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Chromosomal sex determination of *Fukomys mechowii*

Bachelor thesis

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Annotation

This thesis investigates chromosomal sex determination of *Fukomys mechowii*, aiming to elucidate possible differences from the expected mammalian XX/XY chromosomal sex determination system through PCR amplification of Y-associated genes and bioinformatic determination of X-specific sequences.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 15.08.2024 Signature .....

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Abstract

Mammalian chromosomal sex determination system is often outlined to be XX/XY, however different systems are detected in several rodent species. *Fukomys mechowii* is a rodent species in which sex chromosome-autosome translocations occurred, creating large sex chromosomes comprising ancestrally autosomal sequences, and certain females are indicated to have heteromorphic X chromosomes, possibly suggesting a different chromosomal sex determination system. This might be further supported by certain females producing prevalently female offspring. This investigation aimed to determine whether Y-associated genes could be detected in *F. mechowii* females. Additional aims were detecting X-specific and ancestrally autosomal sequences. In order to screen for Y-associated genes polymerase chain reactions (PCR) were performed and followed by Sanger sequencing of PCR products. Moreover, Illumina sequencing of two males and two females was performed and followed with mapping the reads onto a reference genome. The results indicate that the selected Y-associated genes are not present in females as although PCR would amplify a fragment in females, phylogenetic trees created following Sanger sequencing indicated that fragments of X-homologues, *DDX3X* and *KDM5C*, were amplified. While single, bright bands were observed in *SRY* and *UTY* female screening, PCR product sizes of approximately 800 and 2300 bp compared to the expected 216 and 1922 bp, respectively, suggested they did not result from amplification of *SRY* and *UTY* fragments. Bioinformatic analysis revealed likely X-specific sequences, as well as sequences which might be ancestrally autosomal. Thus, future research might aim to further identify those sequences, the sequence amplified with *SRY* primers in females, as well as to elucidate possible effects of heteromorphic X chromosomes.

List of abbreviations

AMH	Anti-Müllerian hormone
DAX1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DDX3Y/DDX3X	DEAD-box helicase 3 Y-linked/X-linked
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Eif2s3y	Eukaryotic translation initiation factor 2, subunit 3
Fgf9	Fibroblast growth factor 9
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HMG	High-mobility group
HMW	High molecular weight
HSFY2	Heat Shock Transcription Factor Y-Linked 2
KDM5D/KDM5C	Lysine-specific demethylase 5D/5C
mRNA	Messenger ribonucleic acid
NGS	Next-generation sequencing
PAR	Pseudoautosomal region
PCR	Polymerase chain reaction
RBMY	RNA-binding motif, Y chromosome
RPS4Y	Ribosomal protein S4 Y-linked
RSPO1	R-Spondin 1
SOX	Sry-box transcription factor
SRY	Sex-determining region Y gene
TSPY	Testis-specific protein Y-encoded
Uba1y	Ubiquitin-activating enzyme, chromosome Y
USP9Y	Ubiquitin specific peptidase 9 Y-linked
UTY/UTX	Ubiquitously transcribed tetratricopeptide repeat on chromosome Y/X
WNT4	Wingless-type MMTV integration site family, member 4
ZFY	Zinc finger protein Y-linked

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1. Introduction

1.1.Sex determination

A number of different definitions of sex have been outlined, explaining it as any exchange of genetic material between different organisms (Beukeboom & Perrin, 2014), recombination with homologous chromosomes segregation (Agrawal, 2009) or a process involving meiosis (Gorelick & Carpinone, 2009). These definitions have certain limitations such as the implication of horizontal gene transfer being classified as sex, or a disregard for the suppression of crossing-over in certain organisms. Hence, the context determines the utility of a definition (Gorelick & Heng, 2011).

Despite the costs of meiotic sex, it is prevalent in eukaryotic lineages (Beukeboom & Perrin, 2014). It has been associated with DNA repair and the reset of epigenetic markers. Furthermore, while it can prevent the creation of extreme genetic variation with possibly harmful effects (Gorelick & Heng 2011), it enables the recombination of genetic material in sexually reproducing organisms, allowing species to adapt to environmental changes (Seidl & Thomma, 2014).

In a large number of cases, the existence of distinct mating types is a requirement for sexual reproduction (Uyenoyama, 2005). While in such systems this type of reproduction is only possible between different mating types, mating types have isomorphic gametes (Geng et al., 2014), whereas different sexes are characterized by the difference in gamete size – anisogamy (Perrin, 2012). Moreover, in organisms belonging to land plants and metazoans anisogamy is extended into oogamy, which implies the production of sperm and egg cells (Geng et al., 2014).

The modes of sex determination are highly diverse (Pennell et al., 2018). A distinction between environmental and genetic sex determination can be made. Genetic sex determination can be performed through either sex chromosomes or autosomal genes. Certain systems are based on the combination of sex chromosomes, whereas in others, X chromosome to autosome ratio determines sex. Additionally, while certain mechanisms require only a difference at one locus, in others a combination of different genes might be required (Haag & Doty, 2005).

Environmental sex determination can depend on factors such as temperature, nutritional status (Charlesworth, 2002) or population density (Haag & Doty, 2005). Furthermore, transitions between different mechanisms can occur (Schwanz, 2013) and in certain cases both genetic and environmental factors can contribute to the outcome (Piferrer et al., 2005).

1.2.Mammalian sex determination

In mammalian species, genetic factors direct gonad development (Gilbert, 2000). Those factors are often associated with heteromorphic sex chromosomes (Valenzuela & Lance, 2004), X and Y. XX and XY combinations are associated with females and males, respectively (Turner et al., 2004), although other combinations such as XXX, X and XXY are possible as well (Sharifi et al., 2021).

X and Y chromosomes are presumed to have evolved from autosomal chromosomes which ceased to recombine (Cīrulis et al., 2022) in the proximity of newly acquired genes related to sex determination. This loss of recombination allowed for mutations and rearrangements to lead to the creation of morphologically different X and Y chromosomes (Olito & Abbott, 2023).

Y chromosome is often largely heterochromatic, with a high content of repetitive DNA and few genes as the majority of the original genes was lost (Bachtrog, 2013), contributing to a decrease in its size (Liu et al., 2019). Despite this, X and Y continue to recombine in the pseudoautosomal region (PAR), which is stated to display high levels of variability across different species (Helena Mangs & Morris, 2007). Furthermore, apart from PAR, Y chromosomes comprise ampliconic regions, containing large palindromes, and a region containing the remains of the autosome preceding the divergence of the Y chromosome, X-degenerate region (Skaletsky et al., 2003).

Unlike in Y chromosomes, the loss of the majority of the ancestral genes has not occurred in the X chromosome. Furthermore, X chromosomes have a high content of euchromatin and often do not significantly differ in size across mammalian species (Graves, 2006; Charlesworth, 1991 as cited in Liu et al., 2019). Moreover, in order to compensate for the

two copies of X-linked genes in individuals with two X chromosomes, X inactivation occurs, leading to a random inactivation of different X chromosomes in different cells, which will be inherited in all future cells (Turner et al., 2004).

These chromosomes are associated with a number of genes related to sex determination. One such gene is *SRY*, an important testis-determining gene, coding for a peptide with the high-mobility group (HMG) box (Gilbert, 2000), which is conserved across species, while other regions are variable (Whitfield et al., 1993). It specifically binds to the minor groove leading to the bending of DNA (Wilhelm et al., 2007), thus affecting the gene expression. In human Y chromosomes, *SRY* is located on the short arm (Gilbert, 2000), although it could be found on the X chromosome, if a non-PAR recombination between X and Y chromosomes during meiosis was to occur (Wilhelm et al., 2007).

Furthermore, certain genes associated with sex determination, such as *SOX9*, are located on autosomes. *SOX9* is another gene, coding for a transcription factor indicated to play a role in testis development. Unlike *SRY*, which is only found in mammals, *SOX9* is present across vertebrates (Gilbert, 2000), in species with Z and W sex chromosomes, as well as in species with environmental sex determination systems (Wilhelm et al., 2007), suggesting a more recent evolution of *SRY* (Gilbert, 2000).

Additionally, a single gene can both, promote and suppress testis development. *DAX1* is shown to be necessary for testis development as well as *SRY* (Meeks et al., 2003), however, testis development is suppressed if *DAX1* is overexpressed (Swain et al., 1998).

The first gene outlined to promote the development of ovaries in humans is *WNT4* (Biason-Lauber, & Konrad, 2008). It is an autosomal gene (Gilbert, 2000) found across vertebrates (Hollyday et al., 1995; Ungar et al., 1995 as cited in Bernard & Harley, 2007). It contributes to the development of oocytes (Biason-Lauber, & Konrad, 2008) and it has been associated with *DAX1* upregulation (Jordan et al., 2001 as cited in Biason-Lauber, & Konrad, 2008). Furthermore, it leads to paracrine signaling through WNT proteins, which bind to the cell membrane receptors (Biason-Lauber, 2012) and have been outlined to regulate numerous developmental processes (Eggers & Sinclair, 2012).

Moreover, it is suggested to act with *RSPO1*, another gene linked to ovarian development (García-Acero et al., 2020), to stabilize beta catenin, which regulates gene

transcription (Eggers & Sinclair, 2012) and inhibits *SOX9* expression (Cederroth et al., 2007; Schuijers & Clevers, 2012 as cited in García-Acero et al., 2020). This pathway stimulates ovaries to develop from an indifferent gonadal rudiment which had the potential to develop into testes as well (Gilbert, 2000). Although both duct system precursors are initially present (Hake & O'Connor, 2008), Müllerian duct, which is the precursor of the uterus, oviducts and cervix, develops. One of the steroid hormones outlined to play a role in this development is estrogen, however it is shown to be an important factor in Wolffian duct development as well (Gilbert, 2000).

Wolffian duct is the precursor of the epididymis and vas deferens (Gilbert, 2000). If the primordial gonad initiates to develop into testes, it secretes testosterone and anti-Müllerian hormone (AMH) (Hake & O'Connor, 2008). Testosterone is outlined as an important hormone for the development of the Wolffian duct, whereas the presence of dihydrotestosterone affects the external genitalia development. Furthermore, AMH is a glycoprotein leading to the Müllerian duct deterioration (Gilbert, 2000).

The molecular pathway that is associated with this process is related to *SRY* which upregulates *SOX9*. Additionally, murine *Sox9* has been shown to act with *Fgf9*, creating a positive feedback loop which inhibits *Wnt4* and leads to testis development (Eggers & Sinclair, 2012). Although the presence of *SRY* is often outlined as the only factor determining whether testis development is going to occur (Camerino et al., 2006), the activity of other genes can negate or substitute it (Turner et al., 2004).

Furthermore, epigenetic factors have been indicated to play a role in mammalian sex determination as well (Kuroki & Tachibana, 2018). One such factor is DNA methylation, regulating gene expression through the transfer of methyl groups on cytosine (García-Acero et al., 2020) at CpG sequences, leading to gene silencing (Kuroki & Tachibana, 2018). It has been associated with *SRY* expression regulation due to the period of hypermethylation of its promoter region at the time when it is not expressed, followed with hypomethylation in tissues expressing *SRY*, whereas, in tissues without *SRY* expression, methylation levels were not altered (Nishino et al., 2004).

Finally, since the idea of sex is constructed to often encompass different factors such as reproductive organs, hormone levels and chromosome combinations (Morgan & Klein, 2019), it

can therefore be discussed at a number of different levels, including gonadal, genetic and chromosomal (Turner et al., 2004).

1.3. Diversity of mammalian chromosomal sex determination mechanisms

Although XX/XY is often outlined as the mammalian chromosomal sex determination system (Smith & Sinclair, 2004), different systems can be found in certain mammalian species (Kuroiwa, 2022). Moreover, despite the presumed stability of mammalian chromosomal sex determination (Furman et al., 2020), reflected in the term "evolutionary trap" referring to the difficulty of chromosomal sex determination mechanism change in systems with vastly heteromorphic sex chromosomes (Saunders & Veyrunes, 2021), transitions to different systems have been observed (Furman et al., 2020).

Instead of an XX/XY system, stated to have evolved in other mammals following monotreme divergence from them (Rens et al., 2007), monotreme mammals display a system which in males involves five X and five Y chromosomes creating a chain during meiosis in *Ornithorhynchus anatinus* (platypus) (Grützner et al., 2004), while male *Tachyglossus aculeatus* (short-beaked echidna) have a total of nine sex chromosomes, four of which are Y (Rens et al., 2007). Moreover, no *SRY* orthologue has been found in these species (Grützner et al., 2004).

A number of therian mammals, which include marsupials and placentals, have a non-XX/XY chromosomal sex determination system as well (Saunders & Veyrunes, 2021). One such system, involving XY females, was first observed in *Myopus schisticolor* (wood lemming). Two different X chromosomes have been found in *M. schisticolor* females (Liu et al., 2001; Akhverdyan & Fredga, 2001; Fredga et al., 2000 as cited in Saunders & Veyrunes, 2021), which display XX/XX*/X*Y chromosome combinations (Fredga et al., 1976; Herbst et al., 1978 as cited in Saunders & Veyrunes, 2021). Moreover, the presence of the Y chromosome in these females has not been associated with a decrease in fertility, rather, an increase of fertility in X*Y females has been reported (Fredga et al., 2000; Bondrup-Nielsen et al., 1993 as cited in Saunders & Veyrunes, 2021).

Similar systems, which include XY females with no reduction in fertility, have evolved independently in a number of other species as well (Saunders & Veyrunes, 2021).

In addition, in certain mammalian chromosomal sex determination systems Y chromosome has been lost (Saunders & Veyrunes, 2021). A recent loss of the Y chromosome and *SRY* has been registered in two of three species in both, *Tokudaia* and *Ellobius* genera (Just et al. 1995; Kuroiwa et al. 2011; Kimura et al. 2014 as cited in Ezaz et al., 2017), however, translocation of certain Y-associated genes on the X chromosome has been suggested to have had occurred in *T. osimensis* (Ryukyu spiny rat) (Arakawa et al., 2002).

Furthermore, loss of the Y chromosome has occurred in *Microtus oregoni* (creeping vole) which has an X0/XX chromosomal sex determination system, with X0 females. The presence of two X-like chromosomes, one of which becomes silenced, has been associated with males. Moreover, Y-associated genes, including functional *SRY*, have been found in males and females. While the expression of *SRY* and *UTY* is found to be limited to male *M. oregoni*, *DDX3Y* and *KDM5D* are found in female transcriptomes as well. The presence these genes typically associated with the Y chromosome suggests either translocation of these genes onto the X chromosome preceding Y loss or the fusion of X and Y (Couger et al., 2021).

Another species with no *SRY* or the Y chromosome is *Lasiopodomys mandarinus* (mandarin vole). Moreover, a clear link between sex determination and chromosome combination has not been found in this species (Romanenko et al. 2020 as cited in Roy, 2021).

Although a possibility of the presence of a non-XX/XY chromosomal sex determining mechanism has been suggested in certain non-rodent species as well (Saunders & Veyrunes, 2021), each of these systems has been outlined in rodent species (Fredga et al. 1976; Fredga 1983, 1988, 1994 as cited in Veyrunes et al., 2009). Additionally, certain sex chromosome properties and reproductive outcomes of *Fukomys mechowii* (giant mole-rat) (Caspar et al., 2021) might be indicating this species' belonging to such rodent species.

1.4. Fukomys mechowii

F. mechowii had previously been categorized under the *Cryptomys* genus (Allen 1939; Ellerman et al. 1953 as cited in Kawalika & Burda, 2007), however, due to DNA and allozyme differences, *Fukomys* was recognized as a distinct genus (Ingram et al. 2004 as cited in Kock et al., 2006) in the *Bathyergidae* family, encompassing *Cryptomys, Fukomys* and four additional genera (Allard & Honeycutt, 1992; Walton, Nedbal & Honeycutt, 2000; Huchon & Douzery, 2001; Faulkes et al., 1997, 2004; Ingram, Burda & Honeycutt, 2004 as cited in Visser et al., 2019) of subterranean rodents (Faulkes et al., 2010; Ingram, Burda & Honeycutt, 2004; Honeycutt, 2017 as cited in Visser et al., 2019).

F. mechowii is significantly larger in comparison to other species in the genus (Kawalika and Burda 2007; Van Daele et al. 2013 as cited in Caspar et al., 2021). This species inhabits southern regions of central Africa (Maree & Faulkes, 2016 as cited in Caspar et al., 2021) and has been observed in a range of different types of soil (Scharff et al., 2001 as cited in Sichilima et al., 2008a). Furthermore, *F. mechowii* are eusocial (Burda and Kawalika 1993; Kock et al. 2006; Kverkovà et al. 2018 as cited in Caspar et al., 2021), with the colony size ranging from eight to 40 (Burda & Kawalika, 1993; Scharff et al., 2001 as cited in Sichilima et al., 2008a).

Eusocial mammalian species, characterized with philopatry and reproductive division of labor (Burda et al., 2000), are rarely found (Heth et al., 2002), however, *F. mechowii* colonies are of comprised of often only one reproducing pair (Begall et al., 2021), with a larger mean lifespan (Dammann & Burda, 2006; Dammann et al., 2011; Schmidt et al., 2013 as cited in Begall et al., 2021), and non-breeding offspring. However, the fertility of the offspring is retained (Bennett et al. 2000).

1.5. Sex determination of Fukomys mechowii

A number of studies present data which might suggest the absence of the expected mammalian XX/XY chromosomal sex determination system in *F. mechowii*.

Macholan et al. (1993) indicate the presence of unexpectedly large X and Y chromosomes, with X and Y chromosomes representing 11.5% and 9.5% of the haploid set length, respectively. No such phenomenon was observed in other species within the genus, in which the percentage X chromosomes constitute can be approximated to 5%. Additionally, Macholan et al. (1993) report the presence of two heteromorphic X chromosomes, one of which was found to be shorter and metacentric, and one larger and submetacentric. Moreover, a pair of heteromorphic homologous autosomes was found in male *F. mechowii*, whereas the female karyotype displayed no such difference of the autosomal pair in question.

Furthermore, as a result of a number of chromosomal rearrangements, the diploid chromosome number in the *Fukomys* genus ranges from 40 to 78, with the lowest number expressed in *F. mechowii* (Macholan et al. 1993; Nevo et al. 1986 as cited in Deuve et al., 2008). Such chromosomal rearrangements involved X and Y chromosomes in *F. mechowii*, which were found carrying homologous autosomal fragments, resulting from X/Y-autosome translocations (Deuve et al., 2006), which have been associated with the emergence of complex systems of sex chromosomes (Fredga, 1970 as cited in Fredga, 1988)

In addition, the ratio of male to female offspring was found to be 0.54 to 1, in laboratory conditions (Scharff et al., 1999). The reasons for this skewed ratio were suggested to be related to the presence of heterogametic *F. mechowii* females (Burda, 2022). However, this has not been confirmed, nor is the nature of X and Y *F. mechowii* chromosomes and their mode of inheritance further elucidated.

Furthermore, in light of the lack of consideration of the plasticity of sex determination mechanisms (Devlin, & Nagahama, 2002) and research directed at non-XX/XY chromosomal sex determination systems (Saunders & Veyrunes, 2021), investigation of species such as *F. mechowii* could provide important insights in mammalian sex determination.

2. Aims

- Establishing whether genes often present in mammalian Y chromosomes are present in the genomes of *F. mechowii* females

- Identifying *F. mechowii* X-specific sequences and determining which sequences, now found on *F. mechowii* sex chromosomes, correspond to autosomal regions in related species

3. Materials and methods

3.1. Gene selection and primer design

In order to investigate the presence of Y-associated genes in the genomes of female *F. mechowii*, a selection of genes to be investigated was required. Furthermore, in order to test their presence, primer design for PCR (polymerase chain reaction) was necessary for amplification of gene fragments, required for Y-associated gene detection.

Genes associated with Y chromosomes were searched in *Mus musculus* (house mouse), *Rattus norvegicus* (brown rat), *Homo sapiens* (human) and *Fukomys damarensis* (Damaraland mole-rat), with the use of Ensembl genome browser (Martin et al., 2023). These species were selected on the basis of the availability of gene sequences and how closely related a species is to *F. mechowii*. This search yielded a list of 12 genes (Table 1).

Table 1: Outline of the presence/absence of Y-associated genes in four selected species

Species	**Presence of genes**												**Reference**
	SRY	*ZFY*	*RPS4Y*	*TSPY*	*USP9Y*	*DDX3Y*	*UTY*	*KDM5D*	*HSFY2*	*Uba1y*	*RBMY*	*Eif2s3y*	
M. musculus	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Martin et al., 2023
R. norvegicus	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
H. sapiens	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	
F. damarensis	Yes	No	No	No	No	No	Yes	Yes	No	No	No	No	

Four genes (*SRY*, *DDX3Y*, *KDM5D*, *UTY*) were selected due to being the most conserved in terms of their presence in these species (Table 1), thus being possibly present in *F. mechowii* as well. Additionally, an autosomal gene, *GAPDH* (Wutz & Jaenisch, 2000), was selected to be the positive control as it is conserved across mammals (Salazar et al., 2021) and can hence be utilized as a control.

Due to the absence of *F. mechowii* genome assembly from public databases (Martin et al., 2023), the primers were designed from *F. damarensis* sequences. Additionally, since there was no *F. damarensis DDX3Y* sequence in the database, *DDY3Y*-like sequence was used

(National Center for Biotechnology Information, 2004). All sequences were retrieved from the GenBank database (National Center for Biotechnology Information, 1988).

Moreover, multiple sequence alignments were generated with the use of Clustal Omega (Madeira et al., 2022), displaying mRNA sequence comparisons of selected Y-associated genes across 4 different species (*M. musculus*, *R. norvegicus*, *H. sapiens* and *F. damarensis*). *DDX3Y* and *KDM5D F. damarensis* sequences were additionally compared to the sequences of the corresponding X homologues, *DDX3X* and *KDM5C* respectively. However, alignments with *SRY* and *UTY* X homologues were not created, as *F. damarensis UTX* sequence was not available (National Center for Biotechnology Information, 1988) and *SRY* retains only HMG box related sequences with its X homologue, *SOX3* (Araujo et al., 2015).

The alignments were used to identify the sequence regions which are most conserved across different species and highly dissimilar in X and Y homologues in the case of *DDX3Y* and *KDM5D*. This would increase the probability of a sequence being amplified in *F. mechowii* and decrease the probability of amplifying X homologues of *DDX3Y* and *KDM5D*.

The selection of primer sequences was based on these alignments, while considering self-complementarity values as well. In order to generate these primer sequences Primer3 (Kõressaar et al., 2018; Koressaar & Remm, 2007; Untergasser et al., 2012) was used with the selected *F. damarensis* sequence regions.

3.2.DNA extraction

3.2.1. DNA extraction for Y-associated genes screening

Moreover, in order to determine whether Y-associated genes are present in female *F. mechowii* genomes, DNA which would later be used in PCR was extracted from tissue samples of previously deceased *F. mechowii.* The tissue samples were collected from 20 *F. mechowii* individuals, obtained from the animal facility of the Faculty of Science, University of South Bohemia. Following cleaning of the working surface and scissors with 70% ethanol solution, the samples from *F. mechowii* fingers, of approximately 3 mm in length, were

collected into labeled 2 mL Eppendorf tubes and kept on ice. The addition of 700 µL of STE buffer (1 M NaCl, 200 mM Tris-HCl, 100 mM EDTA) and 15 µL of proteinase K (10 mg/ml, Sigma Aldrich – Merck) were followed with an incubation period on the heating block at 55 ºC and 900 rpm for approximately three hours. Every hour, mixing by pipetting up and down was performed. The incubation was ended with the inactivation of proteinase K through an increase in temperature to 70 ºC for the duration of five minutes. The Eppendorf tubes were left on ice for additional five minutes, which was followed with centrifugation at 4 ºC and maximum speed for 10 minutes. First a 1000 µL pipette and later a 10 µL pipettes were used in order to collect and transfer only the supernatant, without the remaining pieces of tissue, into a new 1.5 mL Eppendorf tube with 700 µL of isopropanol, followed by gentle mixing in hand, which causes the DNA to precipitate. This was followed by brief vortexing and centrifugation (maximum speed, 4 ºC for five minutes). The supernatant was discarded and the pellet was washed with 400 µL of 70% ethanol. The centrifugation was repeated (maximum speed, 4 ºC for five minutes). The supernatant was discarded and the Eppendorf tubes were left open for approximately two minutes in order for the DNA to dry. Depending on the pellet size 30 – 300 µL of nuclease-free water were added and left on the heating block at 65 ºC and 900 rpm for five minutes in order to increase the DNA dissolution. 300 µL of nuclease-free water, a volume higher compared to the value suggested in the protocol, were added in cases in which there was no DNA pellet following the vortexing and centrifugation with isopropanol, however the DNA could be distinguished from isopropanol as a lower liquid phase.

3.2.2. DNA extraction from live female samples

In order to investigate whether a difference in the presence of the selected Y-associated genes exists between females which produce predominantly female offspring and those which produce male and female offspring equally, DNA was extracted from hair samples collected from five live females. However, due to the unavailability of females which produce prevalently female offspring, four of the samples were collected from the offspring of such females, whereas the fifth sample was obtained from a control female not associated with a skewed ratio of male and female progeny.

DNA was extracted with the use of NucleoSpin Tissue XS, Micro Kit for DNA from cells and tissue (Macherey-Nagel) following manufacturer's instructions. 80 µL of T1 buffer and 8 µL of proteinase K were added to approximately 10 hairs with hair follicles collected from each female into labeled 1.5 mL Eppendorf tubes. Following vortexing, the hair samples, completely submerged in the solution, were left to be mixed and incubated on a heating block at 56 ºC overnight. The incubation was ended with the addition of 80 µL of buffer B3, vortexing and returning the tubes on a heating block at 70 ºC for five minutes. Following the addition of 80 µL of ethanol and vortexing, the samples were loaded onto the columns in collection tubes. They were centrifuged for one minute and the flow-through was removed. The columns were then washed two times with 50 µL of buffer B5, each application of the buffer followed with a one minute centrifugation step. The columns were then placed in new 1.5 mL Eppendorf tubes and elution was performed with 20 µL of nuclease-free water with one minute centrifugation allowing the DNA solution to be collected.

3.2.3. High molecular weight DNA extraction for Illumina next-generation sequencing

Illumina sequencing was required for detecting X-specific sequences, as well as distinguishing the ones which were ancestrally autosomal. In order to obtain high molecular weight (HMW) DNA to be sent for Illumina sequencing approximately 4 mm x 4 mm liver samples were collected from six previously deceased *F. mechowii* and cut to smaller pieces, which were transferred into labeled 2 mL Eppendorf tubes. Furthermore, DNA extraction was performed with the MagAttract HMW DNA Kit (QIAGEN) according to manufacturer's instructions.

The addition of 220 µL of ATL buffer and 20 µL of proteinase K to the samples was followed with vortexing and incubation at 56 ºC and 900 rpm overnight (approximately 14 hours). Following 3 minute centrifugation at maximum speed and room temperature, 200 µL of the lysates were transferred into new 2 mL Eppendorf tubes. Additionally, 4 µL of RNase A was added, and followed by vortexing and incubation at room temperature for the duration of two minutes. Addition of 150 µL of Buffer AL followed and mixing through pipetting up and

down was performed. Moreover, 280 µL of Buffer MB and 40 µL of pre-vortexed MagAttract Suspension G were added. The tubes were then incubated at room temperature and 1400 rpm. Following three minutes of incubation, the tubes were transferred onto the magnetic base for one minute. This led the magnetic beads to concentrate on one side of the tubes, allowing for the removal of supernatant. The supernatant removal was performed slowly and carefully in order to avoid the disturbance of the magnetic beads. The tubes were removed from the magnetic base, and the addition of 700 µL of the Buffer MW1, incubation at room temperature and 1400 rpm for two minutes and careful supernatant removal with the tubes on the magnetic base were performed two times. Furthermore, the identical procedure was performed two times with 700 µL of the Buffer PE. When the entire volume of the supernatant had been removed for the last time, the beads were washed two more times with 700 µL of nuclease-free water. The nuclease-free water was added when the tubes were on the magnetic base, by pipetting onto the side of the tube with no beads. Finally, the elution was performed with 100 µL of nuclease-free water. Following incubation at room temperature and 1400 rpm for three minutes, the tubes were placed onto the magnetic base allowing for the HMW DNA containing supernatant to be collected without the beads and transferred into new 1.5 mL Eppendorf tubes.

The DNA concentration and purity were measured with the use of NanoDrop Microvolume UV-Vis Spectrophotometer. Furthermore, the samples were stored at – 21 ºC and the procedure was repeated for samples which did not reach the sufficient concentration. Following the repetition, two male and two female samples were sent to be sequenced by Illumina next-generation sequencing (NGS) NextSeq 550 system to company Novogene. Paired-end sequencing was performed generating 150 bp reads.

3.3. PCR and gel electrophoresis

Following the acquisition of DNA solutions through DNA extraction, 1:20 dilutions were prepared with nuclease-free water. Those solutions were used with polymerase mixes and PCR primers (Table 2) in order to create PCR mixes. The amounts of each component are outlined in Table 3, for the Y-associated genes screening PCR, and Table 4, for PCR with high-fidelity polymerase, used to obtain the amplified fragments for Sanger sequencing.

Table 2: PCR primers for the amplification of selected Y-associated genes

Gene	Forward primer (5'->3')	Reverse primer (5'->3')
GAPDH	TGTTCCAGTACGACTCCACC	CTGACGATCTTGAGGGAGT
SRY	GCATTCATGGTGTGGTCTCG	CGTCTTAGTCTTCCGGCGA
DDX3Y	CTTCCTCCAAGTGAACGCTT	GCACTGGAGTAGGACGAGTAT
KDM5D	GGGTCAAGATGGTGGTCGTA	CCAGACTTCTCGGCAATGGG
UTY	GCTTTTGTGCGTGTTGTGTC	CAGAAGTCGTTCAGCACACC

Table 3: PCR mixture used for gene screening

Substance	Volume / µL	Manufacturer
2X AMPIGENE *Taq* Mix	25	Enzo Life Sciences
Forward primer (10 µM)	2	Eurofins Genomics
Reverse primer (10 µM)	2	Eurofins Genomics
Nuclease-free water	21	-

4 µL of the mixture prepared according to the Table 3 would be added to 1 µL of the 1:20 DNA solution (approximately 50 ng DNA) or 1 µL of nuclease-free water in the case of the negative control, which was utilized for each PCR run in order to detect possible DNA contamination of the PCR mixture.

Table 4: PCR mixture used for amplification of fragments for Sanger sequencing

Substance	Volume / µL	Manufacturer
2X Phusion High-Fidelity PCR Master Mix with HF Buffer	10	Thermo Scientific
Forward primer (10 µM)	1	Eurofins Genomics
Reverse primer (10 µM)	1	Eurofins Genomics
1:20 DNA solution/nuclease-free water in the negative control	1	-
Nuclease-free water	7	-

PCR was performed in a Biometra thermal cycler (Analytik Jena), with the parameters outlined in Table 5. The number of cycles, ranging from 30 to 35, and annealing temperature would be adjusted depending on the number and intensity of bands, as well as considering the results from male samples and the positive control.

Table 5: PCR program used for gene screening

PCR stage	Temperature / ºC	Time
Initialization	95	1 min
Denaturation	95	15 s
Annealing *GAPDH*	56-58	15 s
Annealing *SRY*	57-61	15 s
Annealing *DDX3Y*	58-61	15 s
Annealing *KDM5D*	63-64	15 s
Annealing *UTY*	64-65	15 s
Extension	72	2 min
Final extension	72	8 min

Following the completion of the PCR cycles, 1 µL of the Gel Loading Dye, Purple (6X) (New England Biolabs) would be added to 5 µL of the PCR mixtures. They would then be loaded into the wells of a 1% agarose gel immersed in a 1X TAE buffer. For the preparation of those gels 0.7 g of agarose would be dissolved in 70 mL of TAE buffer, followed with heating and addition of ethidium bromide. Approximately 30 minutes were required for the gels to solidify. Following the addition of samples and 3 µL of the Quick-Load 100 bp DNA Ladder (New England Biolabs), gel electrophoresis was performed in order to separate DNA fragments of different sizes. A voltage of 100 V would be applied for approximately 30 minutes. Visualization of amplified DNA fragments was achieved through the use of UV light in Azure 280 (Azure Biosystems).

Positions of the bands were compared to the ladder and the sizes of fragments were recorded and compared to the expected values. In the case of detecting a single band in a female sample, PCR would be repeated with high-fidelity polymerase and parameters outlined in Table 6 in order to generate DNA fragments which would be sent for Sanger sequencing.

Table 6: PCR program used for amplification of fragments for Sanger sequencing

PCR stage	Temperature / °C	Time
Initialization	98	30 s
Denaturation	98	30 s
Annealing *SRY*	58-64	15 s
Annealing *DDX3Y*	60-62	15 s
Annealing *KDM5D*	60-64	15 s
Annealing *UTY*	63-66	15 s
Extension	72	40 s
Final extension	72	10 min

3.4. DNA purification and Sanger sequencing

In order to identify the amplified fragments, Sanger sequencing was required, prior to which purification of DNA was necessary. Performing high-fidelity PCR was followed with utilization of the Monarch PCR & DNA Cleanup Kit (New England Biolabs) for DNA purification. Binding Buffer was added to DNA in the 5:1 ratio. The samples were mixed, pipetting up and down, and transferred on a column in a collection tube. Following one minute centrifugation and removal of the flow-through, 200 µL of the Wash Buffer were added to each sample and centrifugation was repeated. Washing step was performed one more time and columns were transferred into 1.5 µL Eppendorf tubes. DNA was eluted with 15 µL of nuclease-free water which was added and left on the column for one minute. This was followed with centrifugation and purified DNA was obtained.

Thus, in order to determine whether bands detected in female samples are indicative of the presence of Y-associated genes in female *F. mechowii*, purified PCR products from a male and multiple female samples were sent to SEQme s.r.o. for Sanger sequencing to be performed with PCR primers as sequencing primers.

Additionally, approximately 15 nucleotides were removed from the beginning and end of the obtained sequences based on the quality of chromatogram signals. In the cases in which the sequencing of male and female PCR products was performed successfully, such sequences were used to create multiple sequence alignments of male and female *F. mechowii* amplified sequences with corresponding Y-associated and X homologue *F. damarensis* sequences with the use of Clustal Omega (Madeira et al., 2022). Moreover, phylogenetic trees were obtained in order to visualize the relationship between those sequences.

However, as the presence of multiple PCR products interfered with Sanger sequencing of certain female PCR products, only the alignments of the male products with corresponding *F. damarensis* sequences were created in order to confirm that the expected sequence was being amplified with PCR.

3.5. Bioinformatic analysis

In order to analyze the data obtained by the Illumina NGS, and to detect X-specific and ancestrally autosomal sequences, MetaCentrum was used, as it provides computing resources (Chudoba et al., 2017) required for such analysis.

Firstly, sequence quality was examined with the use of FastQC (v0.11.5). Trimmomatic (v0.39) with paired-end mode was utilized with the aim of further increasing the sequence quality by removing adapters and low-quality bases. However, a number of quality criteria such as k-mer or GC content indicated no improvement in quality. Moreover, according to certain criteria such as per base sequence quality scores, the quality decreased. Thus, Trim Galore (v5.1.0) was used to improve the sequence quality.

Those sequences were then mapped onto a female *F. mechowii* genome assembly at the contig/scaffold level, produced by Tolar (2023) in our laboratory. Bowtie 2 (v2.3.5.1) was used for mapping. Additionally, coverage of each contig position by the obtained reads was determined with the use of Bedtools genomecov function (v2.26.0). In order to identify X-specific sequences, acquired mean coverage values of contigs were first normalized by division with the total number of reads in order to account for differences in the initial amount of DNA

leading to the production of different number of reads. Furthermore, natural logarithm of the normalized mean coverage by female samples divided by normalized mean coverage by male samples was calculated. Thus, the contigs with the value of zero would indicate that there is no difference between the presence of that sequence between males and females, whereas a value of approximately 0.7 would indicate that a sequence is present twice as much in a female compared to a male genome.

Similarly to Mongue et al. (2017) the obtained values were plotted against the contig length. Additionally, the contigs with the values above zero were mapped onto genomes of *M. musculus* (GRCm39 (GCA_000001635.9)) and *Heterocephalus glaber* (naked mole-rat) (Naked_mole-rat_maternal (GCA_944319715.1)) with D-Genies (Cabanettes & Klopp, 2018) in order to detect possibly ancestrally autosomal regions. This was performed by a member of our lab, who created custom scripts in Python 3.8 required for bioinformatic analysis as well.

4. Results

4.1.Y-associated gene screening

In order to investigate whether *F. mechowii* displays a sex determination system different from the expected XX/XY, screening for the presence of Y-associated genes in *F. mechowii* female samples was performed through PCR amplification of fragments of selected genes using primers designed from *F. damarensis* sequences. Thus, if the targeted sequence would be amplified in *F. mechowii* a band corresponding to the sizes predicted on the basis of the used *F. damarensis* sequence (Table 7) would be visualized following gel electrophoresis. Male samples were used as a control, with an autosomal gene, *GAPDH* screening which indicated whether DNA of sufficient quality was extracted.

Table 7: Expected sizes of amplified fragments

Gene	Size / bp
GAPDH	391
SRY	216
DDX3Y	544
KDM5D	286
UTY	1922

The results of the amplification of the *GAPDH* fragment in 20 samples are outlined in Table 8, whereas screening for Y-associated genes yielded results outlined in Table 9.

Table 8: *GAPDH* PCR results

Sample	Sex	Band size / bp
3563	Female	400
4220	Female	400
8179	Female	400
9757	Male	400
642	Female	400
712	Female	400
724	Female	-
733	Female	400
2771	Male	400
3956	Male	400
7185	Female	400
7316	Male	400
2348	Female	400
2511	Male	400
5496	Female	400
6211	Female	-
7246	Female	400
7569	Female	400
8742	Female	400
8996	Female	-

Table 9: Sizes of gene fragments produced with PCR, with ones detected only with the use of high-fidelity polymerase marked with an asterisk

Sample	Sex	Approximate *SRY* band size / bp	Approximate *DDX3Y* band size /bp	Approximate *KDM5D* band size / bp	Approximate *UTY* band size / bp
3563	Female	900	800	300*, 800	2300
4220	Female	-	-	-	-
8179	Female	900	600*, 800	300*, 800	2300
9757	Male	200, 900	550	300, 800	1900, 2300
642	Female	-	800	-	-
712	Female	-	800	-	-
724	Female	-	-	-	-
733	Female	-	-	-	-
2771	Male	200	550	300	-
3956	Male	200	550	300	1900, 2300
7185	Female	-	800	-	2300
7316	Male	200	550, 800	300	1900, 2300
2348	Female	-	-	-	-
2511	Male	200	550	300	1900, 2300
5496	Female	-	800	-	-
6211	Female	-	-	-	-
7246	Female	800	600*, 800	300*, 800	2300
7569	Female	800	800	800	2300
8742	Female	800	600*, 800	300*, 800	2300
8996	Female	-	-	-	-

No bands were observed in *GAPDH* screening of samples 724, 6211 and 8996, even with the use of undiluted samples, indicating that DNA was not present in sufficient amount for further investigation (Table 8). Hence, screening for other genes yielded no bands for those samples either (Table 9).

However, except in the case of those three samples, fragments of the approximate size of 400 bp were detected in *GAPDH* screening, corresponding to the expected size (Table 7). This indicated successful DNA extraction.

Additionally, in the case of male samples, estimated sizes of the obtained fragments corresponded to the expected values (Table 7). However, in certain cases (Table 9), an additional band would be observed, the size of which would not be in accordance with the predicted sizes (Table 7). However, a band of that size was present in certain female samples as well (Table 9). Only in the case of *KDM5D* screening, certain female samples yielded fragments of the size expected and obtained for the male samples (Table 9).

The results of the PCR screening are depicted in Figures 2 through 6, with the fragment sizes inferred based on the ladder shown in Figure 1. A single 800/900 bp band was observed in *SRY* screening of five female samples (Figure 2), with two faint 900 bp bands and three 800 bp bright bands, indicating the presence of a sequence in certain female DNA samples similar to the targeted *SRY* sequence to a sufficient extent which would allow primers to bind specifically. However, a faint 900 bp band was obtained in one male sample as well with the 200 bp expected product. All other male samples produced only the expected *SRY* band.

DDX3Y (Figure 3), *KDM5D* (Figure 4) and *UTY* (Figure 6) screening yielded similar results, with certain female samples producing bands which would not correspond to the expected product size. In addition, in certain cases in *DDX3Y* and *KDM5D* screening, those bands would be observed for male samples as well, while in the case of *UTY*, a 2300 bp band would be present in every male sample in addition to the expected 1922 bp band.

Moreover, with the use of high-fidelity mix, required for amplifying fragments to be sequenced, bands of the size expected for male samples in the case of *KDM5D* were detected as well as the previously obtained 800 bp bands, whereas in the case of *DDX3Y* smaller products of approximately 600 bp were observed as well, however those were larger compared to the products obtained for male samples as well (Figure 5).

Additionally, while the absence of PCR products in the negative controls indicated that the observed results do not stem from a contamination of the PCR mix, differences in the size of the products obtained in male and female samples indicated that the bands observed in female samples were not caused by contamination with male DNA.

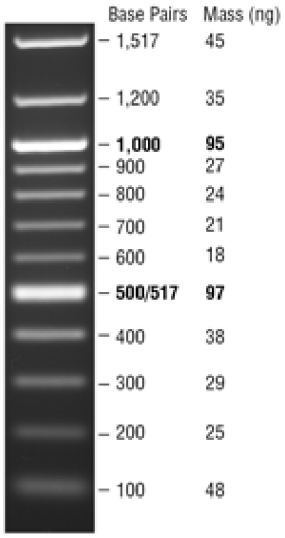

Figure 1: Quick-Load 100 bp DNA Ladder (New England Biolabs, n.d.)

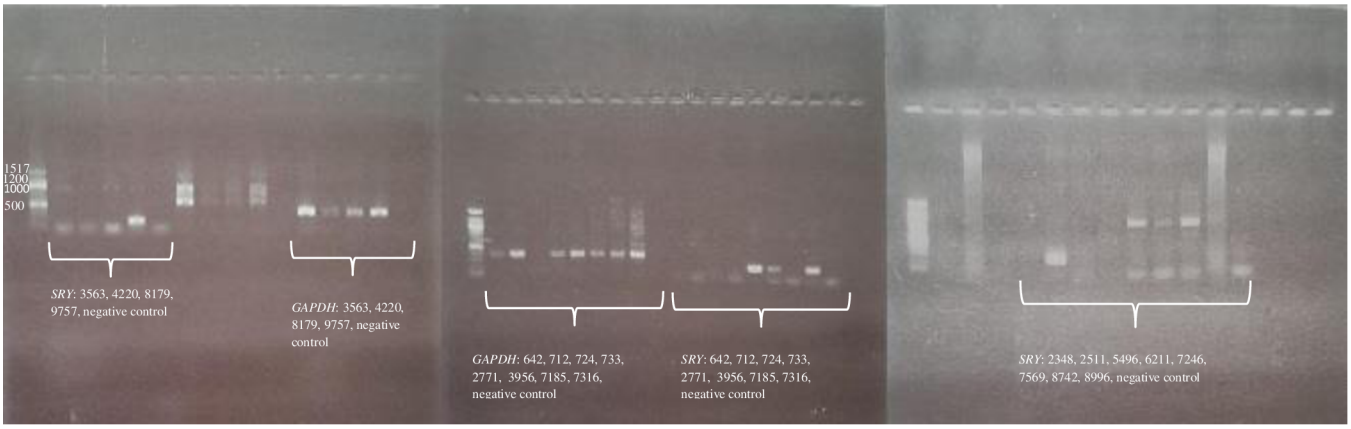

Figure 2: *SRY* screening results

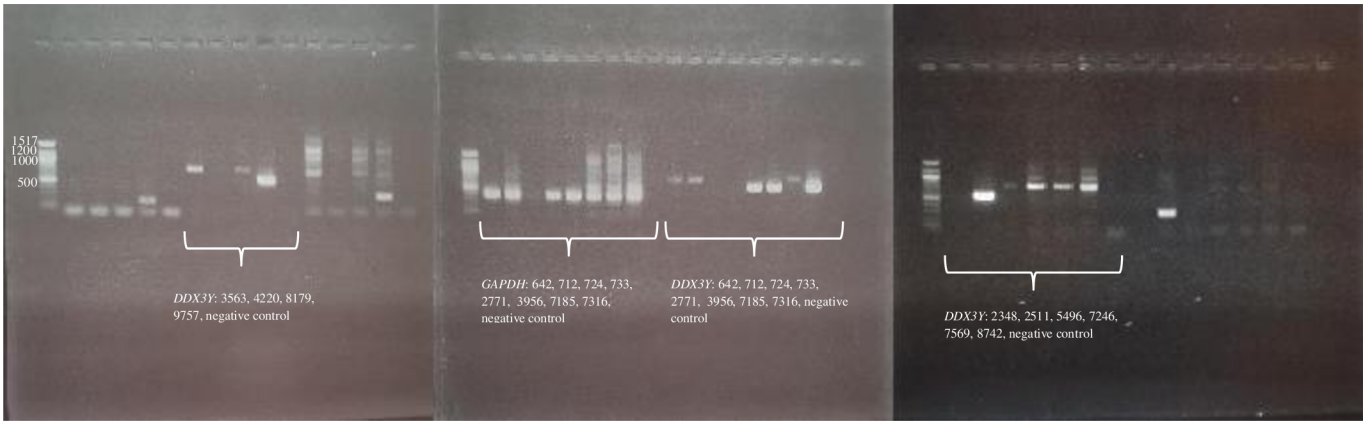

Figure 3: *DDX3Y* screening results

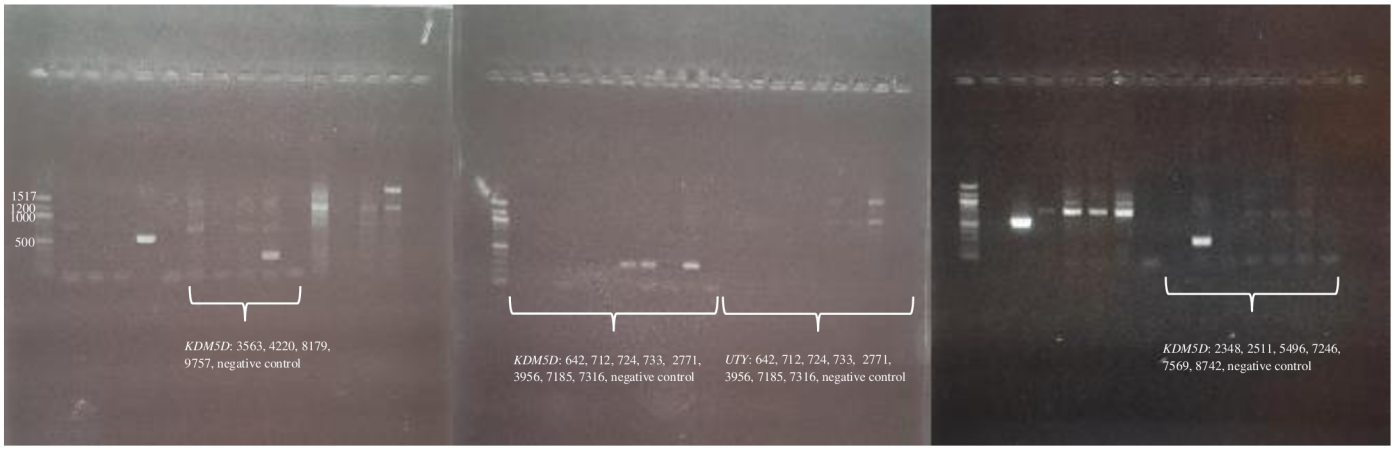

Figure 4: *KDM5D* screening results

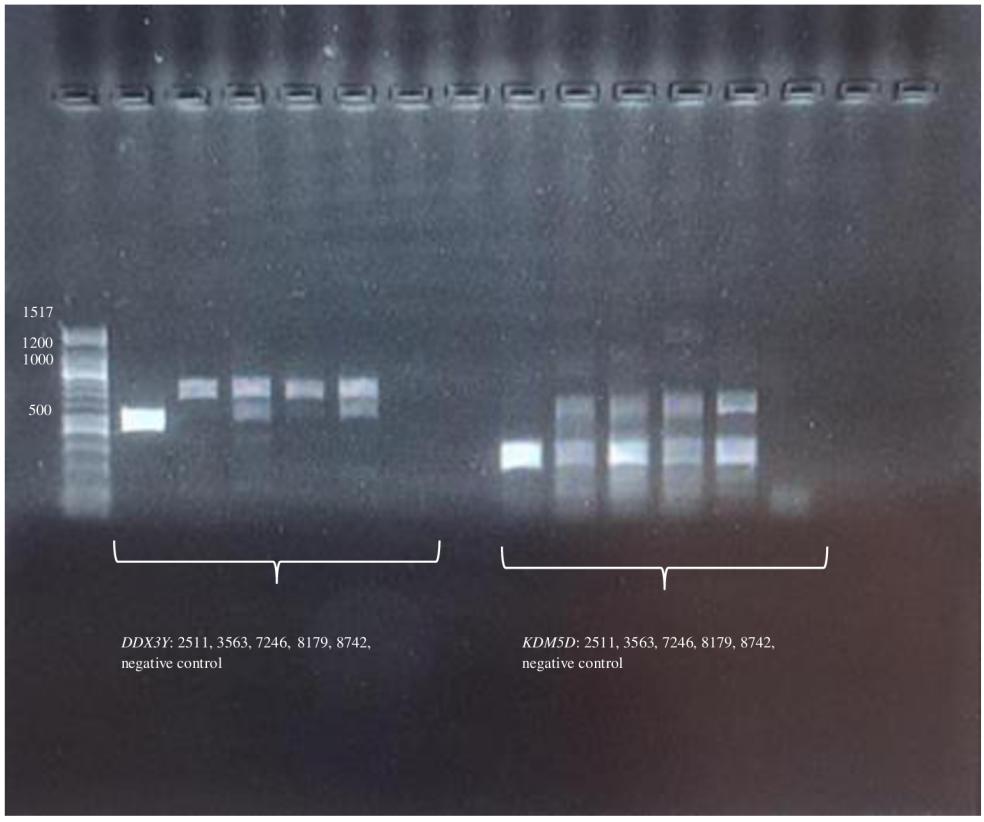

Figure 5: Results of PCR for *DDX3Y* and *KDM5D* performed with high-fidelity polymerase with the annealing temperature of 60 ºC and 33 cycles

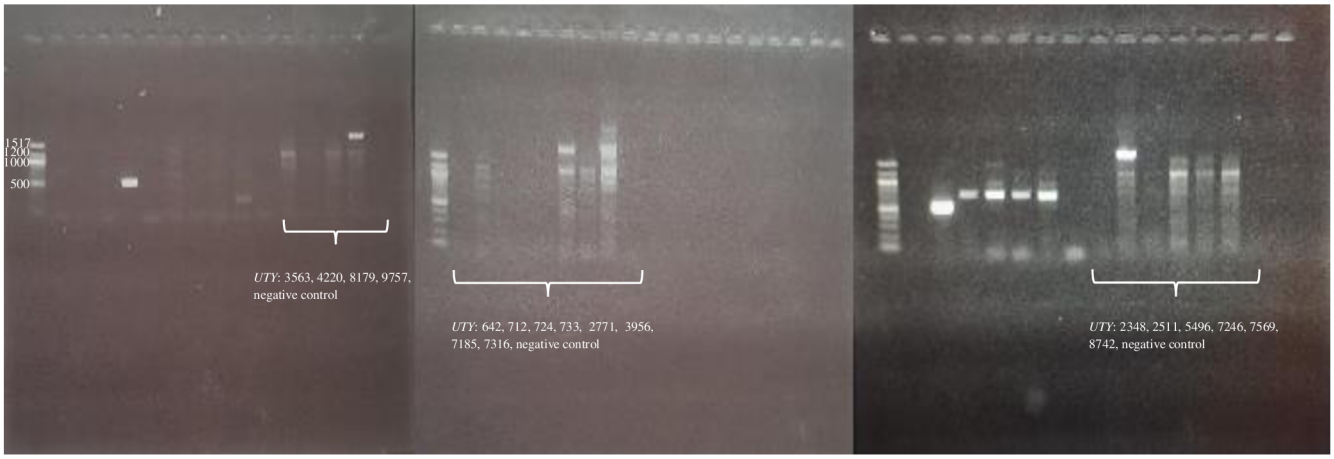

Figure 6: *UTY* screening results

Thus, amplification of a fragment in female samples indicated the presence of sequences similar to those of Y-associated genes. While the difference in sizes compared to the expected values and male PCR products, except in the case of *KDM5D*, suggested the observed bands did not result from Y-associated gene amplification, Sanger sequencing was required to identify the amplified fragments.

In addition, Y-associated gene screening of samples from live females, screened because they are progeny of females producing more female compared to male offspring, which suggested a possible difference in their sex chromosomes compared to the previously screened females, yielded results outlined in Figure 7.

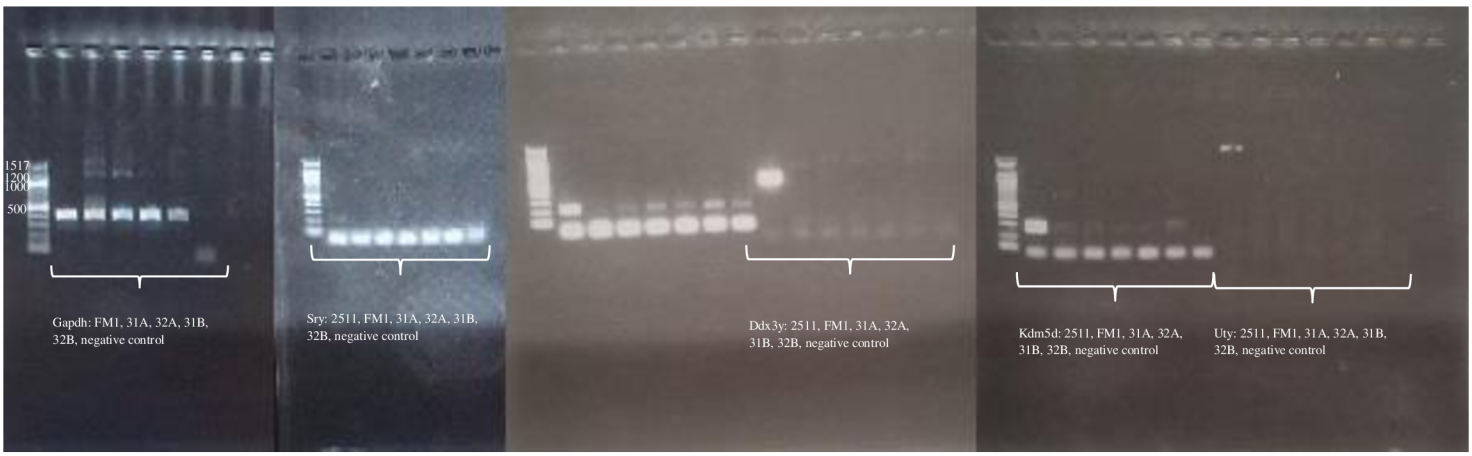

Figure 7: Results of the screening of samples obtained from living females with PCR performed with 33 cycles and annealing temperatures of 58 ºC, 58 ºC, 60 ºC, 63 ºC, 64 ºC for *GAPDH*, *SRY*, *DDX3Y*, *KDM5D* and *UTY* respectively

GAPDH reaction was performed with high-fidelity polymerase, unlike other reactions in the screening of live females, due to the unavailability of *Taq* polymerase, leading to multiple bands in 31A and 32A (Figure 7). This effect had been previously observed and it is displayed in Figure 5 as well. A single band could be obtained with an increased annealing temperature. However, in this case the presence of the product of the expected size (Table 7) indicated successful DNA extraction.

Results of the screening for other genes indicated no difference in the presence of the selected Y-associated genes in the females which are offspring of the females which produce prevalently female progeny and those that are not. PCR created previously detected products in *DDX3Y* and *KDM5D* screening, while no products were observed in females in the case of *SRY* and *UTY*.

4.2. Sanger sequencing

PCR was repeated with high-fidelity polymerase for the purposes of Sanger sequencing of the female and male PCR products and elucidating the cause of the bands observed in female samples. A requirement for a PCR product to be sent for sequencing was the detection of a singular band following gel electrophoresis as depicted in Figure 8.

Figure 8: Examples of products which were purified and sent for Sanger sequencing obtained through PCR with 33, 34, 32, 32 cycles and 60 ºC, 61 ºC, 64 ºC, 66 ºC as annealing temperatures for *SRY*, *DDX3Y*, *KDM5D* and *UTY* respectively

In the case of *KDM5D*, Sanger sequencing was performed despite the presence of faint 800 bp bands (Figure 8) as obtaining only one band was difficult with the use of high-fidelity polymerase, since the use of this polymerase often resulted in the increased number of observed bands. This was the case with *SRY* as well. Due to the presence of multiple bands each time PCR would be performed with high-fidelity polymerase for *SRY*, finally *Taq* polymerase was used to generate the *SRY* product visualized in Figure 8.

The results of the concentration and purity measurement, performed in order to determine whether Sanger sequencing would be possible, are displayed in Table 10 for the samples which were sent to be sequenced due to the highest obtained concentration and purity.

Table 10: Purity and concentration of DNA sent for Sanger sequencing

Sample	Gene	ng/µL	A260/A280	A260/A230
2511	*SRY*	33.7	1.94	2.09
8742		11.1	1.83	1.28
2511	*DDX3Y*	20.8	1.76	1.97
3563		10.9	2.06	0.69
7246		12.0	1.96	1.05
8179		8.6	1.87	0.78
8742		11.5	1.84	1.10
3563		9.3	1.82	1.33
8742		4.0	2.24	0.97
2511	*KDM5D*	14.1	1.87	1.53
3563		11.4	2.01	1.39
7246		18.2	1.94	1.69
8742		19.8	1.99	1.76
2511	*UTY*	35.2	1.89	1.74
3563		15.4	1.85	1.05
3563		3.8	1.80	0.88

However, Sanger sequencing was not performed successfully for female *SRY* and *UTY* products, as the chromatograms indicated the presence of multiple PCR products. Thus, it was only possible to infer that the male PCR products are amplified *SRY* and *UTY* regions on the basis of alignments with *F. damarensis SRY* (Figure 9) and *UTY* (Figure 10) targeted sequences.

```
CLUSTAL O(1.2.4) multiple sequence alignment


Sry_2511                        ----------------------------------------------------------CTT     3
XM_010621263.1_damara_sry       GCATTCATGGTGTGGTCTCGTGACCAGAGGCGCAAAGTGGCTCTGGAGCATCCCAACTTG     60
                                                                                           *


Sry_2511                        GCAAACTCAGAGATCAGCAAGTGGCTGGGGTACCAGTGGAAAATGCTTACTGAAGCCGAA     63
XM_010621263.1_damara_sry       CAAAACTCAGAGATCAGCAAGTGGCTGGGGTACCAGTGGAAAATGCTTACTGAAGCCGAA     120
                                  ************************************************************


Sry_2511                        AAATGGCCATTCTTTCAGGAGGCCCAGAGACTGCGGGCTATGCACAGAAGGAAATATCCT     123
XM_010621263.1_damara_sry       AAATGGCCATTCTTTCAGGAGGCCCAGAGACTGCAGGCTATGCACAGAAGGAAATATCCT     180
                                ********************************** **************************


Sry_2511                        GACTATAAGTATCAACCTCGCCGRAAGACTAAGACGG    160
XM_010621263.1_damara_sry       GACTATAAGTATCAACCTCGCCGGAAGACTAAGACGC    217
                                *********************** ************
```

Figure 9: *SRY* alignment

Figure 10: *UTY* alignment

For the other two genes sequencing was successful. Alignments with *F. damarensis* Y-associated genes and X homologues are displayed in Figures 11 and 12, while phylogenetic trees, indicating relationships between sequenced PCR products and X and Y *F. damarensis* genes, are outlined in Figures 13 and 14.

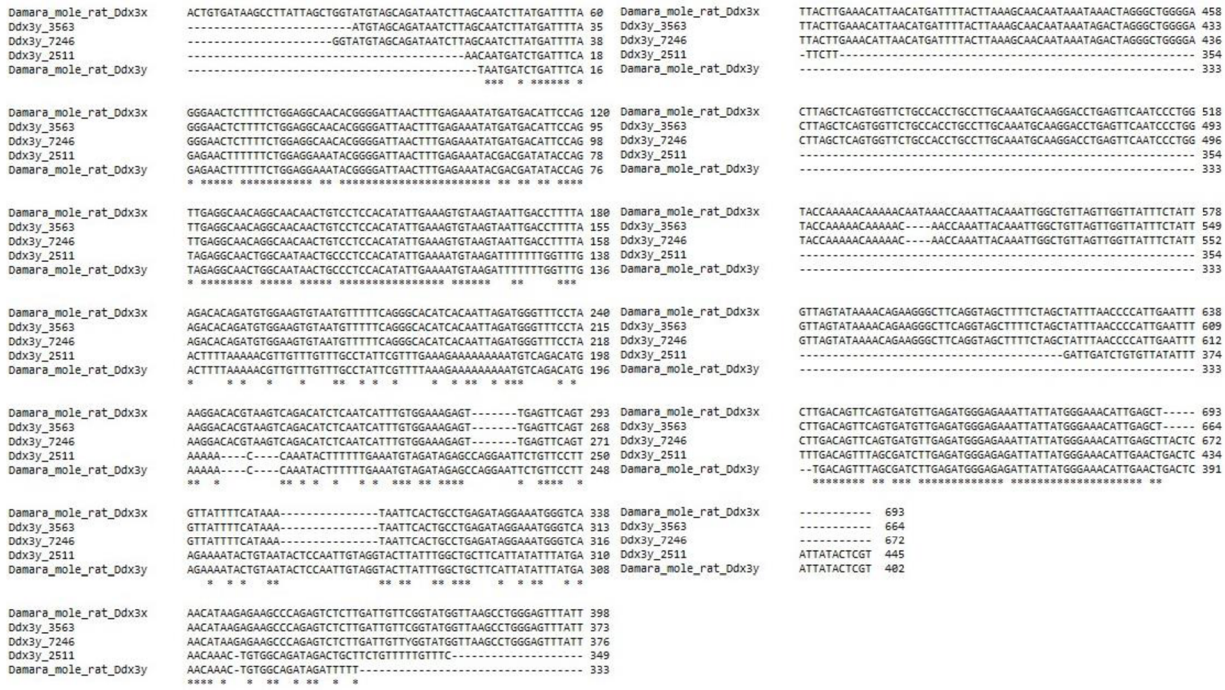

Figure 11: *DDX3Y* alignment

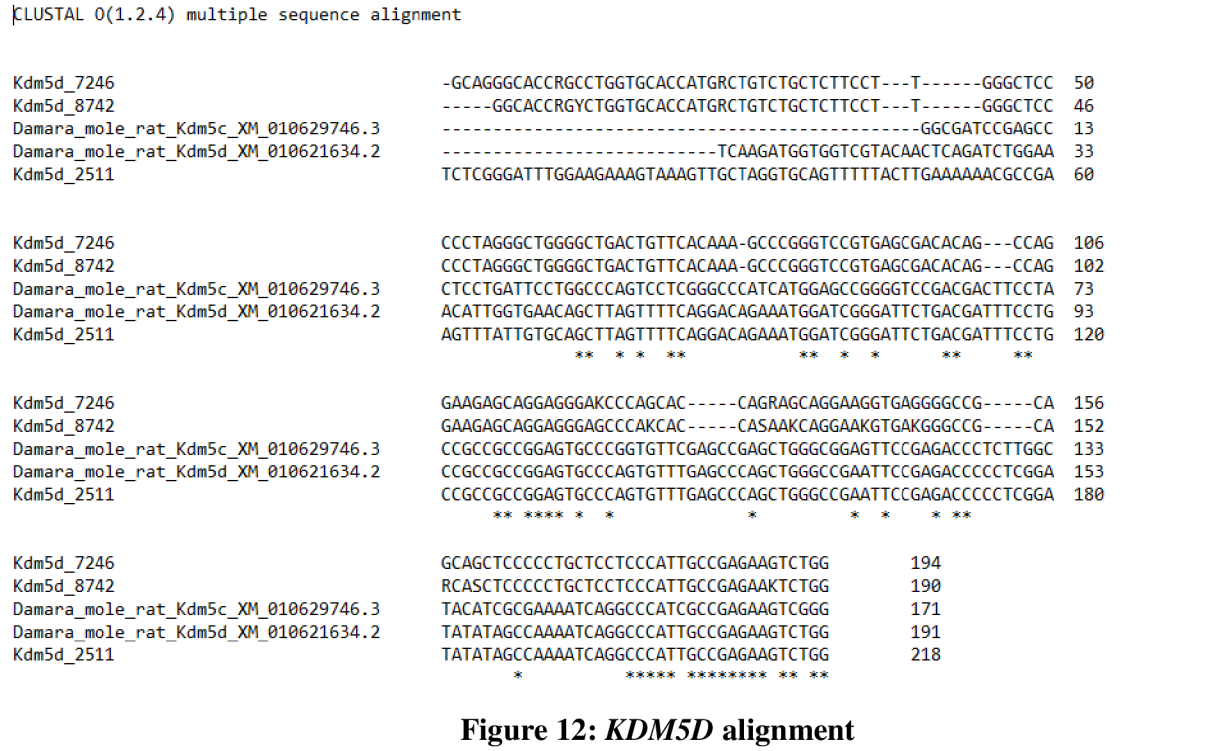

Figure 12: *KDM5D* alignment

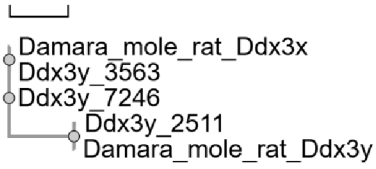

Figure 13: *DDX3Y* phylogenetic tree

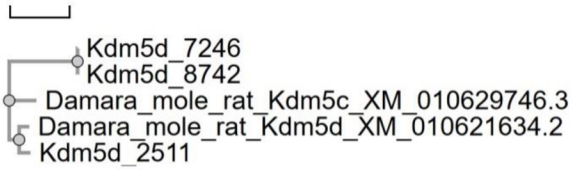

Figure 14: *KDM5D* phylogenetic tree

Figure 13 suggests that successfully sequenced female PCR products, from samples 3563 and 7246, are amplified X homologues of *DDX3Y*, rather than amplified *DDX3Y*, as it is more closely related to *F. damarensis DDX3X* than male sequenced PCR product from sample 2511 and *F. damarensis DDX3Y*. Similarly, Figure 14 indicates that the sequence amplified in 7246 and 8742 females is *KDM5C*, X homologue of *KDM5D*.

Thus, the selected Y-associated genes are shown to be likely absent in *F. mechowii* females since the results indicate that only X homologue amplification occurred in the case of *DDX3Y* and *KDM5D*, while although Sanger sequencing of the fragments amplified *SRY* and *UTY* female screening was not successful, their size suggests they were not Y-associated gene fragments.

4.3.Bioinformatic analysis

A bioinformatics analysis was performed in order to detect X-specific sequences through the analysis of coverage by female compared to male reads. Additionally, mapping sequences with higher coverage by female compared to male reads onto genomes of related species was performed in order to detect possibly ancestrally autosomal sequences.

Following mapping the obtained reads onto a reference and calculating the ratio of the coverage by female and male reads, a graph displaying natural logarithm of those values plotted against the size of contigs was created (Figure 15). It shows that the largest number of contigs has the same coverage by female and male reads, illustrated by the natural logarithm values close to zero, which thus correspond to autosomes or sequences found on both, X and Y

chromosome. Moreover, the second largest cluster can be seen close to the natural logarithm value of 0.7, indicating the coverage by female reads being twice as high as by the male reads, likely because these are X-specific sequences. However, a number of values are found outside of those two clusters, with negative values resulting from technical limitations, and values between zero and 0.7 either resulting from similar technical limitations or indicating ancestrally autosomal sequences on X chromosomes which have not sufficiently diverged from the corresponding ancestrally autosomal sequences on the Y chromosome.

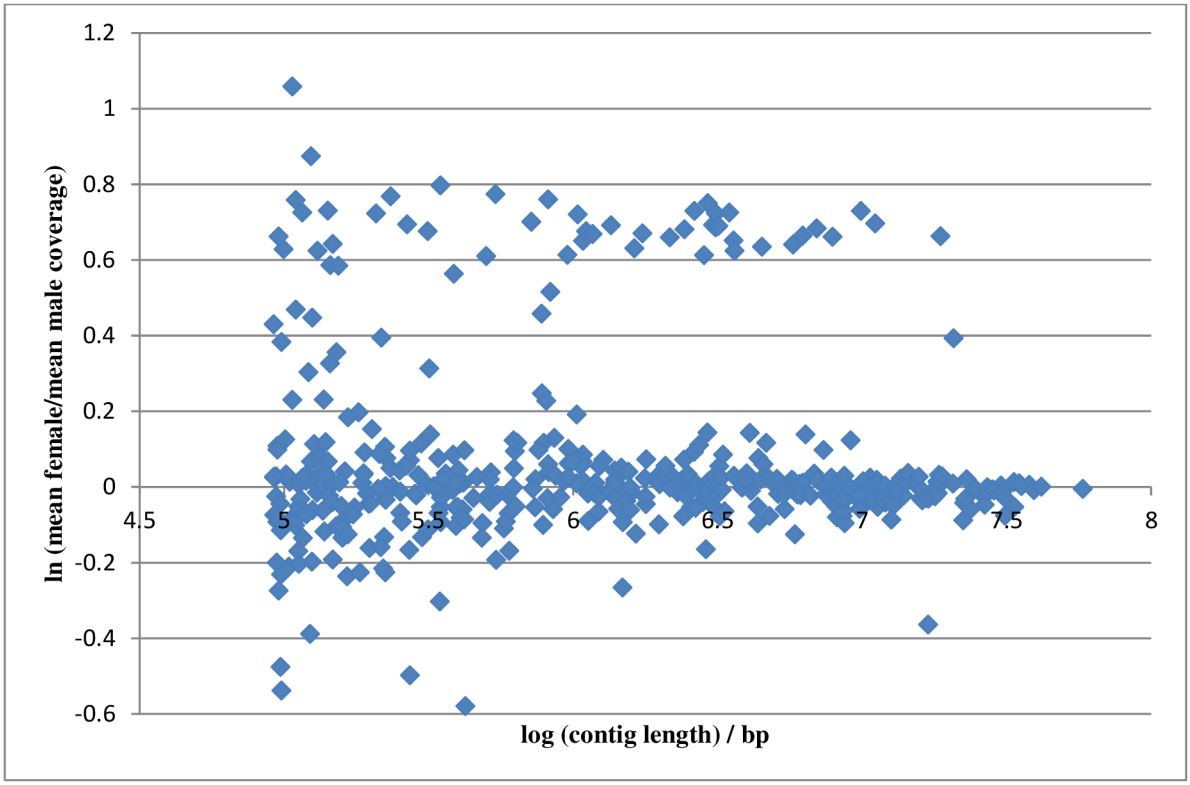

Figure 15: Natural logarithm of the normalized mean coverage by female over male reads plotted against logarithm values of contig lengths

In addition, the mapping of contigs with the values above zero onto a *M. musculus* and *H. glaber* led to the creation of dot plots shown in Figure 16 and 17 respectively, with Figure 16 displaying that several contigs mapped onto chromosome 6 of *M. musculus*, possibly indicating they were ancestrally autosomal sequences that were translocated on *F. mechowii* sex chromosomes, if the obtained values were not caused by technical limitations. Similarly, certain

F. mechowii contigs mapped onto *H. glaber* chromosome 9, again indicating these are likely X-specific ancestrally autosomal sequences (Figure 17).

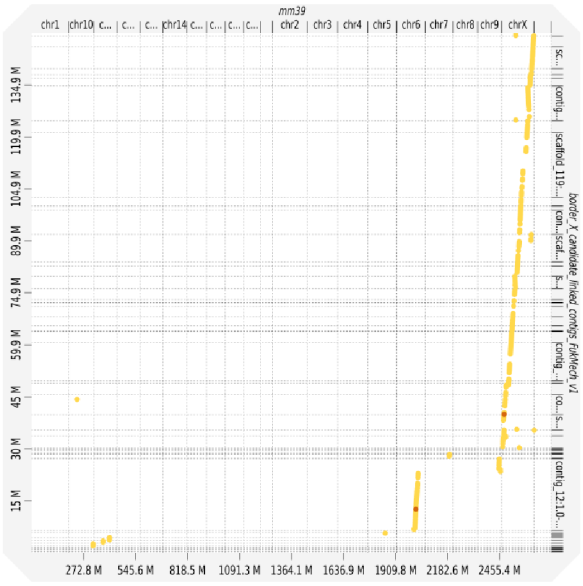

Figure 16: Alignment with *M. musculus*

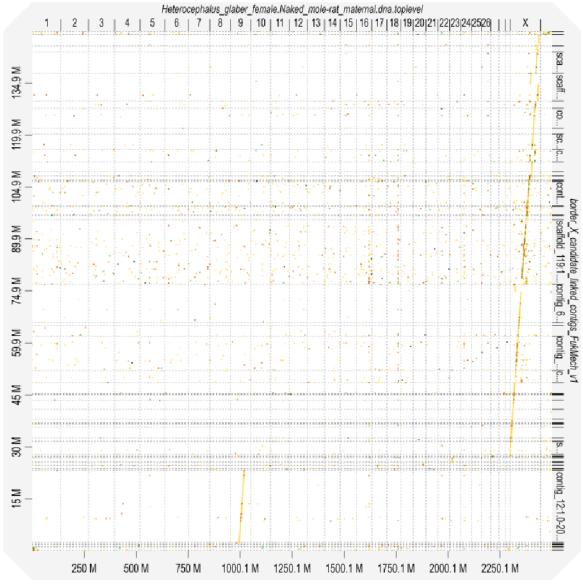

Figure 17: Alignment with *H. glaber*

Thus, the coverage analysis indicated X-specific sequences, as well as sequences with a higher coverage by female reads, however not twice as high as male coverage, indicating sequences which may not have yet sufficiently diversified between X and Y chromosomes to be identified as X-specific. Additionally, mapping those sequences onto genomes of related species suggested possibly ancestrally autosomal sequences, which do not map onto X chromosomes of related species, with several contigs mapping onto *H. glaber* chromosome 9 and *M. musculus* chromosome 6.

5. Discussion

5.1. Y-associated genes screening

Performing PCR with gel electrophoresis in order to screen for the presence of Y-associated genes in *F. mechowii* yielded results showing the presence of bands for both, female and male samples. In the case of male samples, the amplified fragments would be of the predicted size, however in certain cases an additional band would be observed. In the case of the female samples, only *KDM5D* screening yielded bands identical to the ones detected for male samples. However, presence of an additional PCR product was observed in certain PCR runs as well. In the screening process for other selected genes, fragments of a size different to that of the male PCR products were detected.

The bands detected for female samples in *SRY/DDX3Y* screening indicated the presence of a sequence similar to *SRY/DDX3Y* to an extent which would allow the primers to bind and the sequence to be amplified. Detection of only a single bright band indicated that it was unlikely a result of unspecific binding, while the absence of that product in the negative control suggested it did not stem from a contamination of the PCR master mix, primers or water. Moreover, contamination of female DNA with male DNA was not likely as the size of the product differed from that produced by male samples. The specificity of primer annealing during PCR reactions could be resolved by increasing annealing temperature in both, female and male samples, and observing whether the product in the female sample stops to amplify at a lower temperature compared to the male sample.

Furthermore, detection of a PCR product of the identical size for male and female samples in *KDM5D* screening might indicate that the female DNA was contaminated with male DNA, however, detection of different bands for male and female samples upon using that DNA in the screening process of other genes indicated that it was not the case.

In *UTY* screening the band detected for female samples was present in the screening of the male samples as well, possibly indicating that X homologue was being amplified, as the band corresponding to the product of the correct size in male samples was brighter, indicating *UTY* being primarily amplified leading to a higher amount of that product, while *UTX* would be amplified to a lower extent due to the sequence differences partially hindering primer binding.

In addition, screening of females which are offspring of the females with a skewed ratio of the sex of their progeny indicated no difference in the presence of these genes in their genome and the genome of other females, as there is no difference in the observed *DDX3Y* and *KDM5D* bands between these two groups of females. No *SRY* bands were detected, as was the case for the majority of females from the first female group. Moreover, 2300 bp *UTY* bands were not detected for the female samples, which is likely a result of the annealing temperature being too high since it was absent in the male control as well.

Although Macholan et al. (1993) detected the presence of heteromorphic X chromosomes in a female *F. mechowii,* and Burda (2022) proposed heterogametic females as a possible reason for the skewed sex ratio of the offspring, similar to the prevalence of female *M. schisticolor* associated with the polymorphism of X chromosomes (Vorontsov et al., 1980),with 1.46:1 female to male ratio of a population of wild *F. mechowii* (Sichilima et al., 2008b) and 1:0.54 female to male offspring in captivity (Scharff et al., 1999), screening for Y-associated genes did not suggest a difference between the live *F. mechowii* indicated as possibly heterogametic and ones which were not. However, Scharff et al., 1999 reported that the sex ratio of the offspring favoring females was detected for each of the nine studied females, indicating a more common occurrence of heterogametic females if there is a connection between that chromosome combination and the skewed sex ratio of the offspring, which would be supported by the only female studied by Macholan et al. (1993) shown to possess heteromorphic X chromosomes.

While this investigation suggested no difference in the presence of Y-associated genes between females which are offspring of the females with prevalently female progeny and females for which this has not been observed, a future investigation could be conducted with the use of females with the skewed sex ratio instead of their offspring as this was not possible in this investigation due to the unavailability of such females. The offspring of such females was proposed to possibly belong to that group of females as well, however it may not have been the case. An additional limitation of this investigation is that performing karyotyping to identify females with heteromorphic X chromosomes was not possible due to required use of bone marrow (Macholan et al., 1993). Thus, this could be performed in a future study in order for the frequency of occurrence of such females to be determined and compared with the data regarding their offspring sex ratio.

5.2. Sanger sequencing results

Following PCR with high-fidelity polymerase and purification of the obtained DNA, Sanger sequencing was performed in order for the source of the detected bands to be determined. Although detection of only a single *SRY* and *UTY* band suggested that only one sequence was amplified, chromatograms suggested the presence of multiple products. Thus, determining the origin of the *SRY* and *UTY* bands for females was not possible, although in the case of *UTY*, the presence of the product of the identical size in male samples indicates amplification of *UTX*.

DDX3Y and *KDM5D* phylogenetic trees show a closer relationship between female *F. mechowii* PCR products and *F. damarensis* X homologue, compared to the sequenced *F. mechowii* male product and *F. damarensis* Y-associated gene sequences. This indicates that X homologues are likely being amplified in *F. mechowii* females despite primers not annealing perfectly on the DNA sequence. That is supported by a slightly different product size in the case of *DDX3Y* as well. Additionally, this is likely to be the reason for bands becoming fainter and disappearing before the bands in male samples upon increasing the annealing temperature as it increases the specificity of primer binding.

However, if only X homologues are being amplified in females it is unclear why it occurs only in certain females and not others under identical conditions. Low DNA concentration might be the reason as in the majority of cases if a PCR product is detected for one gene in a female it is detected for all genes, except *SRY* and vice versa. However, this is not likely to be the cause due to successful *GAPDH* amplification in those females.

Moreover, if *DDX3X* is amplified with these primers, it is unclear why it is present only in females and not in males as is the case for probable fainter *UTX*, detected with *UTY* bands in males caused by preferential amplification of the *UTY* product. A band corresponding to the female bands in *DDX3Y* screening was detected in one male, thus it might be detected in others as well upon using a lower annealing temperature with *DDX3Y* being more preferentially amplified in males.

In addition, due to the absence of a true *SRY* X homologue, the sequence which is amplified in females is likely only a result of unspecific binding of the primers, although the presence of a singular band would indicate that the binding was specific.

While sex chromosomes of *F. mechowii* are outlined to be exceptionally large (Macholan et al., 1993), possibly indicating a difference to the expected sex determination system, such as in the case of *Microtus* genus in which several species have larger than expected sex chromosomes, including *Microtus cabrerae* (Cabrera vole) for which fertile XY females have been detected (Marchal et al., 2003), Sanger sequencing of *F. mechowii* gene fragments suspected to possibly be Y-associated indicated that only X homologues of those genes could be detected in females. Furthermore, while in this investigation a fragment was amplified in certain females in *SRY* screening, Bullejos et al. (1997) detected a PCR product amplified with *SRY* primers in *M. cabrerae* females and males. However, in that case there was no difference in the size of female and male PCR products, and it was proposed that all different *SRY* copies detected in that species with the exception of one Y-associated copy are pseudogenes. Prior to that, amplified sequences in females were suspected to possibly stem from *SOX3* gene (Bullejos et al., 1997), as it is likely the nearest *SRY* relative and is X-associated (Stevanović et al., 1993). Thus, while it is possible that in *SRY* screening of *F. mechowii* females a copy of *SRY* was amplified, the detected fragment could be a result of *SOX3* or other similar sequence amplification. As Sanger sequencing of that fragment was not successful it was not possible to determine the identity of the amplified female fragment. Hence, a future investigation might elucidate the identity of the amplified *SRY* PCR product in females.

In addition, heteromorphic X chromosomes (Macholan et al., 1993) and autosome-sex chromosome translocations, detected in *F. mechowii* (Deuve et al., 2006), are both outlined as traits of species with a sex determination system differing from XX/XY, with XX/XX*/X*Y females (Saunders & Veyrunes, 2021). However, while this investigation suggested the absence of the Y chromosome and selected Y-associated genes in females, although *UTX* being the amplified fragment in females could not be confirmed due to unsuccessful Sanger sequencing, further research might focus on elucidating the differences in the heteromorphic X chromosomes, the effects they have on *F. mechowii*, and whether a different variant of the X chromosome is advantageous in certain regards. Moreover, Saunders et al. (2014) detected a reproductive advantage in X*Y *Mus minutoides* (African pygmy mouse) females compared to

females with other sex chromosome combinations. Although, females with that chromosome combination have not been detected in *F. mechowii*, future studies might research the possible effects of heteromorphic X chromosomes on reproductive outcomes in *F. mechowii*.

5.3. Bioinformatic analysis

Illumina sequencing of four *F. mechowii* and mapping the reads onto a reference genome followed by a comparison of the coverage by female and male reads yielded results showing the contigs which are most likely autosomal or present on both, X and Y chromosomes, with the coverage ratio value of zero, and X-specific contigs which are covered by approximately twice the number of female reads compared to male reads.

Additionally, comparing contigs with a higher ratio of female to male coverage with *M. musculus* and *H. glaber* genomes showed that certain contigs mapped onto their chromosomes 6 and 9 respectively. This could indicate that those sequences were ancestrally autosomal in *F. mechowii*, although it is possible that the higher ratio of female to male coverage stems only from the technical limitations, especially visible in the case of smaller contigs, similar to those which led to the negative values as negative values would indicate that the coverage of a female genome is higher by male compared to female reads. Similar technical limitations could be the cause of a coverage value slightly higher than zero, however it is possible that contigs in question are ancestrally autosomal sequences on the *F. mechowii* X chromosome which have not yet diversified between X and Y chromosomes.

While certain potentially ancestrally autosomal *F. mechowii* sequences were identified in this bioinformatic analysis, Deuve et al. (2006) utilized chromosome painting to investigate chromosomal rearrangements in *F. mechowii* and identified regions of *F. mechowii* sex chromosomes homologous to *H. glaber* chromosomes 7, 11, 12 and 20, whereas this bioinformatic analysis suggested that the sequences homologous to *H. glaber* chromosome 9 are now located on *F. mechowii* X chromosome. However, Deuve et al. (2006) did not identify *F. mechowii* chromosome regions homologous with that chromosome since the painting probes required to identify regions homologous with chromosome 9 of *H. glaber* are stated to not have been available. In addition, it is not clear whether the numbering of chromosomes is the same in

the genome assembly we used for the analysis and Deuve et al. (2006) publication. Potentially we only discovered the largest ancestrally autosomal block, or the most divergent between X and Y chromosomes, while others were not distinguishable between X and Y and therefore marked as autosomal in our analysis.

Chromosomal rearrangements involving X chromosome and autosomes are stated to likely have adverse effects due to possible interfering with meiosis and spread of heterochromatization from X inactivation to the translocated autosomal region (Dobigny et al., 2004). Thus, Deuve et al. (2008) proposes that the rearrangements are likely to be environmentally or socially advantageous, which could be investigated further in future research.

Thus, Illumina sequencing of two female and two male *F. mechowii* has been successfully performed and X-specific sequences were identified, including some potentially ancestrally autosomal sequences. However, a limitation of this investigation is that if diversification of the ancestrally autosomal sequences on X and Y chromosomes has not yet occurred, such sequences could not be detected as ancestrally autosomal since only potentially X-specific sequences were mapped on genomes of related species. Further investigations might aim to generate more data suggesting which X chromosome sequences were ancestrally autosomal and what are their effects on *F. mechowii*.

Further research focused on studying chromosomal sex determination systems different from XX/XY in mammals might further elucidate sex chromosome evolution and X inactivation mechanisms.

6. Conclusion

This investigation aimed to determine whether genes often associated with the mammalian Y chromosome could be detected in *F. mechowii* females. Furthermore, an additional aim was identifying *F. mechowii* X-specific sequences and sequences which were ancestrally autosomal and are now located on sex chromosomes. The results of the Y-associated genes screening indicated no presence of such genes in females. Although in certain cases a fragment would be amplified in females during PCR with *SRY*, *DDX3Y*, *KDM5D* and *UTY* primers, Sanger sequencing indicated that in the case of *DDX3Y* and *KDM5D* only X homologue fragments were the amplified products. Additionally, *UTY* product size and the presence of an identical band when screening males indicated amplification of the X homologue, *UTX* in this case as well, whereas although single *SRY* bands of high intensity were detected in certain females, the product size did not correspond to the expected value, suggesting that the amplified product is not an *SRY* fragment. Moreover, Y-associated genes screening suggested no difference between female progeny of the females producing prevalently female offspring and females for which this was not the case. Furthermore, X-specific sequences were identified, and certain sequences mapped onto a *M. musculus* chromosome 6 and *H. glaber* chromosome 9, possibly indicating sequences which were ancestrally autosomal. Thus, while Illumina sequencing of two male and two female *F. mechowii* with identification of X-specific sequences was achieved, future research could aim to generate more data regarding which sequences are ancestrally autosomal and how sex chromosome-autosome translocations which led to this type of *F. mechowii* sex chromosomes might have been advantageous. Furthermore, while previous research suggested *F. mechowii* might be one of the mammalian species with a sex determination system different from XX/XY, screening for Y-associated genes within the scope of this investigation suggested no differences from the expected sex determination system in terms of the presence of Y-associated genes in females and males. However, further research is required to elucidate reasons for the skewed sex ratio of the offspring in certain females, differences between two X chromosome variants and the effects of the heteromorphic X chromosomes in this species.

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