Journal of Heredity, 2023, **XX**, 1–10
https://doi.org/10.1093/jhered/esad035
Advance access publication 30 May 2023
Genome Resources

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Chromosome-length genome assembly and karyotype of the endangered black-footed ferret (*Mustela nigripes*)

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Corresponding Editor: Taras Oleksyk

Abstract

The black-footed ferret (*Mustela nigripes*) narrowly avoided extinction to become an oft-cited example of the benefits of intensive management, research, and collaboration to save a species through ex situ conservation breeding and reintroduction into its former range. However, the species remains at risk due to possible inbreeding, disease susceptibility, and multiple fertility challenges. Here, we report the de novo genome assembly of a male black-footed ferret generated through a combination of linked-read sequencing, optical mapping, and Hi-C proximity ligation. In addition, we report the karyotype for this species, which was used to anchor and assign chromosome numbers to the chromosome-length scaffolds. The draft assembly was ~2.5 Gb in length, with 95.6% of it anchored to 19 chromosome-length scaffolds, corresponding to the 2n = 38 chromosomes revealed by the karyotype. The assembly has contig and scaffold N50 values of 148.8 kbp and 145.4 Mbp, respectively, and is up to 96% complete based on BUSCO analyses. Annotation of the assembly, including evidence from RNA-seq data, identified 21,406 protein-coding genes and a repeat content of 37.35%. Phylogenomic analyses indicated that the black-footed ferret diverged from the European polecat/domestic ferret lineage 1.6 million yr ago. This assembly will enable research on the conservation genomics of black-footed ferrets and thereby aid in the further restoration of this endangered species.

Key words: annotation, black-footed ferret, conservation, genome assembly, karyotype, *Mustela nigripes*

Introduction

Black-footed ferrets once occupied the vast grassland habitats across the North American Great Plains. However, starting around the time the species was first described based on a specimen collected near Fort Laramie, Wyoming, USA (Audubon and Bachman 1851), much of these grasslands were being converted to croplands and pastures for agriculture and livestock grazing, which continued into the 20th century (Klein Goldewijk et al. 2011). The consequent habitat loss coincided with the extermination of the ferrets' primary prey, prairie dogs (*Cynomys*), which were further decimated by the introduction and eventual spread of the *Yersinia pestis* bacterium

Received March 6, 2023; Accepted May 27, 2023

in 1900, the causative agent of sylvatic plague (Biggins and Godbey 2003; Adjemian et al. 2007). Habitat and prey loss, along with the ferrets' own morbidity due to sylvatic plague, led to a severe decline in their numbers, such that black-footed ferrets were first listed endangered in 1967. They were then presumed extinct in 1979 when the last ferret in the first small captive breeding colony died (Jachowski 2014).

However, in 1981, a single, small population of ferrets was discovered near Meeteetse, Wyoming. Subsequent studies showed that this population numbered ~129 in 1984 but then declined in 1985 to ~58 individuals, likely as a result of an outbreak of canine distemper as well as sylvatic plague (Forrest et al. 1988). To prevent extinction, 18 of the last remaining ferrets were captured between 1985 and 1987 to commence an ex situ conservation breeding program and Black-footed ferret Recovery Plan coordinated by U.S. Fish & Wildlife Service (USFWS) and multiple Association of Zoos & Aquariums (AZA) facilities (Jachowski 2014). An AZA Species Survival Plan (SSP) was also established to implement a minimization of kinship approach using a studbook pedigree to mitigate the loss of genetic diversity and avoid inbreeding. The SSP was initiated by 7 founders from the remaining 18 ferrets from Meeteetse (Marinari and Lynch 2022). Despite the small founder number, more than 10,500 black-footed ferret kits have been produced since 1986, a large proportion of which were reintroduced at 29 sites across the species' historic range, only 14 of which remain active (USFWS Black-footed Ferret Recovery Program 2019). Current census numbers indicate a total of ~650 ferrets now living in captivity (310 as of 2022) or in the wild (~340 as of 2019) (USFWS Black-footed Ferret Recovery Program 2019; Marinari and Lynch 2022).

Previous genetic analyses based on allozymes, microsatellites, and mitochondrial sequences revealed low levels of genetic variation in black-footed ferrets following the founder bottleneck (O'Brien 1989; Wisely et al. 2002, 2008). Data derived from genomic approaches can provide a more comprehensive understanding of where genetic diversity has been lost or retained across the genome and help inform the conservation management of threatened species like the black-footed ferret (Russello and Jensen 2021). Such analyses are facilitated by annotated reference genome assemblies, which empower investigation of other features important for species recovery. These include inbreeding levels as assessed by runs of homozygosity, mutational load that might affect individual and population fitness, the basis for disease susceptibility, and adaptation to captivity (Brandies et al. 2019; Totikov et al. 2021; Formenti et al. 2022; Paez et al. 2022). Furthermore, having a black-footed ferret high-quality reference genome assembly becomes an essential resource in supporting ongoing efforts to enhance the species' recovery and genetic rescue through biotechnology tools such as interspecies somatic cell nuclear transfer and genome editing (Wisely et al. 2015; Adams and Redford 2021).

Here, we report the chromosomal-level assembly and annotation of a reference genome of the black-footed ferret using a combination of linked-read sequencing, optical mapping, and high-throughput chromosome conformation capture (Hi-C). We integrate the assembly with karyotype data to anchor the chromosome-length scaffolds, thereby providing a scaffotype assembly (*sensu* Lewin et al. 2019) assigned to physical chromosomes. We envision that this annotated draft assembly will provide a rich resource for in-depth studies on the conservation genomics of this iconic species.

Methods

Biological material

Veterinary pathologists in the Center for Animal Care Sciences at Smithsonian's National Zoological Park-Conservation Biology Institute collected fresh tissues during necropsy from a male black-footed ferret named Capone (studbook #6536), aged 7.6 yr (Fig. 1A). Capone was born in 2009 in a litter that was the product of artificial insemination using cryobanked semen collected from Scarface (studbook #18) (Howard et al. 2016), one of the original 7 founders of the conservation breeding program (Marinari and Lynch 2022). Collected pieces of tissues from multiple organs were temporarily stored on dry ice, and then cryopreserved in liquid nitrogen until DNA or RNA isolation.

De novo genome sequencing

Tissues were shipped to the Genomic Services Lab at the HudsonAlpha Institute for Biotechnology in Huntsville, Alabama, USA. High molecular weight (HMW) genomic DNA was isolated using the MagAttract HMW DNA Kit (Qiagen, Germantown, Maryland, USA) following the manufacturer's protocol. The gDNA was quantitated using the Qubit dsDNA HS Assay Kit (Invitrogen, Waltham, Massachusetts, USA) and subsequently profiled on a 0.75% pulsed-field agarose gel, run for 17 h using the 7 to 430 kb protocol on a Pippin Pulse (Sage Science Inc., Beverly, Massachusetts, USA). The samples showed the presence of gDNA fragments >60 kb with minimal to no smearing.

Chromium genomic libraries were prepared and purified with the Chromium Genome Reagent Kit version2 and the microfluidic Genome Chip run in a Chromium Controller instrument according to the manufacturer's instructions (10× Genomics, Pleasanton, California, USA). Fragment sizes of the libraries were quantitated using a 2100 Bioanalyzer with the High Sensitivity DNA Assay (Agilent Technologies, Santa Clara, California, USA) and by qPCR with the KAPA Library Quantification Kit (Roche, Indianapolis, Indiana, USA) following the manufacturer's instructions. Libraries were 150 bp paired-end sequenced on an Illumina HiSeq X Ten instrument to a minimum depth of 60×, generating a total of 849,993,416 reads.

Optical mapping

Tissue samples were fixed in formaldehyde, blended with a rotor-stator homogenizer, treated with EtOH, and pelleted at 1,500 × *g* for 5 min. The resulting pellet was resuspended in Bionano Homogenization Buffer (part no. 20278) and added to prewarmed agarose to make 0.8% agarose plugs. gDNA was extracted from the agarose plugs following the Bionano Prep Animal Tissue DNA Isolation Soft Tissue Protocol, revision C (document no. 30077).

UltraHMW DNA was fluorescently tagged at the recognition sequence Nt.BspQI and Nb.BssSI using the Bionano Genomics NLRS kit (catalog no. 80001), following the Bionano Prep Label—NLRS Protocol, revision K (document no. 30024). Samples were loaded on a Saphyr instrument according to manufacturer specifications (document no. 30247), and run until at least 70× effective coverage (total DNA [molecules >150 kbp] × map rate/reference genome size) and at least 100× raw coverage (total DNA [molecules >150 kbp]/genome size) data were collected, as defined by the manufacturer (document no. 30173, Rev. E).

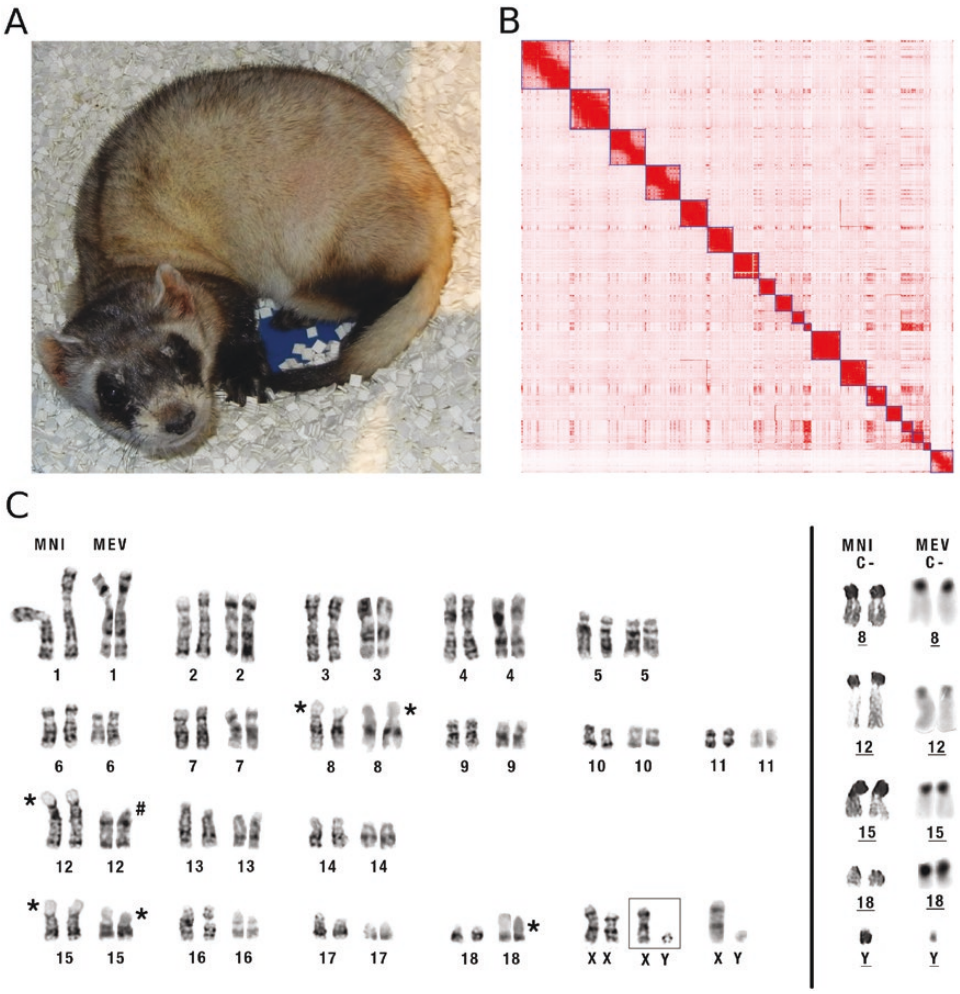

Fig. 1. Genome assembly and karyotype of the black-footed ferret. A) Photograph of Capone (studbook #6536) taken at the Smithsonian's National Zoo & Conservation Biology Institute. B) Hi-C contact density map of the black-footed ferret genome assembly showing 19 chromosome-length scaffolds. C) *To the left*: G-banded karyotype of a wild-caught female black-footed ferret (MNI) "Willa" (studbook #10) aligned with the karyotype of a male steppe polecat, *Mustela eversmanni* (MEV). The X and Y chromosomes of a male black-footed ferret are also shown (black square). *To the right*: C-banding of the chromosomes with additional heterochromatin blocks. The order of G-banded chromosomes follows the nomenclature for mustelids outlined in Graphodatsky et al. (2002). The G-banding for chromosome pairs 6 and 7 depicted in the figure differ from the corresponding karyotypes of *M. nigripes* and *M. eversmanni* in the *Atlas of Mammalian Chromosomes* (Graphodatsky et al. 2020). Asterisk designates the additional heterochromatin blocks forming an entire chromosome arm. The number sign (#) indicates the presence of one more heterochromatin block of smaller size.

Hi-C sequencing

In situ Hi-C library preparation and sequencing were performed following the protocol by (Rao et al. 2014). Briefly, gDNA and proteins in intact nuclei were crosslinked with formaldehyde and the gDNA digested using restriction enzymes. Biotinylated nucleotides were then added to the 5′ end overhangs and the blunt-end fragments ligated. The gDNA was then sheared by sonication and the fragments with biotinylated DNA ligation junctions captured with streptavidin beads. The library was PCR amplified, and 841.4 million of 150 bp paired-end reads were sequenced on an Illumina HiSeq X Ten instrument.

RNA sequencing

RNA sequencing data were generated at the Genomic Services Lab at the HudsonAlpha Institute of Biotechnology from cerebellum, kidney, liver, lung, spleen, and testes tissue samples obtained from black-footed ferret SB#6536. RNA isolation was performed using 350 µl of lysate and the RNAeasy Plus Mini Kit (Qiagen) with DNase digestion. Extracts were quantitated with the Qubit RNA HS and BR Assay Kits (Invitrogen) and evaluated with a HS RNA Fragment Analyzer chip (Agilent Technologies). PolyA RNA-seq libraries were prepared using the TruSeq RNA Library Preparation Kit (Illumina) following the manufacturer's protocol. The libraries were quantitated by qPCR using the KAPA Library Quantification Kit (Roche) following the manufacturer's instructions and then 100 bp paired-end sequenced on an Illumina NovaSeq6000 instrument.

Karyotyping

We generated a karyotype from the cell line of a female black-footed ferret named Willa (studbook #10), which has been stored in liquid nitrogen since 1988 in the FrozenZoo at the Beckman Center for Conservation Research of the San Diego Zoo Wildlife Alliance, USA. Cell culturing, harvesting, and chromosome banding followed the techniques described in Kumamoto et al. (1996). Briefly, metaphase preparations were made from primary fibroblast cell lines grown in minimal essential medium enriched with fetal bovine serum and

antibiotics. Cells were treated with colcemid and hypotonic solution (KCl), followed by fixation with methanol and glacial acetic acid. G-banded chromosomes were produced through trypsin treatment. C-banding followed the method of Sumner (1972) with the modification of 2.5% BaOH and a shortened staining time (20 to 30 min) in Giemsa.

Genome size and assembly

Quality control of reads was performed using FastQC v0.11.9 (Andrews 2010). Distributions of 23-mers after read filtration were counted using Jellyfish v2.2.10 (Marçais and Kingsford 2011) and visualized using KrATER v2.5 (https://github.com/mahajrod/krater) to assess genome size by GenomeScope2 (Ranallo-Benavidez et al. 2020, p. 2) and to check for possible abnormalities. Prior genome size estimation barcodes were trimmed from raw linked reads using the EMA pipeline (Shajii et al. 2017).

Genome assembly was performed in 3 stages. First, raw 10× Genomics linked reads were assembled using Supernova v1 (Weisenfeld et al. 2017) to generate the initial draft. Next, resolution of repetitive regions and scaffolding were performed using the 2-enzyme optical mapping data and Bionano Solve package (Lam et al. 2012). Then Hi-C-scaffolding was performed using Juicer and the 3D-DNA pipeline (Durand et al. 2016; Dudchenko et al. 2017). Finally, the assembly was manually corrected using Juicebox Assembly Tools (Dudchenko et al. 2018). We utilized BUSCO v.5.2.2 (Manni et al. 2021) to evaluate the completeness of the black-footed ferret assembly in relation to the assemblies of 3 other mustelid species (domestic ferret, sea otter, giant otter), using the mammalia_odb10.2021-02-19, laurasiatheria_odb10.2021-02-19, and carnivora_odb10.2021-02-19 single-copy orthologs databases.

Repeat annotation

Dispersed repeats were detected in the assembly using Windowmasker (Morgulis et al. 2006) and RepeatMasker (Smit et al. 2015) with the Carnivora repeat library from RepBase (Jurka 2000; Bao et al. 2015). Tandem repeats were annotated using TRF (Benson 1999) with default options. Bedtools (Quinlan and Hall 2010) was used to softmask detected repeats.

Gene annotation

The annotation of protein-coding genes was performed using a combination of homology-based, transcriptome-based, and de novo predictions. RNA-seq reads from the 6 libraries were aligned to the genome assembly using STAR v2.5.2 (Dobin et al. 2013) followed by extraction of splice site coordinates. Proteins of 3 reference species, *Mustela putorius furo*, *H. sapiens*, and *Canis familiaris*, were aligned to a black-footed ferret assembly using Exonerate v 2.2.4 (Slater and Birney 2005) with the protein2genome model. The obtained alignments were classified into primary and secondary hits; the CDS fragments were cut from each side by 3 bp for the top hits and by 9 bp for secondary hits. Coordinates of these truncated fragments and RNA-seq-based splice sites were used as external evidence for the AUGUSTUS software package v3.2.1 (Stanke et al. 2006) and genes were annotated in a soft-masked assembly. Proteins were extracted from the predicted genes and aligned by HMMER v3.1 (Johnson et al. 2010) and BLAST (Altschul et al. 1990) to Pfam v33.1 (Punta et al. 2012) and Swiss-Prot v2017_8 (The UniProt Consortium 2017) databases, respectively. Only genes supported by hits to protein databases and external evidence were retained. Common gene names were assigned using the EggNOG mapper v1.03 and maNOG database (Huerta-Cepas et al. 2017; Table 1).

Chromosome name assignment

To assign chromosome names to chromosome-scale scaffolds or C-scaffolds (*sensu* Lewin et al. 2019) of the black-footed ferret (*M. nigripes*) and domestic ferret (*M. putorius furo*), we compared whole-genome alignments and cytogenetic data derived from cross-species fluorescent in situ hybridization or ZooFISH. No ZooFISH results have been published for black-footed ferret, however, such data are available for the steppe polecat, *M. eversmannii* (Graphodatsky et al. 2002) and domestic ferret (Cavagna et al. 2000). Steppe polecat and black-footed ferret are closely related (Koepfli et al. 2008; Law et al. 2018), have similar karyotypes (2n = 38), and the karyotype of the black-footed ferret was arranged according to that of the steppe polecat based on G-banding patterns. We first generated a whole-genome alignment between the ferret genomes and the domestic cat genome, *Felis catus* (which was used as reference) using LAST v981 (Frith and Kawaguchi 2015). Next, the chain correspondence between chromosomes of 3 species (domestic cat → American mink [*Neogale vison*] → steppe polecat/black-footed ferret/domestic ferret) was used to identify the correspondence between C-scaffolds and karyotype.

Whole-genome alignment and synteny analysis

To analyze synteny between the genomes of the black-footed ferret and domestic ferret, we generated a whole-genome alignment using Progressive Cactus (Armstrong et al. 2020). Next, synteny blocks were extracted from the whole-genome alignment using halSynteny v2.2 (Krasheninnikova et al. 2020) with the options --minBlockSize 50000 --maxAnchorDistance 50000. Finally, we visualized the results using the draw_synteny.py script from the MACE (https://github.com/mahajrod/mace) package.

Phylogenomic reconstruction

We reconstructed a phylogenetic tree using BUSCO-based gene predictions to unify the quality of the datasets and methodology derived from the assemblies of 15 selected species. These were downloaded from NCBI or www.dnazoo.org: *Panthera leo* (GCA_008795835.1), *F. catus* (GCA_000181335.4), *Canis lupus familiaris* (GCA_000002285.3), *M. putorius furo* (MusPutFur1.0_HiC), *Mustela nigripes* (GCA_022355385.1, this study), *N. vison* (GCA_900108605.1), *Lontra canadensis* (GSC_riverotter_1.0_HiC), *Martes zibellina* (GCA_012583365.1), *Enhydra lutris* (ASM228890v2_HiC), *Mustela nivalis* (GCA_019141155.1), *Ailurus styani* (ASM200746v1_HiC), *Erignathus barbatus* (Erignathus_barbatus_HiC), *Neomonachus schauinslandi* (GCA_002201575.2), *Odobenus rosmarus* (Oros_1.0_HiC), and *Ursus arctos* (ASM358476v1_HiC). Sequences were generated using BUSCO v.5.2.2 (Manni et al. 2021) with the mammalia_odb v.10.2021-02-19 database of 9,226 orthologs. Only single-copy sequences common for all species were included in the analysis. Codon-based sequence alignment was performed separately for each BUSCO using PRANK v.170427 (Löytynoja 2014) followed by filtration of the hypervariable and poorly aligned regions using GBlocks v.0.91b (Castresana 2000; Talavera and Castresana 2007). The processed

Table 1. Tools used in the assembly, annotation, and analysis of the black-footed ferret genome.

Stage of analysis	Software/database	Version	Reference
Data QC	FastQC	0.11	Andrews (2010)
	KrATER	1.2	https://github.com/mahajrod/krater
	Jellyfish	2.2	Marçais and Kingsford (2011)
	EMA	0.6.2	Shajii et al. (2017)
	GenomeScope2	2.0	Ranallo-Benavidez et al. (2020)
Genome assembly	Supernova	1	Weisenfeld et al. (2017)
	Bionano Solve		Lam et al. (2012)
	Juicer	v.2019	Dudchenko et al. (2017) and Durand et al. (2016)
	3D-DNA	v.2019	Dudchenko et al. (2017) and Durand et al. (2016)
	Juicebox Assembly Tools	v.2019	Dudchenko et al. (2018)
Genome assembly QC	BUSCO	5.2.2	Manni et al. (2021)
	OrthoDB*	odb10	Manni et al. (2021)
Repeat detection and masking	RepeatMasker	4.0.7	Smit et al. (2015)
	RepBase*	20181026	Bao et al. (2015) and Jurka (2000)
	Windowmasker	2.9	Morgulis et al. (2006)
	TRF	4.0.9	Benson (1999)
	Bedtools	2.29	Quinlan and Hall (2010)
WGA and visualization	LAST	981	Frith and Kawaguchi (2015)
	ProgressiveCactus	1.0	Armstrong et al. (2020)
	halSynteny	2.2	Krasheninnikova et al. (2020)
	MAVR	0.96	https://github.com/mahajrod/mavr
	MACE	1.1.4	https://github.com/mahajrod/mace
RNA alignment	STAR	2.5.2	Dobin et al. (2013)
Protein alignment	Exonerate	2.2.4	Slater and Birney (2005)
	BLAST	2.9	Altschul et al. (1990)
	HMMER	3.1	Johnson et al. (2010)
Gene prediction	Augustus	3.2.1	Stanke et al. (2006)
	Pfam*	33.1	Punta et al. (2012)
	SwissProt*	2017_8	The UniProt Consortium (2017)
Phylogenetic tree	Prank	170427	Löytynoja (2014)
	Gblocks	0.91	Castresana (2000) and Talavera and Castresana (2007)
	BUSCOclade	0.1	https://github.com/tomarovsky/BuscoClade
	IQ-TREE	2.2.0	Minh et al. (2020)
Dating tree	MCMCtree	4.7	Yang (2007)
	Tracer	1.7	Rambaut et al. (2018)

alignments were then concatenated into a superalignment using a custom script. A maximum likelihood phylogenetic tree was generated using IQ-TREE v.2.2.0 (Minh et al. 2020) with automatic selection of the best-fitting substitution model using ModelFinder (Kalyaanamoorthy et al. 2017). support was evaluated using 1,000 bootstrap replicates. We also quantified genealogical concordance using the site concordance factor based on maximum likelihood (--scfl) for the superalignment (Mo et al. 2022) and the gene concordance factor for the individual gene trees (n = X,XXX) in IQ-TREE v.2.2.0.

For divergence time estimation, we extracted 4-fold degenerate sites from the concatenated sequence alignment and used the MCMCTree tool from the PAML4 package (Yang 2007) with the HKY85+G substitution model and 220,000 MCMC generations, with the first 20,000 generations discarded as burn-in. We used 6 fossil-based priors to calibrate the molecular clock (Supplementary Table S5). The analysis was performed twice according to the recommendation in the MCMCTree manual and no discordance was found between the 2 runs. We verified satisfactory convergence and mixing of the MCMCs for both runs using Tracer v1.7 (Rambaut et al. 2018). All tools and databases used in the study and their versions are listed in Table 1.

Results and discussion

Karyotype

The black-footed ferret karyotype comprises 19 chromosome pairs (2n = 38), including 12 (sub)metacentric, 6 telo/acrocentric autosome pairs and sex chromosomes (Fig. 1C). The diploid chromosome number conforms to the common number found among the Mustelidae and is the same as that found in several close relatives such as the steppe polecat (*Mustela eversmanni*) and the European mink (*M. lutreola*), while differing from the domestic ferret (2n = 40) (Perelman et al. 2012; Graphodatsky et al. 2020). Black-footed ferret

chromosomes are morphologically similar to those of the steppe polecat except for chr12, which is longer in the former species, and chr18, which is longer in the latter species. These differences appear to be due to the presence of heterochromatin blocks that form additional chromosome arms in chr12 and chr18 of the black-footed ferret and steppe polecat, respectively. Furthermore, C-banding (Supplementary Fig. S1) revealed the presence of additional dense (dark) heterochromatin blocks on chr8, chr15, and chrY in both species (right part of Fig. 1C). Telomere-to-telomere assemblies based on long-read sequencing data of these and related species will help elucidate the evolution of these differences, since such assemblies can resolve the sequence of heterochromatin regions (Nurk et al. 2022).

Genome assembly and repeat content

A total of 849,993,416 reads were generated from sequencing the Chromium genomic libraries, with an effective read coverage of 75.4×. The total sequence length of the chromosome-length assembly is 2,498,532,951 bp, which is encompassed in 53,614 contigs and 20,574 scaffolds. The contig and scaffold N50s are 148,763 and 145,433,501 bp, respectively. The assembly size approximates that of other mustelid species such as the domestic ferret (2.4 Gb; Peng et al. 2014), sea otter (2.43 Gb; Jones et al. 2017; Beichman et al. 2019), and wolverine (2.42 Gb; Ekblom et al. 2018).

The chromosome-length assembly of the black-footed ferret includes 19 C-scaffolds with one-to-one correspondence to chromosomes of the karyotype (Fig. 1B, Supplementary Tables S1 and S2). C-scaffolds range between 39.3 Mbp (chr11) and 270 Mbp (chr1) and encompass 2.39 Gbp or 95.6% of the assembly. Total assembly length (~2.5 Gbp) is slightly lower than the genome size estimated from filtered reads (2.79 Gbp, Supplementary Fig. S2). This difference can be explained by the presence of additional heterochromatin blocks which are well known to be an issue for genome assemblies. The completeness of the assembly is similar to other available chromosome-length assemblies of mustelids (Supplementary Table S3). We detected 94.6% to 96.2% complete BUSCOs using the Laurasiatheria_odb or Mammalia_odb databases.

Interspersed repetitive elements comprised 37.35% of the assembly, which is similar to the 36.83% found in the domestic ferret MusPutFur1.0 assembly (Peng et al. 2014). This class of repeats included 20.36% LINEs, 9.8% SINEs, 4.62% Long Terminal Repeats (LTRs), and 2.53% DNA elements. Unclassified repeats constituted 0.03% and tandem repeats made up 1.12% of the assembly.

RNA sequencing and annotation

RNA sequencing generated between 210.3 and 594.2 million reads from the 6 tissue samples (Supplementary Table S4). Using homology-based, RNA-seq-based, and de novo approaches, we predicted 21,406 models of protein-coding genes and 28,268 corresponding proteins. The number of detected coding genes is slightly higher than the 19,910 reported for the initial assembly of the domestic ferret based on similar lines of evidence (Peng et al. 2014). Median support (% of gene elements supported) by external evidence was 50% for exons (CDS), 94.74% for introns and 67.7% for introns + exons (Supplementary Fig. S3). The dramatically higher value for introns implies the crucial role of RNA-seq data for annotation, as in our pipeline homology-based external evidence support CDS predictions only while RNA-seq support intron predictions only, respectively. We detected 91% to 93.2% complete BUSCOs in our annotation (Supplementary Table S3) using the same databases used to assess assembly completeness and successfully assigned common gene names for 18,004 (84.1%) of the gene models (Supplementary File S2). The full annotation of gene models is available in Supplementary File S3.

Synteny

Whole-genome alignment of the black-footed ferret and domestic ferret assemblies revealed a Robertsonian translocation involving chromosomes 17 and 14 in the latter species (Fig. 2A and B), which was previously detected from karyotypic data comparing the domestic ferret and steppe polecat (Graphodatsky et al. 2002) and explains the difference in diploid chromosome number in the 2 species (2n = 38 versus 40). We also detected 3 megabase-scale candidate inversions at the ends of chr5, chr6, and chr17 in the black-footed ferret and dozens of smaller inversions (Fig. 2B, Supplementary File S4). We compared G-banding of corresponding chromosomes, but found no inverted patterns. However, even the largest candidates are relatively small from a cytogenetic perspective and may be located within a single band, making them undetectable.

Phylogenomic analysis

The maximum likelihood phylogenetic tree positioned the black-footed ferret as sister to the domestic ferret along with other Mustelinae species (*Mustela* and *Neogale*) and within the clade containing the sampled mustelid species (Fig. 3). All nodes received 100% bootstrap support out of 1,000 replicates. Gene concordance factors were higher than site concordance factors across all nodes except the one uniting the black-footed ferret and domestic ferret, which were both 86%. Our dating analysis based on 6 fossil calibrations (Supplementary File S5) revealed that black-footed ferret and European polecat/domestic ferret lineages diverged 1.6 Mya (95% confidence interval [CI] 2.4 to 0.9 Mya), followed by a 4.5 Mya (95% CI 6.4 to 2.7 Mya) divergence between the least weasel (*M. nivalis*) and the ferret clade, and a 10.0 Mya (95% CI 11.7 to 7.4 Mya) divergence between the American mink (*N. vison*) and the genus *Mustela*. These divergence times are slightly older compared with previous analyses based on smaller multilocus datasets (Koepfli et al. 2008; Law et al. 2018) but slightly younger relative to analyses based on mitochondrial genome-only data (Hassanin et al. 2021).

Conclusions

We report the first chromosome-length genome assembly for the black-footed ferret. The addition of the karyotype allowed us to anchor chromosomal scaffolds to their respective chromosomes, representing about 96% of the total assembly, thus providing a precise resource enabling in-depth conservation genomic and molecular ecology studies that may aid the ongoing restoration of this well-studied species (Wisely et al. 2015; Totikov et al. 2021). For example, our assembly can be used as a reference for the alignment of whole-genome resequencing data from multiple individuals and the detection of deleterious mutations that may underlie decreased seminal quality due to inbreeding (Santymire

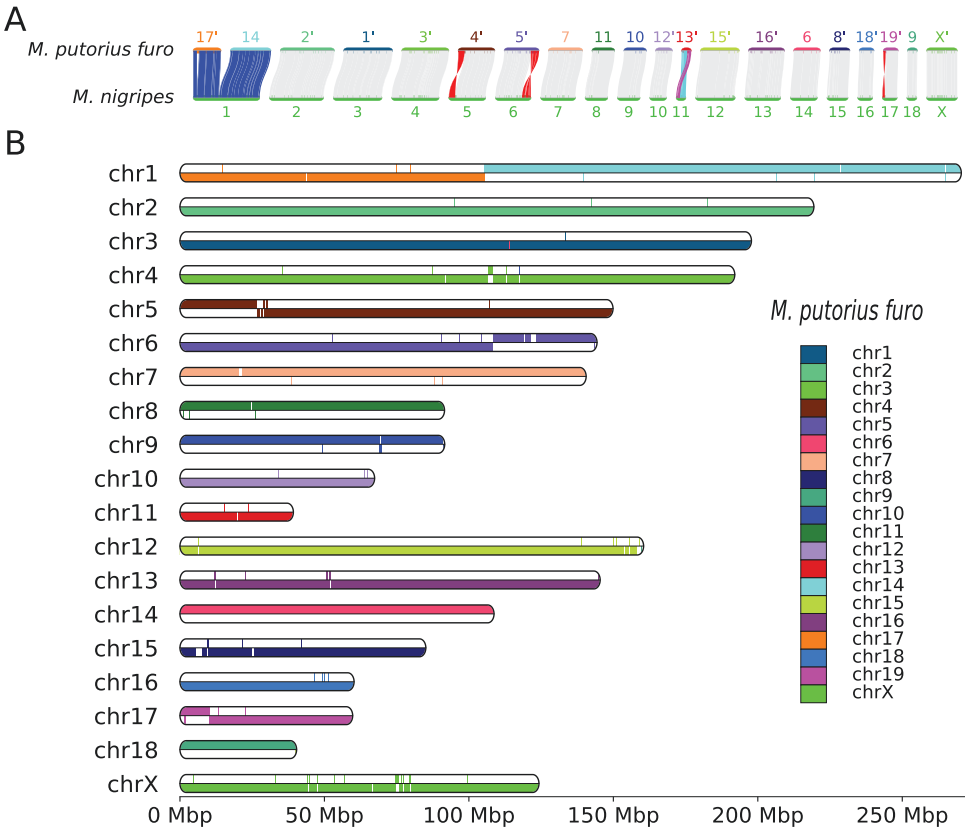

Fig. 2. Synteny between the black-footed ferret and domestic ferret. A) Synteny map between the domestic ferret (*M. putorius furo*) and black-footed ferret (*M. nigripes*) viewed at the macro level. Note that domestic ferret chr1 to chr5, chr8, chr12, chr13, chr15 to chr19, and chrX (labeled with ') were reverse complemented to fit orientation of homologous *M. putorius furo* C-scaffolds. Blue, red, light blue, and magenta colors highlight large-scale chromosome rearrangements between domestic ferret and black-footed ferret. B) Synteny blocks in the genome assemblies of *M. putorius furo* and *M. nigripes* mapped onto the C-scaffolds of *M. nigripes*. Each C-scaffold is split into the top and bottom segment by the line in the middle of the corresponding polygon on the plot. Blocks with the same orientation in both assemblies are in the top segment, with different—below, respectively. Colors of the blocks correspond to the C-scaffold of *M. putorius furo* which they belong to (see legend to the right). Note the large inversions at the ends of *M.nigripes* chr5, chr6, chr17, and multiple small inversion in all C-scaffolds.

et al. 2019) or help understand their high susceptibility to plague relative to their Eurasian congeners (Biggins and Eads 2017). Our assembly might also help elucidate the speciation mechanisms among ferret lineages. We detected 3 candidate megabase-scale inversions between black-footed ferret and domestic ferret (Fig. 2B). Such inversions are known to play a role in at least partial reproductive isolation (Kemppainen et al. 2015; Wellenreuther and Bernatchez 2018) and, therefore, speciation (Cursino et al. 2014). Finally, the contiguity and completeness of our assembly can be improved through the addition of long-read sequencing data. Such data could be used to verify genome rearrangements among species in the Mustelinae lineage, including the candidate inversions mentioned above, as well as provide a foundation for analyses of structural variants and the pangenome of this endangered species (Whibley et al. 2021).

Supplementary material

Supplementary material is available at *Journal of Heredity* online.

Supplementary File S1. Supplementary tables and figures.

Supplementary File S2. Common gene names of the annotated protein-coding genes of the black-footed ferret genome.

Supplementary File S3. Full EggNOG annotations of the protein-coding genes found in the black-footed ferret genome.

Supplementary File S4. Synteny of chromosome locations (start and end sites) between the black-footed ferret and domestic ferret.

Supplementary File S5. Fossil-based calibrations priors used to date the nodes in the phylogenetic tree.

Acknowledgments

We thank Braden Boone, Erin Fledderman, Deanna Fulford, Salina Hall, Angela Jones, John Mote, Nripesh Prasad, Melanie Robinson, Christopher Savell, Geetika Trivedi, and Shawn Levy at HudsonAlpha Discovery, Huntsville, Alabama, for their excellent services in preparing and sequencing the DNA (10× Genomics Chromium) and RNA libraries. The unpublished genome assembly of domestic ferret, North American river otter, sea otter, red panda, bearded seal, walrus, and polar bear was used with permission from the DNA Zoo Consortium (https://www.dnazoo.org/).

Funding

The sequencing and assembly of the black-footed ferret genome were funded by a grant from the Smithsonian Institution

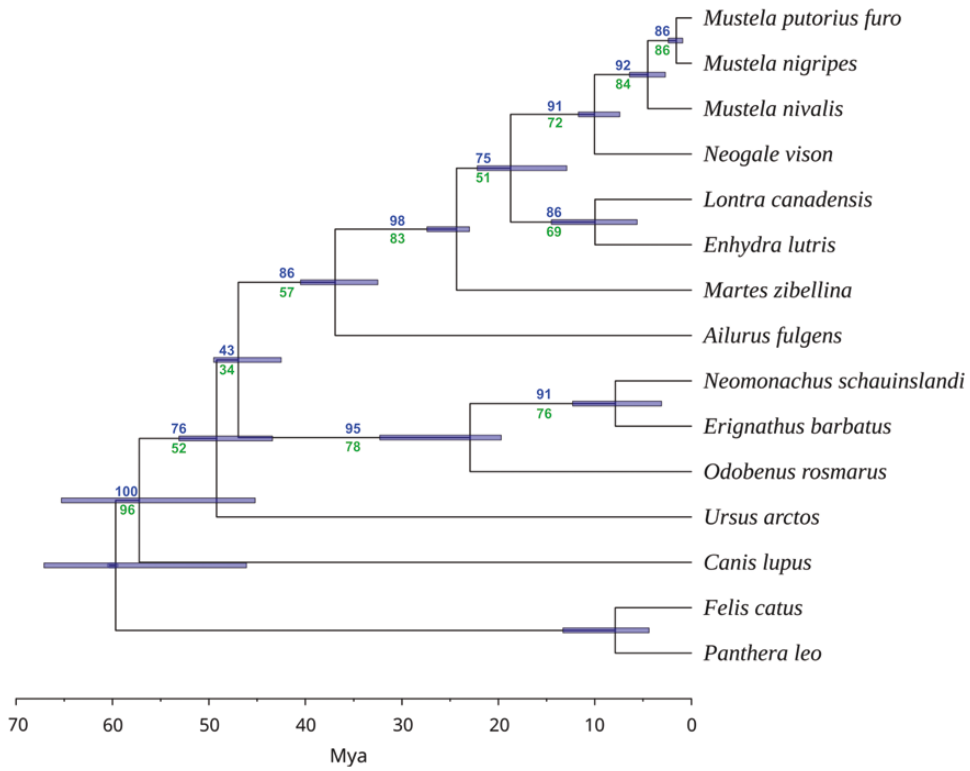

Fig. 3. Timetree among 15 species of Carnivora. Phylogenetic tree showing divergence times among 15 species of Carnivora including *M. nigripes* and *M. putorius furo*. The tree was estimated using MCMCTree with the maximum likelihood topology generated with IQ-TREE v.2.2.0 used as input. All nodes received 100% support based on 1,000 bootstrap replicates. Blue bars indicate 95% CIs for age estimates. The timescale at the bottom corresponds to millions of years ago. Numbers above and below branches correspond to gene concordance factors (blue) and site concordance factors (green), respectively.

Competitive Pell Grants Program in Science awarded to WEJ, K-PK, JEM, PEM, BSP, and DEW. This study was also partly funded by the RSF grant no. 19-14-00034-P. SK was funded from Carlsbergfondet Research Infrastructure Grant CF22-0680. ELA was supported by the Welch Foundation (Q-1866), a McNair Medical Institute Scholar Award, an NIH Encyclopedia of DNA Elements Mapping Center Award (UM1HG009375), a US-Israel Binational Science Foundation Award (2019276), the Behavioral Plasticity Research Institute (NSF DBI-2021795), NSF Physics Frontiers Center Award (NSF PHY-2019745 and PHY-2210291), and an NIH CEGS (RM1HG011016-01A1).

Data availability

The genome assembly, linked reads, and RNA-seq data are available on NCBI from Bioproject PRJNA634921. Corresponding SRA accessions for the DNA and RNA data are listed in Supplementary Table S1. Hi-C reads are available from Bioproject PRJNA512907 under accession SRR8616954.

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