RESEARCH ARTICLE

Circulation, viral diversity and genomic rearrangement in mpox virus in the Netherlands during the 2022 outbreak and beyond

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Abstract

Mpox is an emerging zoonotic disease which has now spread to over 113 countries as of August 2023, with over 89,500 confirmed human cases. The Netherlands had one of the highest incidence rates in Europe during the peak of the outbreak. In this study, we generated 158 near-complete mpox virus (MPXV) genomes (12.4% of nationwide cases) that were collected throughout the Netherlands from the start of the outbreak in May 2022 to August 2023 to track viral evolution and investigate outbreak dynamics. We detected 14 different viral lineages, suggesting multiple introductions followed by rapid initial spread within the country. The estimated evolutionary rate was relatively high compared to previously described in orthopoxvirus literature, with an estimated 11.58 mutations per year. Genomic rearrangement events occurred at a rate of 0.63% and featured a large deletion event. In addition, based on phylogenetics, we identified multiple potential transmission clusters which could be supported by direct source- and contact

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tracing data. This led to the identification of at least two main transmission locations at the beginning of the outbreak. We conclude that whole genome sequencing of MPXV is essential to enhance our understanding of outbreak dynamics and evolution of a relatively understudied and emerging zoonotic pathogen.

KEYWORDS
evolution, monkeypox, mpox virus, phylogenetics, surveillance, whole genome sequencing

1 | INTRODUCTION

Mpox is a zoonotic disease caused by mpox virus (MPXV) which belongs to the genus *Orthopoxvirus* and was first reported in the Democratic Republic of Congo in 1970.[1] MPXV is a double-stranded DNA virus of approximately 200,000 bp and encodes 195–200 genes.[2] Human MPXV (hMPXV) is enzootic in Central and West Africa and most likely transmitted to humans through direct or indirect contact with rodents. In recent decades, hMPXV infections have been widely associated with zoonotic transmission with short estimated human-to-human transmission chains.[3–5] Despite increasing cases in enzootic regions since its first detection, many unanswered questions remain regarding the epidemiological situation, risk factors and animal reservoirs.[3,6,7]

Since 2018, there have been reports of a few travel-associated mpox cases in the United States of America, the United Kingdom, Singapore and Israel after travel to Nigeria.[4,8] In May 2022, the UK Health Security Agency reported hMPXV in a traveler returning from Nigeria followed by several cases without travel history to known endemic countries.[8,9] Within several weeks, hMPXV infections were detected in 111 countries, including 104 non-enzootic countries leading to declaration of a Public Health Emergency of International Concern by the World Health Organization on July 23, 2022. As of August 2023, more than 89,500 cases have been reported globally.[10] Transmission during the 2022 outbreak was predominantly through sexual activity among men who have sex with men (MSM) in their thirties with primarily single genital, oral mucosal and anal lesions.[7,11] This is in contrast to case descriptions from endemic mpox cases which typically presented with multiple vesiculopustular and papular lesions/rash, affecting the entire body, particularly in the face.[4] Additionally, during the 2022 outbreak, infectious hMPXV could be detected in semen, sparking a debate to classify hMPXV as a sexually transmitted disease.[12,13]

Due to continuous technical advancements and decreasing costs of sequencing techniques, whole genome sequencing (WGS) is increasingly being used as a tool to provide insight into the evolutionary dynamics, transmission and genomic diversity of RNA viruses during local and global outbreaks.[14–16] Additionally, the SARS-CoV-2 pandemic has greatly increased the capacity to perform viral WGS with data sharing platforms and practices.[17] These capabilities have resulted in an efficient global response to sequence hMPXV genomes associated with the 2022 outbreak to track viral evolution.[18] The hMPXV outbreak provides an example of how WGS can be applied to provide insights into using pathogen genomics for a large DNA virus with comparatively low evolutionary rates (compared to, for instance, SARS-CoV-2 or influenza A virus).

Analysis of the first hMPXV outbreak genomes in April 2022 revealed that the strains had evolved from previous cases linked to Nigeria between 2017 - 2019 and belonged to Clade IIb, which is thought to be less virulent compared to Clade I strains. Replication in the human host appears to be ubiquitous, with detection of viral DNA in multiple sample types.[19] The wide host spectrum of MPXV in multiple mammalian species raises concerns that continuous circulation and evolution by genomic rearrangement events could result in the adaptation of MPXV to the human host.[18,20]

The Netherlands reported the first mpox case on the 20th of May 2022 and had one of the highest cumulative incidence rates worldwide per million population during the height of the outbreak.[21] As of the 31st of August 2023, there were a total of 1,266 laboratory-detected mpox cases in the Netherlands.[10] Although mpox cases have decreased towards the end of 2022, with few global mpox detections in early 2023,[10] reports of continuous low-level detection, re-infections and new clusters highlight the possibility of re-emergence and warrant the continuous genomic surveillance of hMPXV.[22,23] In this study, we generated and analyzed 158 near-complete hMPXV genomes (12.4% of nationwide cases) between the 6th of May 2022 and the 15th of August 2023 from six provinces in the Netherlands.

2 | MATERIALS AND METHODS

2.1 | Epidemiological and sample collection data

In the Netherlands, all suspected and confirmed hMPXV infections were notifiable to the regional Public Health Service and subsequently to the National Institute for Public Health and the Environment (RIVM). For this study, clinical patient data and source and contact tracing data were mainly collected by the Municipal Health Services of Amsterdam and Rotterdam in close collaboration with the two sexual health clinics of both cities. Anonymized minimal metadata (sampling date, sex and sample type) were provided to both sequencing centers: the Erasmus Medical Center (EMC) and the Amsterdam University Medical Center (AUMC). Additional anonymized epidemiological data for mpox cases outside of the Amsterdam and Rotterdam region were provided by the RIVM.

2.2 | Sample selection

Samples (pharyngeal swabs, skin lesions and/or anal swabs) were selected for sequencing following detection of hMPXV through real-time PCR.[24–26] Initially, samples with low Ct value (<20) were selected for metagenomic sequencing and when amplicon-based sequencing assays became available, samples with Ct value of <30 were additionally included.[27,28] If multiple hMPXV positive materials were present from a single patient, the sample with the lowest Ct value was selected for sequencing. A random selection of samples from male patients were used for sequencing while all available samples collected from female patients were selected, if within the Ct value margins, as female samples could potentially offer insights into a different patient group (Table S1).

2.3 | Metagenomic nanopore sequencing

For metagenomic sequencing, 110 µL of sample material was centrifuged at 10,000×g for 5 min and 100 µL of the supernatant was treated with 20 U of Turbo DNase (Invitrogen) for 30 min at 37°C. Nucleic acids were isolated using the High Pure Viral Nucleic Acid Kit (Roche). Lysis buffer was used as a negative control. Sequencing libraries were generated using the PCR Barcoding Sequencing Kit (SQK-PBK004) (ONT). A maximum of four metagenomic samples were run per flow cell (FLO-MIN106D, R9.4.1) (ONT). If abnormal read coverage was observed after amplicon sequencing, metagenomic sequencing was performed using the Rapid PCR Barcoding Kit (SQK-RPB004), while increasing the input DNA to 7 ng and amplification cycles to 25.

2.4 | Amplicon-based nanopore sequencing

For WGS, two amplicon-based approaches were utilized, (i) a protocol with an amplicon size of 2,500 bp with 88 primer pairs (doubling the concentration of primer pairs 50 and 57 to increase the coverage depth),[27] and (ii) a protocol with an amplicon size of 375 bp with 682 primer pairs.[28] Amplicon-based libraries were generated using the Ligation Sequencing Kit (SQK-LSK110) (ONT) and Native Barcode Expansion (EXP-NBD196) (ONT), according to the manufacturer's recommendations. A total of 16–20 amplicon-based libraries were run on a flow cell (FLO-MIN106D, R9.4.1) (ONT). For low quality samples (e.g., old samples with multiple freeze thaw cycles or retrospective screening of previously extracted DNA), both amplicon approaches were combined to obtain high quality hMPXV genomes.

2.5 | Genomic data analysis

Sequencing reads were basecalled on a GridION (ONT) with Guppy v6.2.7 (ONT) using the high-accuracy model, a minimal read length of 200 bp and a phred cut-off of 9 using fastp (https://github.com/OpenGene/fastp). Nanopore reads were de-multiplexed with Guppy and trimmed with Porechop v0.2.4 using --require-two-barcodes --discard_unclassified options (https://github.com/rrwick/Porechop). Trimmed reads were mapped to a reference genome from GenBank (ON563414.3) using Minimap2 v2.16 (https://github.com/lh3/minimap2) and primer sequence coordinates were obtained using Geneious v2023.1 (Dotmatics). Consensus sequences were obtained with a coverage cutoff of at least 25× (for amplicon sequencing) and 10× (for metagenomic sequencing) using Samtools v1.7 (https://github.com/samtools/). The sequence depth was obtained using Samtools v1.7. Bam files were visually inspected using UGENE v40.1 for genome coverage patterns. Following any observation of abnormal coverage patterns, de novo assembly using metaFlye v2.9.1 (https://github.com/fenderglass/Flye) and polishing of the assembly using Medaka v1.7.3 (ONT) was applied. Sequence quality checks, mutation calling and clade assignment was performed using NextClade v2.14.1.[29] All hMPXV genomes were uploaded to GISAID (Table S2).

Phylogenetic analysis was performed using Nextstrain CLI v7.2.0 with the Nextstrain MPXV build (https://github.com/nextstrain/mpox) (accessed August 28, 2023) and visualized with auspice[30,31] (Table S3). Nextstrain masks several challenging genomic regions in the MPXV genome that contain homopolymeric and repetitive regions of variable lengths to reduce bias in consensus sequences derived from different workflows and sequencing platforms. Meta-data was used to identify and characterize genetic clusters (≥2 sequences diverging from the B.1 hMPXV basal level by one SNP or more) within the Dutch sequences.[32] Sequences were annotated using Geneious with MT903345 as reference.

2.6 | Ethical statement

The Public Health Service has legal permission, provided by Dutch national public health law (Wet Publieke Gezondheid (WPG) (https://wetten.overheid.nl/BWBR0024705/2021-07-17), to process patient information for surveillance and outbreak analysis of notifiable diseases and can request microbiological laboratories to perform additional testing (including whole genome sequencing) on available clinical materials (article 25 Section 5 WPG). Therefore, separate medical ethical clearance for this study was not legally required. However, all patient information was deidentified before sequencing analysis to ensure anonymity and confirmation of the nonmedical research status was reviewed and confirmed by the Amsterdam University Medical Centre Ethical Committee (reference letter: W22_257 # 22.313, dd July 14, 2022).

2.7 | Data availability statement

All hMPXV sequences of this study (n = 158) have been deposited to GISAID (www.gisaid.org) under the virus names: hMpxV/Netherlands/NH-AUMC-NL001–076/2022 and hMpxV/Netherlands/NH-AUMC-NL001–003/2023; hMpxV/Netherlands/un-EMC-NL001-058; 063-088/2022 (Table S2).

3 | RESULTS

In this study, we generated 158 (near-) complete MPXV genomes (12.4% of nationwide cases) detected between the 6th of May 2022 and the 15th of August 2023 to track circulation, identify genomic changes and monitor viral evolution. The two applied amplicon-based approaches performed comparably and enabled the generation of high quality near-complete MPXV sequences, even for samples with high Ct values previously not feasible for shotgun metagenomic sequencing (Figure S1, Table S4).

3.1 | hMPXV sample cohort and phylogenetic analysis

3.1.1 | hMPXV samples and demographics

Most sequenced cases originated from the provinces of North Holland (Amsterdam; 47%, 75/158) and South Holland (mainly Rotterdam and the Hague; 44%, 70/158), with other cases (8%, 13/158) occurring in the northern and eastern provinces of the Netherlands (Figure 1). The majority of samples were obtained from male patients between 31 and 40 years, typically from anal swabs and skin lesion swabs (Table S1).

3.1.2 | Genomic epidemiology of MPXV

Phylogenetic analysis included an additional 1737 global hMPXV reference sequences and revealed that the majority of the reference outbreak strains originated from the Clade IIb B.1 lineage (Figure 2A, Figure S2). All 158 sequenced genomes from the Netherlands belonged to Clade IIb B.1 lineages, linking all cases in the Netherlands to the global outbreak. Phylogenetic analysis suggests that several of the B.1 lineage clusters were introduced into the Netherlands and then spread locally in the community (Figure 2B). A total of twelve different B.1 sub-lineages were detected consisting of predominantly B.1 (72.2%), followed by B.1.3 (4.4%), B.1.2/B.1.4/B.1.7/B.1.8/B.1.14 (3.2%), B.1.6 (1.9%), B.1.1/B.1.10/B.1.19 (1.3%) and three single detections of B.1.5/B.1.11/C.1 (0.6%) (Figure 2C, Table S5). A total of 54/158 (34.2%) sequenced genomes could not be linked in the phylogenetic tree to any other Dutch sequences.

Phylogenetic analysis indicated (sustained) transmission based on genomic features (Figure 3). A monophyletic group of 22 Dutch sequences (all detected in Amsterdam) within the B.1 lineage was detected (Figure 3A). All sequences shared the SNP: T70477C. The first case sequenced of this cluster was detected in Amsterdam on the 5th of June with the last case detected on the 14th of June. Source and contact tracing data suggested that the majority of these cases visited one of two specific locations in Amsterdam.
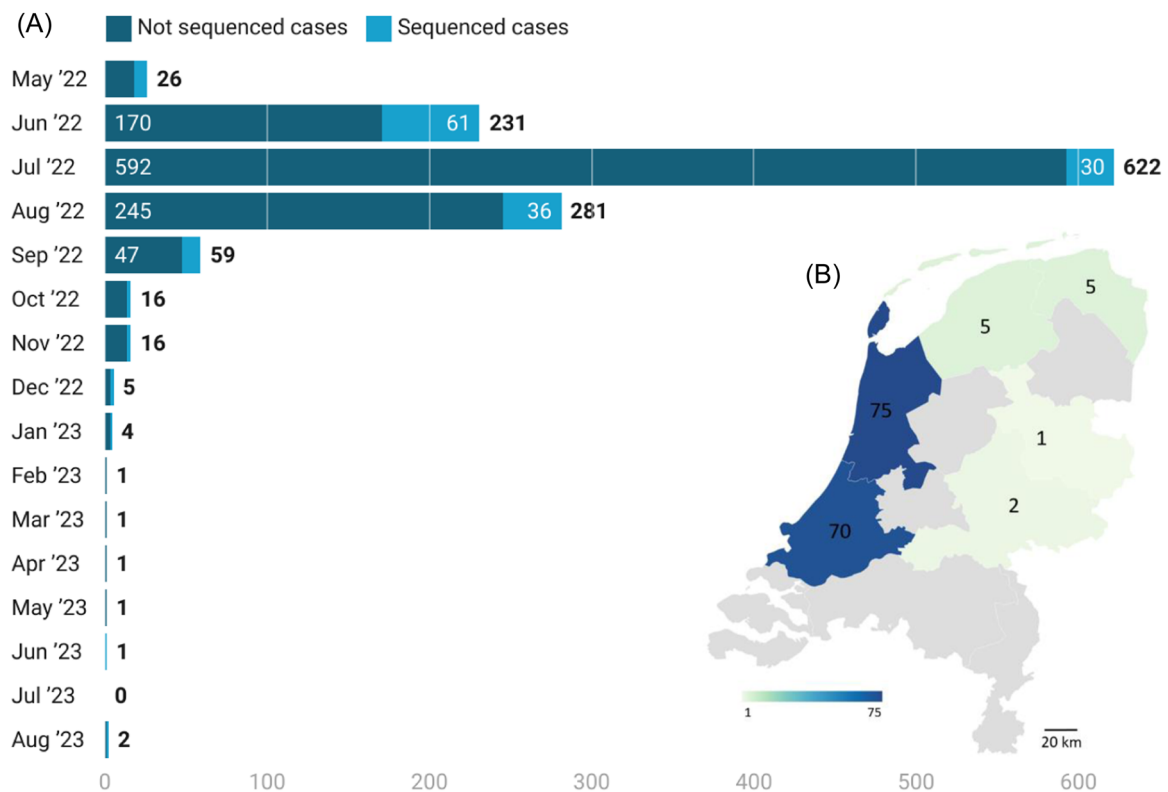

FIGURE 1 (A) The total number of confirmed hMPXV cases (dark blue n = 1266) and sequenced hMPXV genomes (light blue n = 158) per month between May 2022 and August 2023 in the Netherlands. The total case data were retrieved from WHO data (https://github.com/owid/monkeypox). (B) Map of the Netherlands indicating the provinces of the sequenced hMPXV genomes (n = 158) in this study.
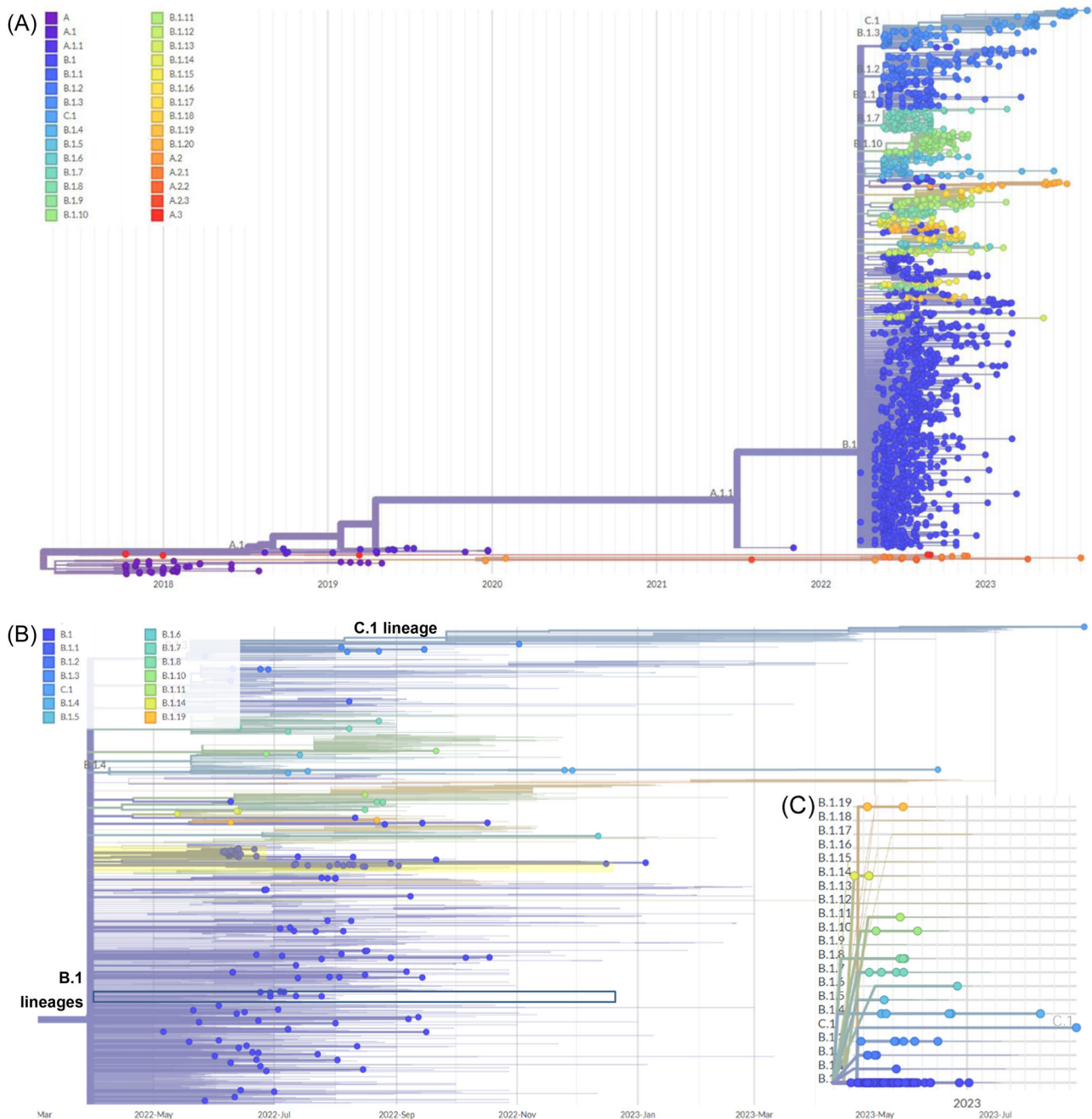
FIGURE 2 (A) Phylogenetic tree showing 1895 global hMPXV genomes between October 2017 and August 2023. (B) Phylogeny tree of 1806 Clade IIb B.1 and C.1 lineages. Dutch sequences (158 genomes) are highlighted by enlarged circles. The yellow boxes highlight two clusters of interest, indicating local transmission within the Netherlands. (C) Scatter plot of Dutch MPXV lineages detected from October 2017 to August 2023.

Two similar sequences were later also detected in Germany at the end of June and July. Another cluster with multiple Dutch sequences was first detected in the Netherlands from a patient visiting a sexual health clinic in Rotterdam with similar sequences later detected in the USA (Figure 3B). A further 10 sequences, obtained from 10 different patients, were subsequently detected in six different Dutch cities in a period of two months. All sequences within this cluster shared the SNP: T70389G (AA mutation OPG089: M175R). This cluster also contained 3 out of the 5 sequenced samples obtained from female patients and a man who

reported having been infected from unprotected sex with an unknown female. This could represent a transmission chain or cluster where hMPXV was not primarily transmitted through MSM contact. There were several additional smaller clusters ($n = 23$) which included less than six sequences.

Molecular clock analysis estimated an evolutionary rate for the entire hMPXV Clade IIb at 11.44 SNPs per year (5.71×10^{-5} substitutions per site per year) (Figure S3A). The evolutionary rate for the global outbreak Clade IIb B.1 and C.1 lineages was 11.46 SNPs per year (5.73×10^{-5} substitutions per site per year with all 1806 B.1 lineages)
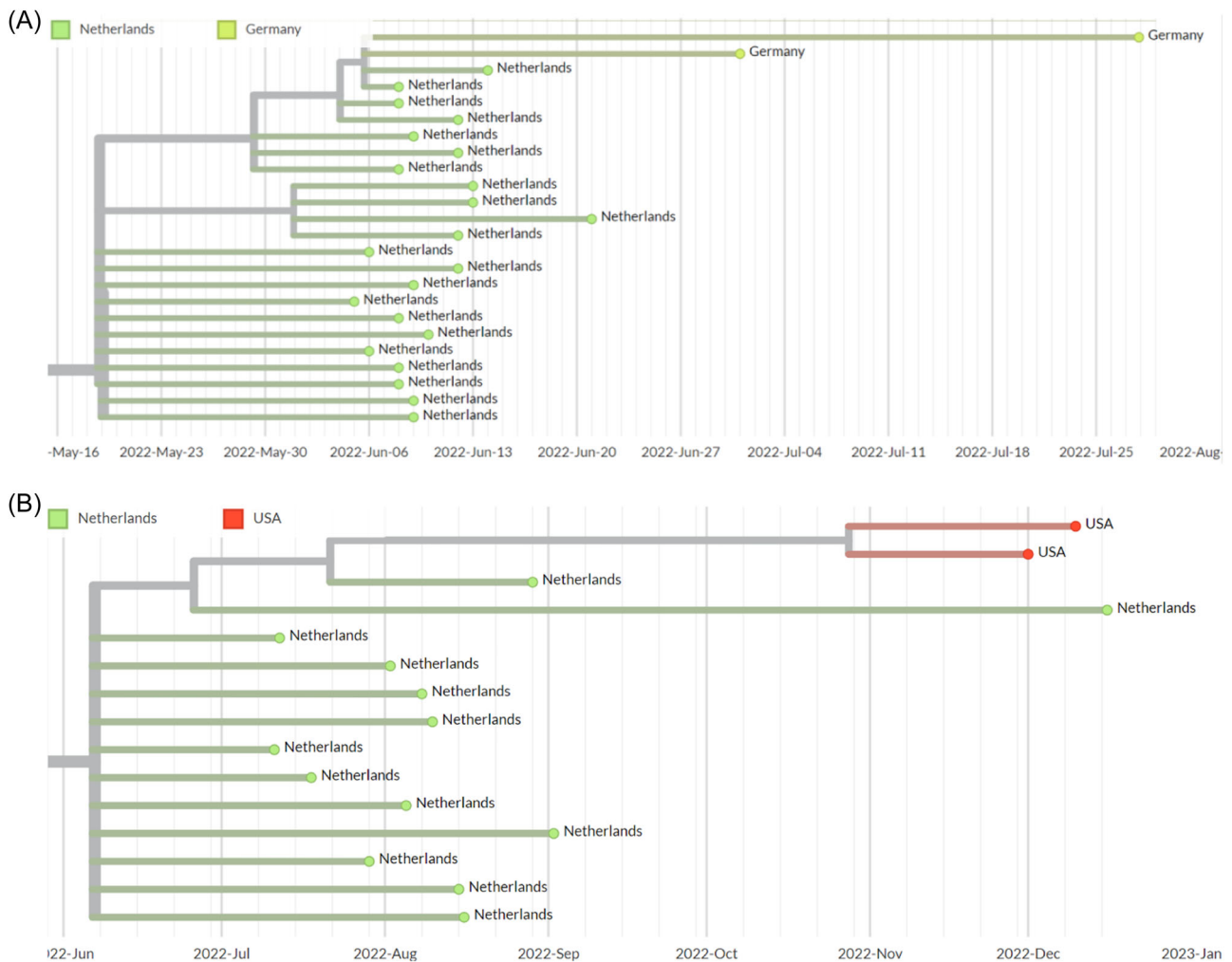
FIGURE 3 Phylogenetic data (time-scaled tree) of Dutch hMPXV sequence clusters. (A) Phylogenetic tree depicting hMPXV transmission in Amsterdam. (B) Phylogenetic tree showing prolonged hMPXV transmission covering multiple cities and three out of five sequences obtained from female patients in the Netherlands.

(Figure S3B) was comparable to the 158 Dutch Clade IIb B.1 and C.1 lineages with 11.58 SNPs per year (6.10×10^{-5} substitutions per site per year) (Figure S3C). The comparable rates of hMPXV sequences from this study (Nanopore) and other available reference sequences (Illumina and Nanopore) indicate comparable consensus sequence accuracy (Figures S3B and S3C). Analysis of the genetic diversity of the entire Clade IIb hMPXV sequences ($n = 1895$) indicated that SNPs occurred across the genome, demonstrating that WGS is essential for monitoring the evolution and spread of hMPXV (Figure S4).

3.2 | Genomic rearrangement

Genomic rearrangement was observed in 1 out of the 158 (0.63%) sequences. The sample originated from a skin lesion (Ct 17.7) of an unvaccinated immunocompetent man in his thirties (MSM) where a gap of 13,542 bp shortly after the 5′-ITR region (6440 – 19,981) was observed in the read mapping (Figure 4A). The gap was flanked by high read coverage plateaus. To determine if the gap resulted from

primer target mismatches, untargeted metagenomic sequencing was performed which confirmed the same gap and included multiple reads spanning the genome region with the insertion and deletion (N50 = 3519 bp with 93.1× average sequence depth) (Figure 4A). Subsequently, metagenomic *de novo* assembly was performed resulting in a 159,992 bp contig that confirmed the major deletion event after the 5′-ITR again. Annotation revealed the deletion of 13 CDS and truncation of one CDS (Table 1, Figure 4B). Furthermore, a 128 bp insertion in the left/right ITR (intergenic region between gp003 and gp004) was detected increasing the size of the ITR to approximately 6,567 bp. This genomic rearrangement resulted in a hMPXV genome size of approximately 183,791 bp.

4 | DISCUSSION

During the 2022 mpox outbreak, the Netherlands reported one of the highest incidence rates in Europe. In this study, we examined the emergence and spread of MPXV in the Netherlands. We applied two
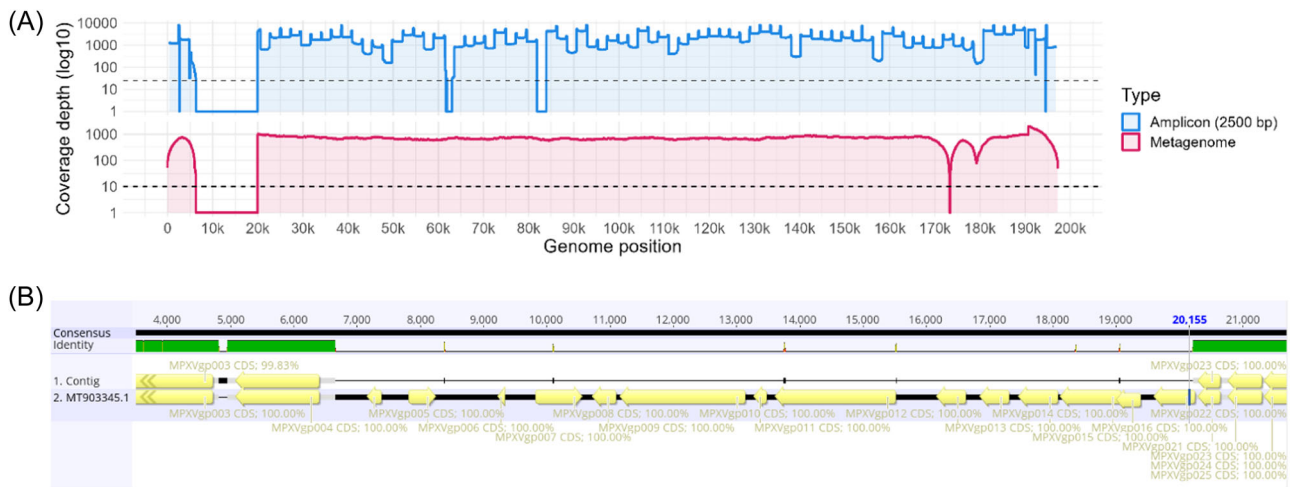
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FIGURE 4 Read mapping and alignment highlighting genomic rearrangement of a hMPXV genome. (A) Mapping coverage pattern of metagenomic (red) and amplicon-based (blue) sequencing approaches indicating the deletion event at position 6,440–19,981. The dotted line indicates the coverage cutoff (10× for metagenomics and 25x for amplicon). Amplicon dropouts of primer pairs 50 and 57 (genome positions ~60k and ~85k) were observed in the initial sequencing runs which resulted in doubling of the primer concentration as a refinement in follow-up runs. (B) Alignment of annotated de novo assembled contig and MPXV reference highlighting a small insertion in the ITR (position 4822–4,949) and the deletion downstream of the left ITR (position 6440–19,981).

TABLE 1 Insertion and large deletion event in a single Dutch hMPXV sequence isolated from a skin lesion.

	Status	Locus tag[a]	Gene	Protein type	Function (predicted)
ITR	Present	Gp001	J1L	Chemokine binding protein	CC-chemokine binding
	Present	Gp002	J2L	Soluble TNF receptor CrmB	
	Present	Gp003	J3L	Ankyrin-like protein	
	Insertion	-		Intergenic region (+128 bp)	
	Present	Gp004	D1L	Ankyrin-like protein	
5′ end/left	Deletion	Gp005	D2L	Ankyrin	Host range protein
	Deletion	Gp006	D3R	Secreted growth factor	ERK2 and NF-kB activation
	Deletion	Gp007	D4L	IL-1 receptor antagonist	Blockade of IL-1 receptors
	Deletion	Gp008	D5R	Zn-finger like protein	Inhibition of apoptosis induced by UV irradiation
	Deletion	Gp009	D6L	Soluble IL-18 binding protein	Binds to IL-18 and inhibits its activity
	Deletion	Gp010	D7L	Ankyrin-like protein	
	Deletion	Gp011	D8L	Ankyrin-like protein	
	Deletion	Gp012	D9L	Ankyrin-like protein	Type I IFN resistance
	Deletion	Gp013	D10L	Type-I IFN inhibitor	Inhibits SMAD9 domain protein
	Deletion	Gp014	D11L	IFN-B inhibitor	Inhibits cellular response to type 1 IFN
	Deletion	Gp015	D12L	BTB kelch-like protein	
	Deletion	Gp016	D13L	IL-1 receptor antagonist	Inhibits NF-kB activation
	Deletion	Gp021	D18L	POZ/BTB kelch-like protein	
	Truncation	Gp022	D19L	TLR signaling inhibitor (603 bp)	
	Present	Gp023	P1L	Antiapoptotic Bcl-2-like protein	Secreted virulence factor

[a]Annotations were transferred from GenBank accession: MT903345.

different amplicon-based approaches, along with direct metagenomic Nanopore sequencing to generate 158 hMPXV sequences from 158 patients (12.4% of nationwide cases) since the beginning of the outbreak in May 2022. We detected 14 different viral lineages, suggesting multiple introductions followed by rapid initial spread within the country. Genomic rearrangement events occurred at a rate of 0.63% with one large scale genomic rearrangement event. In addition, based on phylogenetics, we identified multiple potential transmission clusters which could be supported by direct source- and contact tracing data. This led to the identification of at least two main transmission locations at the beginning of the outbreak.

All sequenced Dutch hMPXV genomes appear to have originated from Clade IIb, lineage B.1. This was observed in almost all other countries, with only a few non-endemic countries, such as Portugal, the United Kingdom, Slovenia and India, reporting cases infected with viruses from other lineages.[20,33] However, these cases were mostly associated with travel to Nigeria.[34] This reinforces the current hypothesis that the circulation of hMPXV in 2022 most likely originated from a single (outbreak) source, which then rapidly spread throughout the global MSM community and international travel.[35]

Currently, the epidemic Clade IIb B.1 lineage has 20 sublineages, each containing one to four unique nucleotide mutations.[18] Additionally, nucleotide changes and mutations that define the clades are spread throughout the MPXV genome, resulting in significant variability. However, this diversity is still relatively limited, and therefore sequencing of the whole genome is required for sufficient phylogenetic signal.[36,37] WGS revealed the presence of 13 different B.1 sub lineages and a C.1 lineage, indicating a diverse set of viral introductions in the Netherlands, likely reflecting the international network of highly active MSM.[38] Over half of the hMPXV detections in the Netherlands originated from Amsterdam, likely because Amsterdam has the unique combination of (i) a large MSM community, (ii) being a major travel and party hub, and (iii) hosting one of the largest testing facilities for sexually transmitted diseases, as part of the national municipal health services centers of sexual health.[39]

Phylogenetic and epidemiological analysis indicated that a large cluster was associated with two specific geographical locations in Amsterdam. Sequences belonging to this cluster were also detected in Germany. This highlights the importance of educational and response efforts, by actively involving the MSM community by communication about safe sex practices in clubs where transmission can occur. In total, we identified nine (larger) clusters based on sequence similarity indicating further spread in the country, despite active campaigns to reduce risk of transmission (e.g., vaccinations, education, social media campaigns, self-isolation and surveillance).

Large-scale genomic rearrangement is an evolutionary mechanism by which poxviruses counteract their relatively low SNP rates.[18] Surprisingly, the initial outbreak strains diverged from related 2017–2019 MPXV from Nigeria by 42 nucleotides, which is 6–12 fold higher than previous estimated mutation rate of 1–2 SNPs per site per year for orthopoxviruses before the global outbreak of MPXV in 2022.[18,20,40] The majority of these changes follow a distinctive pattern

of the human APOBEC3 enzyme activity (TC > TT and GA > AA) likely reflecting prolonged circulation in the human host (Figure S5).[20]

It is hypothesized that current orthopoxviruses have emerged from a common ancestor and evolved through the loss of accessory genes, including host adaptation-related genes.[41] Deleted genes may be associated with the loss of a specific antigenic signal or continuous evolutionary adaptation through the reduction of disease severity.[42] In our cohort, we detected one large-scale genomic rearrangement event: a minor insertion into the inverted repeats and a major deletion event in the flanking region of the left ITR. Deletion events covering parts of the same region have been previously reported, which affect multiple ankyrin or ankyrin-like repeats, similar to our case.[18,43] Furthermore, frequent nonsense mutations have been reported in ankyrin domains previously.[44] As ankyrin has previously been reported to inhibit antiviral signaling within the host (possibly also in other intermediate hosts, such as rodents), it could suggest that during the adaptation of MPXV to the human host, the persistence of some ankyrin or ankyrin-like repeat-containing proteins could have become functionally redundant.[45] The low Ct value (Ct 17.7) obtained in the immunocompetent patient does indicate the virus was viable in our case.

There have been limited reports of genomic rearrangements in the hMPXV Clade IIb genome, with rates in other studies ranging between 1.5% and 3.4%.[18,43,44,46] The impact of deletion events in hMPXV is currently unclear, and to date, there have been no reports of detecting the same genomic rearrangement event twice, indicating that it was not transmitted or advantageous.

Genome rearrangement and SNPs could also result in the loss of current PCR target genes.[18] Gene loss and duplication events occur at a higher rate at the genomic termini, which contain virulence and non-essential genes. Imperfect matching of repeat regions could also explain the high frequency of deletion and duplication events in ITRs.[18,43,44,47] As current real-time PCR assays target genes in the conserved central region of the genome, they are unlikely to be affected by these events.[24–26]

This study identified several challenges. Firstly, linking genomic sequences with epidata was not always possible because (i) MPXV WGS for public health investigation is (currently) not validated for health service providers (such as sexual health clinics, the public health service (GGD) and general practitioners) and the interpretation of the General Data Protection Regulation limited the sharing of metadata, and (ii) the circulation of hMPXV in a specific risk group. This has resulted in increased data protection and stigmatization concerns. As a result, precise locations and other metadata were not linked to sequences in this study to prevent potentially identifying information. Secondly, merging and transferring of MPXV data between different (public) database platforms remains challenging and, as a result, divides datasets for genomic classification and surveillance efforts. Finally, the phenotypical impact of increased mutation rates and putative mutations remains mostly unclear as MPXV replication mechanisms and genome organization is currently poorly understood.

By September 2022, there was a sharp decrease in the number of mpox cases, likely due to temporary behavior change, acquired

immunity (either by infection or vaccination) and education.[48,49] Additionally, there were few reports of significant or sustainable changes in transmission or virulence at the genomic or symptom level.[50] Furthermore, the mode of transmission (close physical contact) could also have played a role in the limited transmission into the general public and among family members, with no reported detections in healthcare workers in the Netherlands. However, at the same time, the detection of lineage A hMPXV viruses in multiple non-endemic countries, the lack of (active) surveillance in many resource-limited countries and increase in reports of MPXV human-to-human transmission since 2016 can be an indicator of undetected circulation.[4,20] Furthermore, other lineages are being detected and small outbreaks have been reported, indicating continuous spillover and subsequent further spread of MPXV.[20,35] Additionally, reports of re-infections and weekly detection of hMPXV more than a year after the initial outbreak are still a reminder of the possibility of re-introduction or upsurges and further human-to-human transmission in non-endemic (and endemic) countries of a historically considered zoonotic disease.[10,20] It is important to learn from outbreaks and enhance our understanding of the epidemiology and evolution of an understudied and emerging zoonotic pathogen.

AUTHOR CONTRIBUTIONS

Study conception and design: Leonard Schuele, Marion Koopmans, Bas Oude Munnink and Matthijs Welkers. *Sample collection, processing and data collection*: Marjan Boter, Leonard Schuele, Hannelore Götz, Ewout Fanoy, Henry de Vries, Bruno Vieyra, Roisin Bavalia, Elske Hoornenborg, Richard Molenkamp, Margarida Simões, Mariken van den Lubben, Matthijs Welkers and Bas Oude Munnink. *Data analysis*: Leonard Schuele, David Nieuwenhuijse, Matthijs Welkers, Bas Oude Munnink. *Amplicon primer design*: Anton van den Ouden, Marcel Jonges and Matthijs Welkers. *Draft manuscript*: Leonard Schuele, Matthijs Welkers, Bas Oude Munnink. All authors have reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

Anton van den Ouden is employed by Molecular Biology Systems B.V. All other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in hMpxV/Netherlands/NH-AUMC; hMpxV/Netherlands/un-EMC at https://gisaid.org/.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.