymerase
NY_014-057	Mur-061	YKV044	96	96	Sulfhydryl oxidase (FAD-linked) (Cop-E10R)
NY_014-058	Mur-062	YKV045c	129	129	Virion core protein (Cop-E11L)
NY_014-059	Mur-063	YKV046c	662	662	Unknown (Cop-O1L)
NY_014-060	Mur-064	YKV047c	110	110	Glutaredoxin 1 (Cop-O2L)
NY_014-061	Mur-065	YKV048c	33	33	Virus entry/fusion complex component
NY_014-062	Mur-066	YKV049c	310	310	DNA-binding core protein (Cop-I1L)
NY_014-063	Mur-067	YKV050c	68	69	IMV membrane protein (Cop-I2L)
NY_014-064	Mur-068	YKV051c	268	269	ssDNA-binding phosphoprotein (Cop-I3L)

Table 1 continued

NY_014	Murmansk	Reference	NY_014 AA Size	Murmansk AA Size	Product
NY_014-065	Mur-069	YKV052c	763	763	Ribonucleotide Reductase large subunit
NY_014-066	Mur-070	YKV053c	78	78	IMV protein VP13
NY_014-067	Mur-071	YKV054c	383	383	Telomere-Binding protein
NY_014-068	Mur-072	YKV055c	422	422	Virion Core Cysteine Protease
NY_014-069	Mur-073	YKV056	672	673	RNA helicase, DExH-NPH-II domain
NY_014-070	Mur-074	YKV057c	590	590	Metalloprotease (Cop-G1L)
NY_014-071	Mur-075	YKV058c	111	111	Entry/fusion complex component (Cop-G3L)
NY_014-072	Mur-076	YKV059	220	220	VLTF (late transcription elongation factor Cop-G2R)
NY_014-073	Mur-077	YKV060c	124	125	Disulfide Oxidoreductase (Cop-G4L)
NY_014-074	Mur-078	YKV061	441	441	FEN1-like nuclease (Cop-G5R)
NY_014-075	Mur-079	YKV062	63	63	RNA polymerase (RPO7)
NY_014-076	Mur-080	YKV063	158	158	NLPc/P60 superfamily protein (Cop-G6R)
NY_014-077	Mur-081	YKV065c	381	380	Virion phosphoprotein, early morphogenesis (Cop-G7L)
NY_014-078	Mur-082	YKV066	260	260	VLTF-1 (Cop-G8R)
NY_014-079	Mur-083	YKV067	339	339	Entry-fusion complex component, myristylprotein
NY_014-080	Mur-084	YKV068	249	249	IMV membrane protein (Cop-L1R)
NY_014-081	Mur-085	YKV069	102	102	Crescent membrane and immature virion formation (Cop-L2R)
NY_014-082	Mur-086	YKV070c	355	375	Internal Virion Protein (Cop-L3L)
NY_014-083	Mur-087	YKV071	250	250	ss/dsDNA-binding protein (VP8; Cop-L4R)
NY_014-084	Mur-088	YKV072	128	128	Entry and Fusion IMV protein (Cop-L5R)
NY_014-085	Mur-089	YKV073	152	147	Virion morph (Cop-J1R)
NY_014-086	Mur-090	YKV074	180	180	Thymidine kinase
NY_014-087	Mur-091	YKV075	333	333	Poly(A) polymerase small subunit (VP39)
NY_014-088	Mur-092	YKV076	185	185	RNA polymerase (RPO22)
NY_014-089	Mur-093	YKV077c	133	133	Putative late 16 kDa membrane protein (Cop-J5L)
NY_014-090	Mur-094	YKV078	1286	1286	RNA polymerase (RPO147)
NY_014-091	Mur-095	YKV079c	171	171	Tyr/Ser phosphatase, IFN-gamma inhibitor
NY_014-092	Mur-096	YKV080	192	192	Entry-fusion complex essential component (Cop-H2R)
NY_014-093	Mur-097	YKV081c	324	324	IMV heparin binding surface protein
NY_014-094	Mur-098	YKV082c	795	795	RAP94 (RNA pol assoc protein)
NY_014-095	Mur-099	YKV083	199	202	VLTF-4 (late transcription factor 4)
NY_014-096	Mur-100	YKV084	313	313	DNA Topoisomerase type I

Table 1 continued

NY_014	Murmansk	Reference	NY_014 AA Size	Murmansk AA Size	Product
NY_014-097	Mur-101	YKV085	144	144	Crescent membrane and immature virion formation (Cop-H7R)
NY_014-098	Mur-102	YKV086	839	839	mRNA capping enzyme large subunit
NY_014-099	Mur-103	YKV087c	144	144	Virion Core (Cop-D2L)
NY_014-100	Mur-104	YKV088	233	233	Virion core (Cop-D3R)
NY_014-101	Mur-105	YKV089	218	218	Uracil-DNA glycosylase, DNA polymerase processivity factor
NY_014-102	Mur-106	YKV090	788	788	NTPase, DNA primase
NY_014-103	Mur-107	YKV092	636	636	Morph, VETF-s (early transcription factor small)
NY_014-104	Mur-108	YKV093	161	161	RNA polymerase (RPO18)
NY_014-105	Mur-109	YKV094c	304	305	Carbonic anhydrase, GAG-binding IMV membrane protein
NY_014-106	Mur-110	YKV095	209	209	mRNA decapping enzyme (Cop-D10R)
NY_014-107	Mur-111	YKV096	251	251	mRNA decapping enzyme (Cop-D9R)
NY_014-108	Mur-112	YKV097c	632	632	ATPase, NPH1
NY_014-109	Mur-113	YKV099c	287	287	mRNA capping enzyme small subunit
NY_014-110	Mur-114	YKV100c	549	549	Trimeric virion coat protein (rifampicin res)
NY_014-111	Mur-115	YKV101c	150	150	VLTF-2 (late transcription factor 2)
NY_014-112	Mur-116	YKV102c	224	224	VLTF-3 (late transcription factor 3)
NY_014-113	Mur-117	YKV103c	75	75	S–S bond formation pathway protein (Cop-A2.5L)
NY_014-114	Mur-118	YKV104c	642	641	P4b precursor
NY_014-115	Mur-119	YKV105c	309	309	39 kDa virion core protein (Cop-A4L)
NY_014-116	Mur-120	YKV106	164	165	RNA polymerase (RPO19)
NY_014-117	Mur-121	YKV107c	372	372	Virion morphogenesis, core protein (Cop-A6L)
NY_014-118	Mur-122	YKV108c	719	719	VETF-L (early transcription factor large)
NY_014-119	Mur-123	YKV110	289	289	VITF-3 34kda subunit (Cop-A8R)
NY_014-120	Mur-124	YKV111c	88	104	Viral membrane associated, early morphogenesis (Cop-A9L)
NY_014-121	Mur-125	YKV112c	904	903	P4a precursor
NY_014-122	Mur-126	YKV114	316	316	Viral membrane formation (Cop-A11R)
NY_014-123	Mur-127	YKV115c	181	180	Virion core and cleavage processing protein (Cop-A12L)
NY_014-124	Mur-128	YKV116c	62	62	IMV membrane protein, virion maturation (Cop-A13L)
NY_014-125	Mur-129	YKV117c	91	91	Essential IMV membrane protein (Cop-A14L)
NY_014-126	Mur-130	YKV118c	53	53	Non-essential IMV membrane protein (Cop-A14.5L)
NY_014-127	Mur-131	YKV119c	94	94	Core protein (Cop-A15L)

Table 1 continued

NY_014	Murmansk	Reference	NY_014 AA Size	Murmansk AA Size	Product
NY_014-128	Mur-132	YKV120c	373	373	Myristylprotein, essential for entry/fusion (Cop-A16L)
NY_014-129	Mur-133	YKV121c	208	210	IMV membrane protein (Cop-A17L)
NY_014-130	Mur-134	YKV122	481	481	DNA Helicase, transcript release factor
NY_014-131	Mur-135	YKV123c	73	74	Zinc finger-like protein (Cop-A19L)
NY_014-132	Mur-136	YKV124c	116	116	IMV membrane, entry/fusion complex component (Cop-A21L)
NY_014-133	Mur-137	YKV125	426	426	DNA polymerase processivity factor
NY_014-134	Mur-138	YKV126	187	187	Holliday junction resolvase
NY_014-135	Mur-139	YKV127	382	382	VITF-3 45kda subunit (Cop-A23R)
NY_014-136	Mur-140	YKV128	1166	1166	RNA polymerase (RPO132)
NY_014-137	Mur-141	YKV129c	1155	1199	A type inclusion protein (CPXV)
NY_014-138	Mur-142	YKV130c	515	516	P4c precursor
NY_014-139	Mur-143	YKV131c	117	119	IMV surface protein, fusion protein (Cop-A27L)
NY_014-140	Mur-144	YKV132c	146	145	IMV MP/Virus entry (Cop-A28L)
NY_014-141	Mur-145	YKV133c	303	303	RNA polymerase (RPO35)
NY_014-142	Mur-146	YKV134c	74	75	IMV protein (Cop-A30L)
NY_014-143	Mur-147	YKV135c	46	45	Hypothetical protein
NY_014-144	Mur-148	YKV136	135	143	Unknown (Cop-A31R)
NY_014-145	Mur-149	YKV137c	270	278	ATPase/DNA packaging protein
NY_014-146	Mur-150	YKV139	185	185	EEV membrane, C-type lectin-like domain (Cop-A33R)
NY_014-147	Mur-151	YKV140	169	169	C-type lectin-like IEV/EEV glycoprotein (Cop-A34R)
NY_014-148	Mur-152	YKV141	175	175	MHC class II antigen presentation inhibitor (Cop-A35R)
NY_014-149	Mur-153	YKV142	220	234	IEV transmembrane phosphoprotein (Cop-A36R)
NY_014-150	Mur-154	YKV143	263	261	Unknown (Cop-A37R)
NY_014-151	Mur-155	YKV144c	275	274	CD47-like, integral membrane protein
	Mur-156	CPXV176		389	Semaphorin
	Mur-157	CPXV178[a]		231	Chemokine binding protein
NY_014-152	Mur-158	YKV145	133	133	Profilin-like protein, ATI-localized (Cop-A42R)
NY_014-153	Mur-159	YKV146	190	191	Type I membrane glycoprotein
NY_014-154	Mur-160	YKV147	62	67	Hypothetical protein
NY_014-155	Mur-161	YKV148	300	301	2,3-sialyltransferase
NY_014-156	Mur-162	YKV149c	347	347	3 beta-hydroxysteroid dehydrogenase/delta 5- > 4 isomerase
NY_014-157	Mur-163	YKV150	108	108	Inactive Cu–Zn superoxide dismutase-like virion protein
NY_014-158	Mur-164	CPXV184	227	227	IL-1/TLR signaling inhibitor
NY_014-159	Mur-165	YKV151c	289	291	Immunoprevalent protein (Cop-A47L)

Table 1 continued

NY_014	Murmansk	Reference	NY_014 AA Size	Murmansk AA Size	Product
NY_014-160	Mur-166	YKV152	205	205	Thymidylate kinase
NY_014-161	Mur-167	YKV153	159	159	Putative Phosphotransferase/anion transport (Cop-A49R)
NY_014-162	Mur-168	YKV154	550	550	ATP-dependent DNA ligase
NY_014-163	Mur-169	YKV155	333	333	Unknown (Cop-A51R)
NY_014-164	Mur-170	YKV156	185	185	Toll/IL-1 receptor-like, IL-1, NFkB signal inhibitor (Cop-A52R)
NY_014-165	Mur-171	RCNV-Herman-171	179	179	Hypothetical protein
NY_014-166	Mur-172	YKV157	402	404	Ornithine decarboxylase
NY_014-167	Mur-173	YKV158	564	565	BTB Kelch-domain containing protein (Cop-A55R)
NY_014-168	Mur-174	CPXV195	197	197	Guanylate kinase
NY_014-169	Mur-175	YKV159	302	302	Ser/Thr Kinase (Cop-B1R)
NY_014-170	Mur-176	YKV008[a]	247	247	Zinc Finger-Like Protein
NY_014-171	Mur-177	YKV160	193	193	Schlafen (Cop-B2R)
NY_014-172	Mur-178	YKV161	319	320	EEV type-1 membrane glycoprotein, protective antigen
NY_014-173	Mur-179		235	234	IL-1 receptor, type 2
NY_014-174	Mur-180	YKV162	183	186	Ankyrin-like protein (Cop-B6R)
NY_014-175	Mur-181	YKV163	270	262	Soluble interferon-gamma receptor-like protein
NY_014-176	Mur-182	YKV185[a]	217	218	Virulence factor (Cop-B9R)
NY_014-177	Mur-183	YKV164	286	287	Ser/Thr Kinase (Cop-B12R)
NY_014-178	Mur-184	YKV165	364	339	Serpin 1,2,3
NY_014-179	Mur-185	YKV166	152	152	Unknown (Cop-B22R)
	Mur-186	CPXV209		272	IL-1 beta inhibitor
NY_014-180	Mur-187	CPXV210	335	335	Unknown
NY_014-181	Mur-188	YKV167	357	354	IFN-alpha/beta receptor glycoprotein
	Mur-189	YKV167		342	IFN-alpha/beta receptor glycoprotein
NY_014-182	Mur-190	CPXV213	815	796	Ankyrin (Bang-B18R)
NY_014-183	Mur-191	YKV168	408	569	Kelch-like protein (EV-M-167)
NY_014-184		YKV169	347		Ankyrin/NFkB inhibitor (Cop-K1L)
NY_014-185	Mur-192	YKV170	222	221	NFkB inhibitor (Cop-M2L)
	Mur-193	YKV171		148	Type 1 IFN inhibitor (Cop-C7L)
	Mur-194	YKV172		550	Ankyrin (Cop-C9L)
	Mur-195	CPXV016		783	Ankyrin
NY_014-186	Mur-196	YKV173	337	339	MHC class I protein
NY_014-187	Mur-197	CPXV025[a]	438	438	Ankyrin/Host Range (Bang-D8L)
NY_014-188	Mur-198	YKV174	86	108	Unknown (CPXV-GRI-D13L)
	Mur-199	CPXV014		89	TNF receptor (CrmB)
	Mur-200	CPXV013		568	BTB Kelch-domain containing protein
NY_014-189		YKV175	227		NFkB inhibitor (Cop-M2L)
NY_014-190	Mur-201	YKV003c	124	142	C-type lectin (FPV-V-008)
NY_014-191	Mur-202	YKV177	351	351	Serpin 1,2,3
NY_014-192	Mur-203	CPXV222	148	152	Unknown

Table 1 continued

NY_014	Murmansk	Reference	NY_014 AA Size	Murmansk AA Size	Product
NY_014-193	Mur-204	CPXV220	567	563	Ankyrin
	Mur-205	CPXV221		317	TNF receptor (CrmD)
NY_014-194		CPXV-AUS-028	123		Type 1 IFN inhibitor (Cop-C7L)
NY_014-195		CPXV-AUS-027	183		Unknown
NY_014-196		CPXV-AUS-026	615		Ankyrin
NY_014-197	Mur-206	CPXV-AUS-209	166	166	TNF-alpha receptor (CrmE)

[a] Indicates that paralog listed due to the absence of ortholog. Blanks in Reference column indicates no poxvirus counterpart of gene

constructed using approximately 25 kbp of the most conserved core of the Chordopoxvirus genomes (equivalent to VACV-Cop-A2L to VACV-Cop-A24R). This greatly reduced the number of gaps required for the alignments, which are a common source of errors. The phylogenetic tree is shown in Fig. 1, and Table 1 provides the percent nucleotide identity for the individual genes through the aligned regions. For a frame of reference, NY_014 and Murmansk are more similar (98.2% nt identity) than variola and ectromelia viruses (97.0% nt identity) and almost as similar as variola and camelpox viruses (98.5% nt identity). Predicting dates of divergence for poxviruses is difficult, especially when recombination events are suspected, but it has been estimated that variola and camelpox viruses diverged 3–4000 years ago [18]. Within the core alignment region, for the most part, these novel viruses were syntenic with Yoka poxvirus, their closest relative, and much of this is also in common with the Orthopoxviruses. However, both ends of these genomes are much more variable than the core, not only in the order of genes, but also in their relationship to other genomes (discussed below). This emphasizes that it is essential to construct the viral phylogenetic tree using sequences that have a single common evolutionary history and not with regions that may have been targets of recombination. The variation beyond the left and right extremes of the core is best illustrated by a Dotplot [17] of the Yoka poxvirus and Murmansk genomes (Fig. 2). At the resolution provided by this dotplot, no indels can be observed within the core, but, in fact, numerous small indels are present. It is notable that the phylogenetic tree generated by the 25-kbp core is closely mirrored by a tree built using only the conserved RNA polymerase (RPO147) gene (data not shown), which supports its utility as an indicator of accurate relationships between poxviruses.

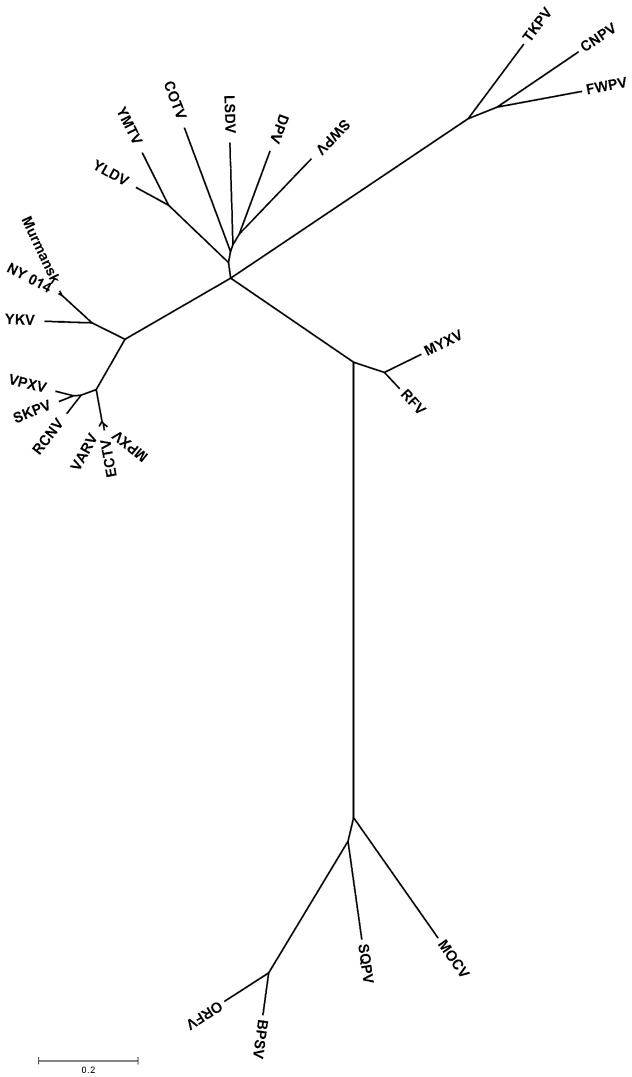

Fig. 1 Maximum likelihood phylogenetic tree of the subfamily *Chordopoxvirinae* constructed using MEGA 7 with a 25-kbp segment of the genome core (VACV-Cop-A2L to VACV-Cop-A24R). All branches except five: MOCV origin branch (67), SWPV origin branch (57), DPV origin branch (72), VACV origin branch (71), and VARV/CMLV/TATV origin branch (49) had bootstrap scores of 95 or higher. These values were generated from 500 bootstrap replicates. The scale bar represents nucleotide substitutions per site

Novel poxvirus genes in the NY_014 and Murmansk genomes

Our annotation of the genomes (Table 1) revealed 5 pairs of orthologs common to these 2 viruses, but absent from

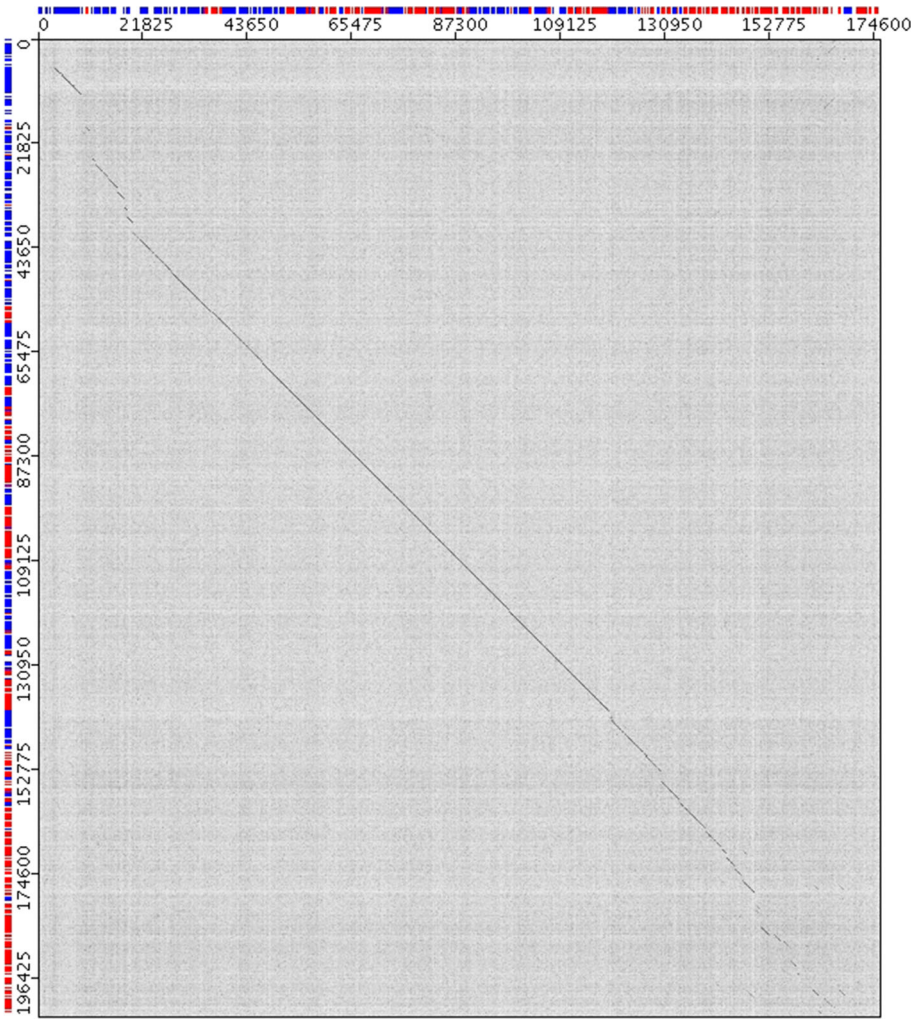

Fig. 2 Dotplot of Murmansk (*vertical*) and Yoka (*horizontal*) poxvirus genomes. Blue and red boxes represent genes transcribed towards the *left* and *right*, respectively

other poxviruses. Murmansk and NY_014 each contain three MHC class I-like homologs (Murmansk-037, -038, and -196 and NY_014-033, 034, and 186), while the Yoka genome contains a single MHC class I homolog (YKV173). All 6 of these poxvirus MHC class I-like proteins are predicted to possess a signal sequence and a C-terminal transmembrane domain and possibly function as MHC class I mimics. The Murmansk-196 and NY_014-186 pair are most similar to YKV173, suggesting that this gene was likely present in an ancestor of all three viruses. The remaining 2 pairs of MHC class I-like homologs present only in Murmansk and NY_014 are more similar to the MHC class I proteins of vertebrates than they are to any poxvirus protein. Each of the poxvirus orthologous protein pairs is >95% aa identical as expected; however, the pairs of paralogs from a single virus are only 36% aa identical. Interestingly, the NY_014-033/Murmansk-037 pair were 54–55% aa identical to rodent MHC class I proteins, whereas the NY_014-034/Murmansk-038 pairs were 37–42% aa identical to the same set of rodent proteins. A comparison of the group of rodent MHC class I proteins revealed them to be 66–80% aa identical among themselves. For the 2 sets of poxvirus genes, proteins from different rodent species were the top matches; however, a "best database match" does not imply these were the hosts from which the poxvirus genes were acquired especially since the top scores were very similar. These results suggest that a poxvirus, ancestral to Murmansk and NY_014, first acquired one MHC class I-like gene and this was subsequently duplicated a long time very much before these two viruses diverged.

BLASTP failed to match the proteins encoded by 2 of the pairs of orthologs (Murmansk-033 and -048) with proteins in the non-redundant protein database leaving these with an "unknown function" annotation. However, the final novel poxvirus gene NY_014-173/Murmansk-179 encodes a product with very low similarity to an IL-1 receptor-like protein, mostly matching to an immunoglobulin-like domain. Although a similarly annotated protein exists in some Capripoxviruses, these share only 21% aa identity to

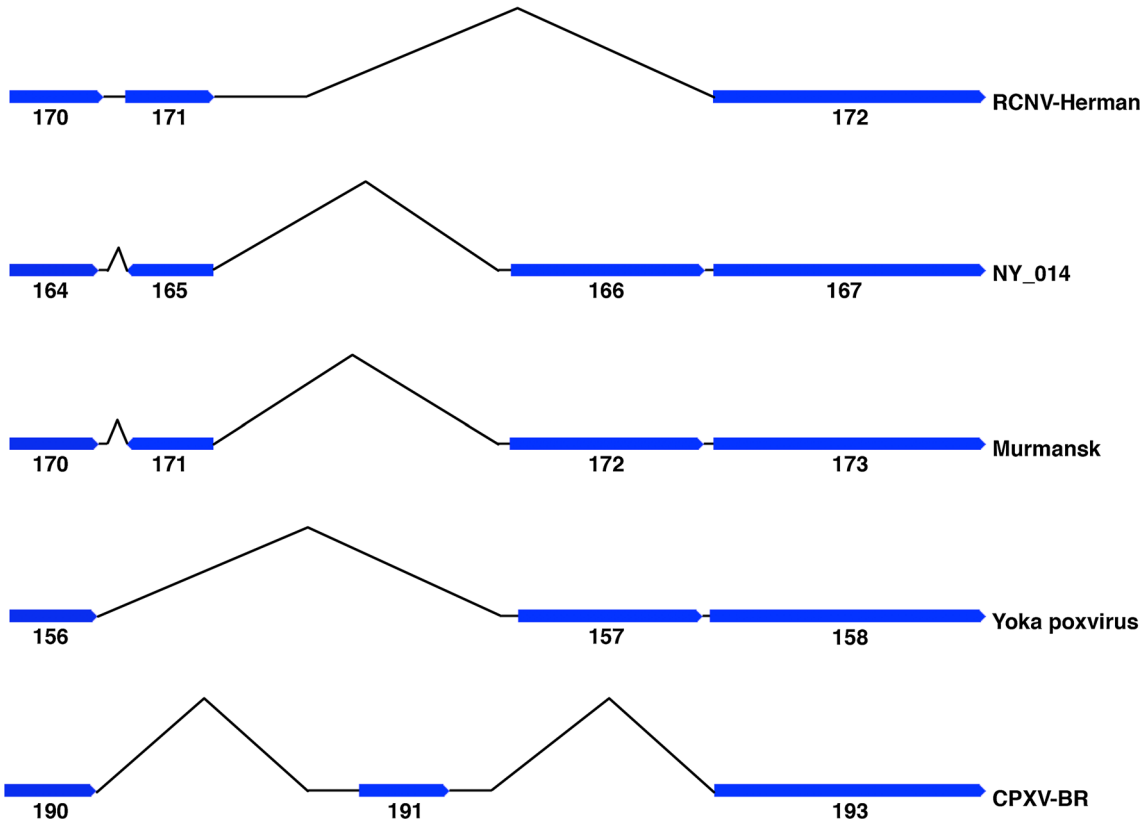

Fig. 3 Organization of the variable region at right end of viral genomes. Direction of gene transcription is illustrated by the *blue arrows* representing the genes

the NY_014- and Murmansk-predicted proteins. Since the host proteins that match these 2 distantly related poxvirus proteins share very few residues, within an immunoglobulin-like domain, it is unlikely that the NY_014 and Murmansk orthologs described here share a common ancestral protein within the poxvirus lineage.

The NY_014-165 and Murmansk-171 genes were found to be orthologous (47% aa identity) to RCNV-Herman-171, which previously was unique to the NAOV. Interestingly, the position of the gene is syntenic with RCNV; however, it is encoded by the opposite DNA strand in NY_014 and Murmansk (Fig. 3). Additionally, in this same region NY_014 and Murmansk genomes possess a gene (NY_014-166 and Murmansk-172), which is immediately to the right of the previously discussed gene, that they share only with Yoka poxvirus (YKV-157).

Unusual features within the terminal regions of the NY_014 and Murmansk genomes

The left and right terminal regions are more variable than the central conserved core of the poxvirus genomes in several ways: (1) the position of orthologous genes, (2) the particular genes present, and (3) the percent nucleotide identity between orthologous genes. Usually, such lower DNA similarity is simply due to the types of proteins encoded by the genes in these regions. For example, comparing the DNA polymerase and RNA polymerase (RPO147) genes shows that the NY_014 and Murmansk orthologs are 98–99% identical (nt), whereas the large surface glycoprotein genes are only 90% identical. Therefore, it was a surprise when we examined the regions from the termini that are present in only one or the other of the NY_014 or Murmansk genomes and discovered that they have very unusual relationship patterns with other poxvirus genomes.

Left terminal region

DNA and protein alignments first revealed that some of the NY_014 proteins from the viral termini were much more similar to RCNV proteins than the conserved core proteins. It was also apparent that these NY_014 proteins were even more similar to RCNV proteins than the Yoka poxvirus orthologs contradicting the pattern of the phylogenetic tree produced with the genome cores (Fig. 1). For example, the NY_014 and Murmansk DNA polymerases are approximately 86 and 83% (aa) identical to the Yoka poxvirus and

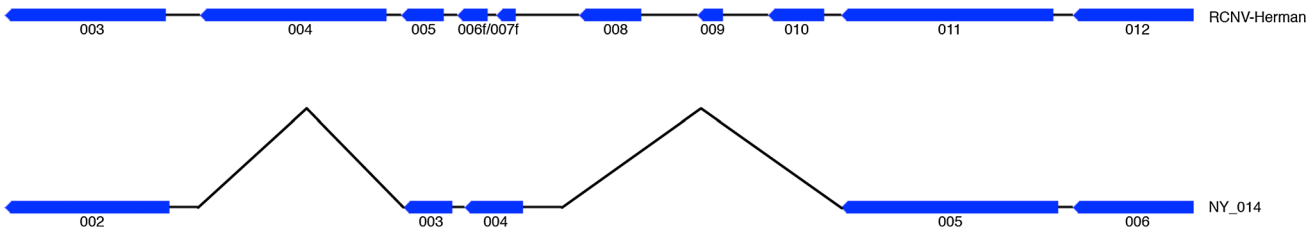

Fig. 4 Alignment of the left terminal regions of the NY_014 (*bottom*) and RCNV-Herman (*top*) genomes. The MAFFT alignment generated 90% nucleotide identity over aligned regions

RCNV orthologs, respectively, whereas the NY_014-005 (encoding an Ankyrin-like protein (Cop-B18R)) has 94% aa identity with the RCNV ortholog. Although this switching between different viruses as "most similar ortholog" appears complex and at odds to a straightforward evolutionary trajectory, it is often simplified when the presence or absence of genes is considered. For example, if genes have been lost from Yoka poxvirus, then the closet match to NY_014 and Murmansk genes is likely to be RCNV. If the genes are also absent from RCNV, then the nearest neighbor most likely becomes another orthopoxvirus. Also since NY_014 and Murmansk are approximately evolutionarily equi-distant from the various orthopoxvirus species, then minor differences between orthologs can result in different species appearing to be the closest relative. However, a closer examination of similarity scores between these orthologs clearly reveals that some genome segments have been exchanged between viruses. Terminal genes are generally quite variable in poxviruses, for example, terminal genes NY_014-020, -021, and -022 have only 62–69% aa identity to their orthologs in Yoka poxvirus. In contrast, orthologs of the genes NY_014-002 to -006 (Fig. 4 and Supplemental Fig. 1), which are absent from the Murmansk and Yoka poxvirus genomes, are 83–96% identical (aa) to corresponding orthologs in RCNV. This high percent identity suggests a recombination event that resulted in an exchange of genes from an RCNV-like virus to an ancestor of NY_014. Using a comparison of these genes to CPXV orthologs as a control indicates that the NY_014 genome acquired the DNA from an RCNV-like ancestor, and not the other way around. For several other genes such as Murmansk-002/003, the closest ortholog is in CPXV (78 and 69% aa identity, respectively). However, given the location of the genes and the predicted rarity of recombination events compared to deletion events, this arrangement may be the result of the previously described recombination followed by loss of genes from RCNV and NY_014 ancestors (Fig. 4 and Supplemental Fig. 1).

Figure 4 illustrates the synteny between the NY_014 and RCNV genomes, but several genes are missing from the NY_014 genome, which could be achieved by 2 deletion events following the introduction of the region into the NY_014 genome. Within this region, one of the genes has also been lost from RCNV, but in this instance gene loss was due to mutation that fragmented the gene to produce RCNV-006f/007f. Both scenarios are consistent with the high variability that is observed for the terminal regions of poxvirus genomes, especially with respect to the generation of indels.

Yet another category of gene organization is represented by Murmansk genes -010 to -021, most of which are also present in NY_014 (Supplemental Fig. 1). The most similar orthologs of these genes are found in CPXV; however, the percent identity is relatively low (28–50% nt identity), suggesting that this region was acquired from a virus not represented by the currently known poxvirus species.

There are 3 pairs of adjacent genes in the Murmansk genome 010/011 (unknown function), 015/016 (IL-18 binding protein), 188/189 (interferon alpha/beta binding protein) that may be the result of gene duplication events (Supplemental Figs. 1 and 2). Although the NY_014 genome contains only one member of each pair that is likely to be functional, an alignment of the genomic sequences suggests that the gene duplications occurred before these 2 viruses diverged. One member of each of the 3 NY_014 pairs has been disrupted in a slightly different manner. The ortholog of Murmansk-010 is mostly deleted and the ortholog of Murmansk-016 has been disrupted by mutations that destroy the start of the coding region. A large deletion in NY_014 has created NY_014-181 from the Murmansk-188 and Murmansk-189 gene pair, generating a fusion of the N-terminal 2/3 of Murmansk-188 and the C-terminal 1/3 of Murmansk-189 (Supplemental Fig. 2). The nt identity between these Murmansk gene pairs is 63, 52, and 59%, respectively. Since the Murmansk proteins 010/011 have only approximately 33% aa identity with a CPXV hypothetical protein, they are unlikely to be orthologs.

Right terminal region

Examination of the right terminal regions of the NY_014 and Murmansk genomes revealed that these too have a complex relationship with each other and the

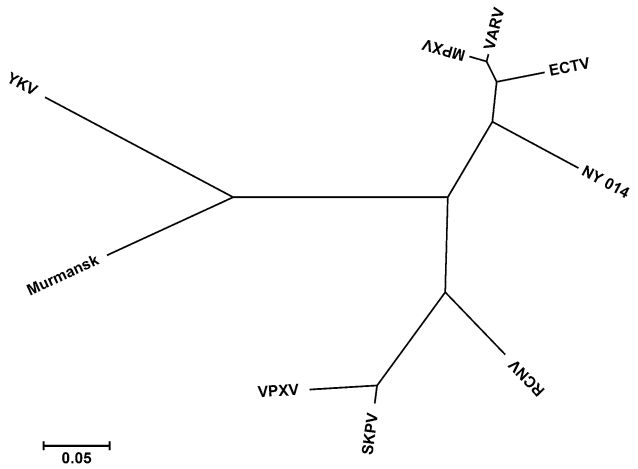

Fig. 5 Maximum likelihood phylogenetic tree of orthologs of NY_014-194 (Type 1 IFN inhibitor) constructed using MEGA 7. The *scale bar* represents nucleotide substitutions per site

orthopoxviruses. Within this region, 8 Murmansk genes are absent from NY_014 and 5 NY_014 genes are absent from Murmansk (Table 1). Furthermore, there are no Yoka poxvirus or RCNV counterparts of several of the NY_014 and Murmansk genes in the right region. This results in the most similar homolog being in the orthopoxvirus group (CPXVs in Table 1). However, the relationship between the genomes is not that simple because although some of the Murmansk and NY_014 proteins are in the order of 40–60% identical to the CPXV homologs, which would be expected for the relationship shown by the phylogenetic tree in Fig. 1, several of the Murmansk and NY_014 proteins are more than 70% identical to the CPXV protein homologs. Similar to the relationship with RCNV in the left terminal region, NY_014 has three genes with high aa identity to CPXV orthologs: NY_014-194 and C7L (Type 1 IFN inhibitor) 93% aa identity, NY_014-195 and C8L (unknown protein) 92% aa identity, and NY_014-196 and C9L (Ankyrin-like protein) 79% aa identity. These three genes are also absent from Murmansk in this region. For example, the C7L-like gene in NY_014 (194) is not syntenic with the C7L orthologs Murmansk-193 and Yoka poxvirus-171, and sequence alignment reveals that a gene syntenic with Yoka/Murmansk poxvirus C7L-like genes may have lost function in NY_014 (Supplemental Fig. 2). Phylogenetic analysis of C7L revealed obvious rearrangement compared to phylogenies created using more conserved genes, supporting the idea of horizontal gene transfer (Fig. 5).

Host range and virulence genes in Murmansk and NY_014

Poxviruses typically encode many genes that are known to target different aspects of the host immune response, and different clades of poxviruses tend to have somewhat distinct repertoires of host range and virulence genes simply by virtue of their evolutionary history. As might be predicted because of their position on the phylogenetic tree, the repertoire of these genes in the genomes of the Murmansk and NY_014 viruses is most similar to the orthopoxviruses, including the presence of orthologs of genes almost exclusively found in the orthopoxvirus genus such as K1L and CrmB-E. However, gene duplication and loss through mutation or out-right deletion have generated complex patterns (presence/absence) for this type of gene in poxviruses. Additionally, since the molecular mechanisms by which most of these proteins function are unknown, it is impossible to gauge the contribution that the different genes make in the different hosts.

Discussion

We have presented the genome sequences of two novel poxviruses that are likely new species within the genus typified by Yoka poxvirus. Five genes, present in each of these viruses, are otherwise unique in poxviruses. Two of the genes encode MHC class I-like proteins and another has low similarity to an IL-1 receptor-like protein. The other 2 unique genes are also likely to encode some sort of virulence protein, adding to the repertoire of processes that the poxviruses, as a family, have acquired to overcome host defense mechanisms. Although much of the genomes of the two viruses presented here show synteny with their closest relative, Yoka poxvirus, a close examination of the similarity of the genomes across the entire length revealed the results of several ancient recombination events. Based on the similarity of these exchanged regions, they appear to have been acquired from an ancestor of the North American Orthopoxvirus RCNV. This finding strengthens the notion that recombination has played an important role in the evolution of the poxviruses [19–22], including variola virus [23].

Poxvirus genomes typically encode many virulence genes that are known to target different aspects of the host immune response. The host range gene repertoire of Murmansk and NY_014 is very typical of what has been reported for orthopoxviruses, but there are a few notable similarities to clade II poxviruses. We observed several differences in host range genes between the individual isolates of the Yoka-Murmansk-NY_014 lineage including the expansion of ANK-containing proteins, the presence of two K1L orthologs in NY_014, the disruption or loss of several host range genes in Yoka poxvirus, and an expansion of TNF receptor-like genes in Murmansk. Similar differences in the host range gene repertoire have been correlated with differences in the host range of other poxviruses (for a recent review, see [24]).

One of the perennial fears regarding poxviruses is the possibility of emergence of a virus capable of creating a new smallpox-like disease. NY_014, presented here, may represent a special risk since it was isolated from an immunocompromised human. Natural processes that could lead to such a catastrophic event include (1) changes to a known zoonotic poxvirus that leads to persistence and rapid spread in humans, (2) introduction of a novel poxvirus to humans from an unknown animal reservoir, and (3) recombination among poxviruses that currently infect animals to create a variant able to infect humans. It is immensely difficult to predict the likelihood of any of these events. However, given that there has been a steady discovery of novel poxviruses and evidence supporting recombination among the various viral genomes, such possibilities are real. Furthermore, the geographic isolation of each of these three viruses on three different continents from an immunocompromised human (NY_014), vole (Murmansk), and mosquito pool (Yoka) only contributes to the question of the natural host and geographical range of these isolates. Further studies on these isolates, as well as collection and identification of more poxviruses, will help us understand their host range/evolution and potential for more serious and widespread zoonotic infections.

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Author contributions HM, ID, and YL conceived of, or designed study; JG, HZ, and DB performed research; CS, CG, and CU analyzed data; and CS, HM, CU, and YL wrote the manuscript.

Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent No human subjects were involved in this study.

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