Exploring the Biology of the Trioecious Nematode *Auanema rhodensis*

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List of Abbreviations

A, C, G, T Nucleotide bases. Adenine, Cytosine, Guanine and Thymine, respectively.
AIL Advance Intercross Line.
ATP Adenosine triphosphate.
Aut Autosome(s).
BAM Binary Alignment Map (File format).
BCF Binary variant Call Format.
BLAST Basic Local Alignment Search Tool.
BP Biological Process (Gene Ontology category).
CC Cellular Component (Gene Ontology category).
CF or Conv. Fem. Converted Female (hermaphrodite-fated larvae chemically converted into a functional female).
cDNA Complementary DNA.
CDS CoDing Sequence.
Chp. Chapter.
Chr or Chrom. Chromosome(s).
DA Dafachronic Acid. used to convert hermaphrodite-fated larvae into females.
DAPI 4',6-diamidino-2-phenylindole. Dye used to visualise DNA.
DB `BLAST` Database (created with the program `makeblastdb` of `BLAST+`).
DDT Dithiothreitol. Used to synthesise dsRNA.
DEG Differentially Expressed Gene.
DEPC Diethyl pyrocarbonate.
DNA Deoxyribonucleic acid.
DSB Double Stranded Breaks.
dsRNA Double Stranded RNA.
ESD Environmental Sex Determination.
ESS Evolutionary Stable Strategy.
EST Expressed Sequence Tag.
F1, F2 Filial generation 1, 2.
FC Fold Change.
FDR False Discovery Rate.

Fem. or F Female(s).
FR Forward Reverse (usual orientation of pair-end reads).
GC content Guanine Cytosine content.
GO Gene Ontology.
GPS Global Positioning System.
GSD Genetic Sex Determination.
Herm. or H Hermaphrodite(s).
InDel Insertion Deletion (a type of polymorphism).
L1, L2, L3, L4 Larval stages. The progressing from one larval stage to the next is marked by a moulting event.
LG Linkage group.
LOD Logarithm of the odds. A statistical estimate of whether two markers on a genome are likely to be located near each other on a chromosome. The higher the LOD score, the most likely the 2 markers are close to each other and the more likely they will be inherited together.
M9 Nematode buffer.
M Male.
MF Molecular Function (Gene Ontology category).
miRNA Micro-RNA.
MMLV RT Moloney Murine Leukemia Virus Reverse Transcriptase.
MP Mate-pair reads.
mRNA Messenger RNA.
NCBI National Center for Biotechnology Information.
NGM Nematode Growth Medium. Agar based medium used to culture the nematodes.
P0 Parental generation.
PCR Polymerase Chain Reaction.
PE Pair-end reads.
PSSC Premature Separation of Sister Chromatids (during meiosis).
qPCR Quantitative Polymerase Chain Reaction.
RAD-seq or RAD-tag Restriction site associated DNA Sequencing / Tags.
RF Reverse Forward (usual orientation of mate-pair reads).
RNA RiboNucleic Acid.
RNAi Interfering RiboNucleic Acid.
RNAPII RNA Polymerase II.
RNAse Enzyme used to degrade RNA.
RNA-seq RNA sequencing.

rRNA Ribosomal RNA.
rNTPs ribonucleoside tri-phosphate. Used to synthesise RNA.
RSD Random sex Determination.
RT Reverse Transcriptase.
SAM Sequence Alignment Map (file format).
siRNA small interfering RNA.
SL Splice Leader.
SNP Single Nucleotide Polymorphism.
snoRNA small nucleolar RNA.
snRNA small nuclear RNA.
SRP Signal Recognition Particle (part of the signal recognition particle ribonucleoprotein complex).
SYBR Green Cyanine dye, used to stain nucleic acids during quantitative PCR.
TAGC plot Taxon-Annotated-GC-Coverage plot.
tRNA Transfer RNA.
tncRNA tiny noncoding RNA.

List of Units

bp Base pair.
°C Degree Celsius.
cm centimetre.
$g\,l^{-1}$ gram per litre.
h Hour(s).
kb Kilobase.
Mb Megabase.
$\mu g\,ml^{-1}$ Microgram per millilitre.
µL Microlitre.
mL Millilitre.
$mg\,l^{-1}$ Milligram per litre.
mm Millimetre.
mM MilliMolar.
min Minute(s).
nM Nanomolar
s Second(s).

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Declarations

This thesis has been submitted to the University of Warwick towards the earning of the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted to support the application of any other degree.

Some of the work presented in this thesis has been published or made available as described below:

- The empirical data on the population dynamics of *A. rhodensis* (Chapter 2) was included in Chaudhuri J, Bose N, Tandonnet S, Adams S, Zuco G, Kache V, Parihar M, von Reuss SH, Schroeder FC, Pires-daSilva A (2015). Mating dynamics in a nematode with three sexes and its evolutionary implications. Scientific Reports, 5, 17676. `http://doi.org/10.1038/srep17676`

- The crosses and genetic work on the X chromosome inheritance (Chapter 4) was published as: Tandonnet S, Farrell MC, Koutsovoulos GD, Blaxter ML, Parihar M, Sadler PL, Shakes DC, Pires-daSilva A (2018). Sex- and Gamete-Specific Patterns of X Chromosome Segregation in a Trioecious Nematode. Current Biology, 28(1), 93–99.e3. `http://doi.org/10.1016/j.cub.2017.11.037`

- The genome and genetic map and their analysis (Chapter 3) was made available on the BioRxiv as: Tandonnet S, Koutsovoulos G, Adams S, Cloarec D, Parihar M, Blaxter M, Pires-daSilva A (2018). Chromosome-wide evolution and sex determination in a nematode with three sexes. bioRxiv, 466961. `https://www.biorxiv.org/content/early/2018/11/09/466961`

Contributions

The work presented (data acquisition, analyses and presentation) was carried out by the author except in the cases outlined below:

- The coding of the simulator (See section 2.2.3, on page 27) was joint work with Tássio Naia dos Santos.
- The *dumpy* mutant line used in section 4.2.3, on page 79 was created by Talal Al Yazeedi.
- The schematic of meiosis dynamics (Figure 4.5, on page 86) was created by André Pires da Silva.
- The genome assembly and structural annotation (See sections in 3.2, on page 41) was joint work with Georgios Koutsovoulos, who taught me how to conduct these bioinformatics experiments.
- The sequences used (RNA-seq, RAD-seq and DNA-seq) come from original RNA and DNA extractions performed by Manish Parihar (See sections in 3.2, on page 41).
- The phylogenetic tree (Appendix C.1, on page 110) was constructed by Sandhya Dhawan.

Abstract

The free-living nematode *Auanema rhodensis* is a rare example of a species with three sexes, in which males, females and hermaphrodites coexist. Hermaphrodites (XX) reproduce primarily by selfing, and females (XX) and males (XO) by crossing, generating a mixed system in which selfing and outcrossing entwine.

In *A. rhodensis*, females and hermaphrodites follow different developmental trajectories, as hermaphrodites always pass through a resistant larval stage called dauer, whereas females (and males) never do. Here I report that the meiosis programme controlling the segregation of the X chromosome in XX animals also varies according to the sex and type of gametogenesis, as well as within the same gametogenesis. Females undergo a conventional meiosis, yielding predominantly haplo-X (1X) oocytes. Hermaphrodites, on the other hand, produce diplo-X sperm and nullo-X oocytes. These results complement previous findings in males, in which an atypical meiosis also occurs, resulting in the exclusive production of haplo-X sperm.

Consequences of these unusual patterns are that (i) aneuploidy can be readily observed in most gametes of self-fertilising hermaphrodites, which is then 'rescued' during self-fertilisation as the nullo-X oocytes are fertilised by the diplo-X sperm, (ii) the X homologues do not recombine in hermaphrodites and (iii) crosses between hermaphrodites and males result exclusively in male progeny.

The X chromosome, apart from following atypical segregation patterns, presents various differences compared to autosomes (e.g., it is smaller, more polymorphic and has fewer genes). These characteristics can be, at least partially, explained by major removals of X material to autosomes in the lineage leading to *A. rhodensis* and by the lower recombination rate of the X resulting in the maintenance of heterozygosity.

The sex determination mechanism controlling the production of females *versus* hermaphrodites is unknown. Here, I present the first steps undertaken to understand the female-hermaphrodite specification. Through empirical studies, I found that the females are generated mainly by hermaphrodite mothers and are always produced early in the brood, suggesting a maternal sex determination mechanism.

Chapter 1

Introduction

1.1 Mating systems and their evolution

1.1.1 The broad spectrum of reproductive strategies

Reproductive strategies and sex determination systems are remarkably diverse in nature. They can be studied by focusing on (i) the sex determination mechanisms (e.g., presence/absence of sex chromosomes, heteromorphic sex chromosomes, environmental sex determination systems), (ii) the mating per se (monogamy, polygamy, promiscuity as well as, behavioural and physiological aspects of mating), (iii) which gametes are produced by an individual and how they combine (mating types and contrasting male/female, hermaphroditic and parthenogenetic species) and (iv) outcrossing *versus* selfing strategies. These different categories overlap in a given species and are, in many cases, tightly linked within the biology of an individual. In this introduction, I will focus on the outcrossing *versus* selfing aspect of mating systems, using, as a background, the sexual morph of the individual (i.e., male, female, hermaphrodite, parthenogenetic female).

In this sense, mating systems encompass dioecy (separate males and females), hermaphroditism (males and female gametes produced by the same individual), pseudogamy (development via sperm stimulation of an unfertilised egg) and parthenogenesis (reproduction through females only). Some promote outcrossing (allogamy) while others favour self-fertilisation (autogamy).

Dioecy (gonochorism) is a purely sexual mating system in which organisms produce gametes with a single set of chromosomes (haploid) used for reproduction, and cells with a double set of chromosomes (diploid) for the soma. Dioecy enforces outcrossing (although it does not exclude high inbreeding rates[1]).

[1]The cheetah, for example is a male/female species with high inbreeding rates due to severe population bottlenecks.

Dioecy can be readily observed in animals [87]. However, it is important to note that the extent of dioecy throughout the phylogeny of animals is not straightforward as a balanced sampling of the different animal groups is needed to make unbiased assertions of how common dioecy truly is. Dioecy can be observed in plants such as the kiwi or the ginkgo as well.

Strict or preferential outcrossing can also be observed in hermaphroditic species, which have evolved mechanisms to limit self-fertilisation. Sequential hermaphroditism or, self-incompatibility mechanisms in the case of simultaneous hermaphroditism can promote outcrossing in both plants and animals [67, 87, 88, 122, 149].

On the other end of the spectrum, we can find species that practice self-fertilisation on a regular basis. This is the case of pure selfing hermaphroditism, obligate parthenogenesis as well as pseudogamy (sperm-dependent parthenogenesis). Obligate selfing can be observed in parthenogenetic (and more generally unisexual) vertebrate species such as lizards, salamanders and geckos [148] although there are examples of obligate parthenogenesis in invertebrates (e.g., bdelloid rotifers, marbled crayfish) [92, 202]. Some examples of predominantly selfing hermaphrodite species, which have however conserved a small level of outcrossing, are the classical model *Caenorhabditis elegans* and its most prominent satellite model *Pristionchus pacificus*, oligochaetes, some fish such as the killifish [125, 196] and 20% of the flowering plants [14], which include the model organism *Arabidopsis thaliana* and partial cleistogamous species (individuals with closed flowers which reproduce through self-fertilisation).

Between obligate outcrossing and pure selfing, we can find mixed reproductive strategies (sometimes referred to as 'facultative selfing' species). Most of these systems have been studied in plants in which it has been estimated that at least one third of all species surveyed use a mixed mating strategy [14]. Indeed, the diversity and flexibility of reproductive strategies and sexual morphs is especially rich in angiosperms (flowering plants). Most angiosperms are hermaphroditic with flowers simultaneously displaying male (anthers producing pollen) and female (stigmas with ovules) parts [13, 14, 16]. However, some species have separate male and female flowers, which can be found on the same individual (monoecy, a sort of hermaphroditism) or on distinct individuals (dioecy) [13, 14, 16]. Systems combining hermaphroditic flowers with flowers of one sex only, such as gynodioecy (hermaphrodite and females individuals), androdioecy (hermaphrodite and male individuals), gynomonoecy (hermaphrodite and female flowers on the same individual) and andromonoecy (hermaphrodite

and male flowers on the same individual) can also be observed [13, 14, 16]. Plant parthenogenesis also exists [79] and, of course, clonality, via adventitious roots for example, is widespread. The self-incompatibility mechanisms of plants (or their absence) are the best studied mechanisms preventing self-reproduction and the transition from outcrossing to principally selfing has occurred in many taxa and is arguably the most common evolutionary transition observed [178]. Given the flexibility of plant systems, intermediate levels of outcrossing and selfing can be readily observed.

In animals, mixed reproductive strategies also occur frequently [87]. Excluding insects, hermaphroditism has been found to occur in around one third of animal species and self-fertilisation was reported in all animal phyla displaying some hermaphroditic species (especially Porifera, Cnidaria, Platyhelminthes, Mollusca, Urochordata, and Annelida) [87]. However, few species practice exclusively self-fertilisation [87]. Partial and facultative parthenogenesis (development of an unfertilised egg cell) is also present across many animal groups including haplodiploid insects (bees), root-knot nematodes (genus *Meloidogyne*), water fleas (*Daphnia*), stick-insects, aphids and, to a larger extent, in vertebrates (fish, sharks, reptiles, amphibians, birds and mammals) [31, 102, 110, 139].

Mixed strategies have long been considered as unstable evolutionary intermediates [111]. Predominant outcrossing or selfing are indeed predicted to be the most evolutionary stable strategies [111] due to disruptive selection favouring one extreme or the other. However, a growing body of empirical and theoretical work, taking into consideration ecological factors, points towards the possibility of a stable maintenance of these strategies [69, 87] (see Section 1.1.4, on page 9).

The broad range of reproductive systems in nature, with varying degrees of outcrossing and selfing, provides an ideal framework to understand the origin, evolution and consequences of alternative mating systems. Furthermore, they enable us to study the costs and advantages of sex/selfing, which remains one of the most fundamental questions in biology.

1.1.2 Outcrossing *versus* selfing: costs and advantages

Sexual reproduction is widespread [152]. Thus, the consensus is that sex is generally advantageous (at least in the long term). Indeed, through recombination and random assortment of the chromosomes, new and potentially advantageous

genotypes can arise (increased diversity) while deleterious mutations can be removed. The increased diversity due to sex enables a higher efficacy of selection as the most advantageous combination will be selected for, thus allowing for an enhanced adaptability of the populations to changing environments.

However, sex is extremely costly [152]: individuals must necessarily find a mating partner to reproduce. In the case of dioecious species with a 1:1 sex ratio of males and females, this means that an individual can mate with at most 50% of the adult population as male-male or female-female encounters are reproductively null. Furthermore, the meiotic machinery, necessary to produce gametes, as well as the mating itself (specific behaviours, visual displays, attractive chemicals, special morphologies and organs) requires a big investment of energy [88, 152]. Recombination, although increasing diversity, can also break advantageous allelic combinations. Moreover, sex is risky as it exposes mating partners to diseases and predators [88, 152]. Finally, in outcrossing species, each parent contributes only half of the genetic material to its offspring, contrary to selfing species. Another way to see this cost is that, in a sexually reproducing population, only females effectively produce offspring (50% of the individuals when the sex ratios are 1:1). By contrast, in a selfing population (composed of parthenogenetic females or selfing hermaphrodites, for example), all the individuals produce the next generation. As a consequence, an asexual population grows exponentially (doubling its population size at each generation) whereas a sexual one remains constant, all other factors being equal (fitness, developmental rate...) (Figure 1.1). This cost of sex is widely referred to as 'the two-fold cost of sex' or 'the two-fold cost of males'.

Selfing strategies, besides providing an increased rate of population growth, also offer 'reproductive assurance', as selfers can reproduce on their own without the need of a mating partner [88]. They are also much cheaper energetically (no resources go into expensive sexual/reproductive traits). However, selfing reduces genome-wide diversity and can increase homozygosity as populations can arise from a single founder (severe bottleneck) [34]. Additionally, because recombination is absent or ineffective in selfing populations, the genome tends to accumulate deleterious mutations as there is no active mechanism to remove them (Muller's ratchet) [33, 34, 70]. The increased homozygosity and accumulation of deleterious alleles in selfing species can lead to inbreeding depression, i.e., the reduced survival and fitness of progeny produced by closely related or selfing organisms [88]. On a macro-evolutionary scale, the low diversity can reduce the population's ability to adapt to environmental (biotic

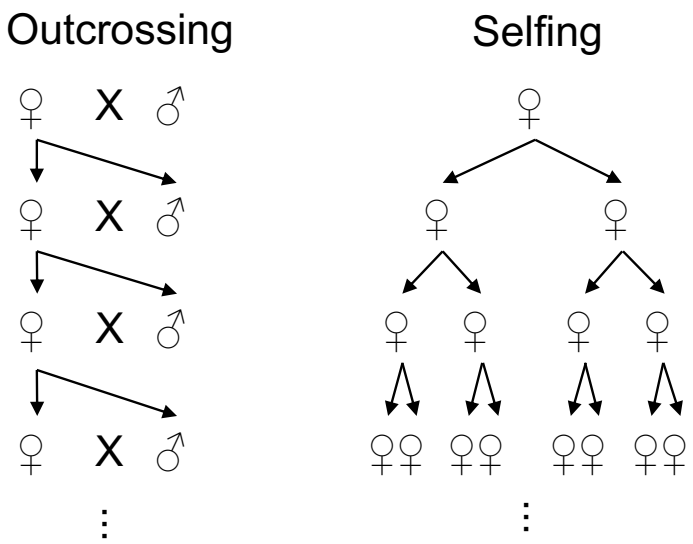


Figure 1.1: **Schematics representing the 'two-fold cost of sex'.** In this example, an outcrossing female or a selfing female produce two offspring per generation. In the outcrossing scenario, males are needed for reproduction and a 1:1 sex ratio of males and females is depicted. Consequently, half the offspring from an outcrossing female is male. The selfing population double at each generation, whereas the outcrossing population remains constant.

and abiotic) challenges and therefore lead to population extinction in the long term (dead-end hypothesis) [178]. Nonetheless, successful selfing species can purge the deleterious alleles and, by doing so, reduce inbreeding depression. Indeed as selfing increases homozygosity, recessive deleterious alleles are more exposed to natural selection. As a consequence, individuals homozygous for a recessive deleterious allele will be selected against. This decreases the number of recessive deleterious alleles in the population. Thus purging diminishes the prevalence of deleterious alleles through natural selection and increases the overall fitness of the population (reduces inbreeding depression). Additionally, as recombination is effectively non-existent, advantageous coupling of alleles is preserved [88].

To understand the evolution of mating systems, it is essential to assess the relative costs and advantages of selfing *versus* outcrossing, not only on the short term but also the long term; it is also crucial to comprehend the ecological and genetic factors favouring one reproductive strategy over the other and the extent of mixed strategies combining outcrossing and selfing.

1.1.3 Causes and consequences of mating system transitions

Selection forces such as some ecological/environmental conditions can either favour selfing or outcrossing. Low population density, marginal habitats, as well as lack of motility (sessile and slow animals) favour self-reproduction [195]. Indeed, when mating partners are rare or far away, the emergence of a selfer, capable of efficiently passing on its genetic material will be selected for.

On the other hand, antagonistic biotic interactions (pathogens/parasites *versus* host defence systems) and, more broadly, rapidly-changing environments usually favour outcrossing [54, 56, 120, 143]. By increasing genetic diversity through meiosis (recombination and random assortment of alleles) and fertilisation (the fusion of two gametes from distinct individuals), new genotypes can emerge, and, through natural selection, allow for faster adaptation and evolution of populations.

The mating system itself can also impact on various aspects of the evolution of populations. Selfing is predicted to reduce the effective population size, decrease the appearance of novel allelic combinations (ineffective or absent recombination) and increase linkage disequilibrium (i.e. the non-random association of alleles at nearby loci) and genetic hitchhiking (selective sweeps and background selection), which leads to an overall reduction in the level of polymorphism [34]. Also at the genomic level, the dynamic of selfish genetic material (such as transposable elements) is affected by the mating system [20, 209]. Transposable elements can go through an increased activity and accumulation in selfers [19, 34]. This can be due to a less efficacious selection against the proliferation of these elements. The selfing *C. elegans*, for example, has higher Tc1-like insertions than *C. remanei* [52], its outcrossing relative. The opposite, i.e., fewer transposable elements in selfing species, can also be argued [20]. Non-homologous (ectopic) recombination and deleterious insertions of transposable elements can be strongly selected against in homozygous selfers and thus, result in a reduced activity and abundance of these elements [141, 209].

These genomic consequences of selfing can cause population extinction, since low effective population size combined with low genetic diversity decreases the efficacy of selection to remove deleterious alleles and to adapt to new environments [178, 187, 208]. Selfing has, thus, been predicted to be an evolutionary 'dead-end' [178], as mentioned in the previous section, which

would explain the lack of old selfing lineages (reviewed in [83, 187]). However, it is to note that some ancient asexual lineages, such as the the selfing bdelloid rotifers, exist [92] and that direct testing of the 'dead-end hypothesis' remains challenging [83, 187].

Speciation can also be impacted by mating system transitions [208]. Indeed, the emergence of a new reproductive strategy can either create reproduction isolation within a population or loosen existing interspecific reproductive barriers. For example, it was shown that transitions to selfing in the wild tomato (*Solanum habrochaites*) can weaken the reproductive barriers between species [25]. In the flowering species genus *Capsella*, the transition to selfing, concomitant with a severe bottleneck, lead to speciation along with major morphological changes (reduced floral organs and petals) [65]. The emergence of parthenogenesis (through autopolyploidisation) in the marbled crayfish led to speciation, as mating with its parent species does not result in cross progeny [201].

Speciation and change of mating system can also arise when two closely related species hybridise [43]. This is the case for the parthenogenetic lizard *Cnemidophorus neomexicanus*, which comes from the crossing between the sexual parent species *C. inornatus* and *C. tigris* [43].

Demographic, biogeographic and population dynamics can also be affected by the mating system [15]. Indeed, in selfing species, new populations can originate from a single individual or appear in low density conditions. Sex allocation can also be distorted from the 1:1 equal resource allocation to female and male functions due to the mating system. For example, it is well documented, in plants, that selfing species allocate less resources to male functions than to female functions while most outcrossing species maintain a 1:1 ratio between the male and female functions [172].

Phenotypic traits can also be shaped by the mating system, especially those subject to sexual selection, such as courtship displays and other ritualised behaviours, ornaments (e.g., flashy feathers, big antlers), pheromones or, in angiosperms, attractive flowers (colours, forms, sizes and nutrients for pollinator attraction). These traits are important for sexual reproduction but are superfluous for a selfing species. They are therefore not observed in selfers and lost in the transition from outcrossing to selfing as they are costly and put individuals at a greater predatory and disease risk [27, 172].

Ecological and life history traits have also been seen to correlate with the mating system strategy. For example, in nematodes of the genus *Pristionchus*, predominantly hermaphroditic species were short-lived compared to females of

sister dioecious species [203].

We have seen, previously, that parasites and pathogens play an important role in the evolution of mating systems, usually by favouring sexual selection and outcrossing. The reverse, i.e., the role of the mating system on the evolution of parasitism, and more generally, on macro-evolutionary patterns (e.g., defence mechanisms) is also possible. In the Solanaceae, transitions to self-compatibility is accompanied by an increased induced-resistance strategy to specialist herbivores [30]. In the evening primrose family (Onagraceae), generalist herbivores were more successful on asexual plants than sexual plants, although this pattern was reversed for specialist herbivores [90]. Mixed mating strategies (intermediate levels of selfing and outcrossing) have been predicted to especially well suited to parasitic and parasitoid species [121]. In these systems, selfing is favoured to efficiently colonise the host but sexual reproduction provides recombination and enhanced adaptability to the host's defence strategies [121]. This is the case of some parasitic nematodes, as we will see in more detail in the following section.

1.1.4 Mixed reproductive strategies: the best of two worlds?

Considering the drawbacks and advantages of selfing and outcrossing, mixed reproductive strategies offer perhaps the best compromise allying the benefits of reproductive assurance and efficient colonisation with increased genetic diversity and adaptability.

Mixed reproductive strategies come in a variety of flavours such as haplodiploidy (observed in bees and ants), mixture of clonal and sexual reproduction (facultative parthenogenetic species; also commonly observed in plants using a clonal strategy via roots or cuttings and sexual reproduction via pollen and ovules[2]) and mixed mating systems (androdioecy, gynodioecy and trioecy).

One can even consider that depending on the conditions (e.g., presence of pathogens, absence of mating partners, highly fluctuating environments) the proportion of selfing and outcrossing can dynamically adjust to the optimal level [84].

Mixed mating strategies have originally been predicted to be unstable and genetic models have shown that disruptive selection (i.e., the favouring of

[2]The animal equivalent to this would be the species that can regenerate large parts of their body such as sea stars. However, this ability is not considered a reproductive strategy.

extreme traits over intermediate ones) would lead to the evolution of either pure outcrossing or pure selfing as evolutionary stable strategies (ESS) [14, 111]. The rationale behind these models is that the advantages of selfing interplay with inbreeding depression, which can tip a mixed system to evolve towards pure outcrossing (heterosis with strong inbreeding depression) or selfing (reduction of inbreeding depression through purging of deleterious alleles) [111]. This view has dominated how we consider mating systems and is partially supported by empirical data, in which more species are predominantly selfers or outcrossers (U-shaped distribution of number of species along a selfing to outcrossing continuum) [69, 87].

However, mixed mating systems are common in nature and very few species are purely selfing [10, 69, 88, 123]. Although one cannot discard the possibility that these mixed systems are transitioning to one or the other extreme, it has been argued that such strategies can be evolutionarily stable. In angiosperms, for example, it has been estimated that a third of all species examined use a mixed mating system with significant levels of both selfing and outcrossing [14]. In particular, animal-pollinated species do not present a bimodal distribution of outcrossing and selfing species but are rather found to be spread more or less evenly along the selfing-outcrossing continuum. The diversity of mating systems for animal-pollinated plants can even be observed within a same species with different populations displaying varying degrees of outcrossing and selfing [14]. Interestingly, this observation does not seem to hold true for wind-pollinated plants, which are found to be predominantly selfing or predominantly outcrossing, in accordance with the theoretical models. This bimodality is possibly due to a strong selection to avoid selfing, in outcrossing species, which would inevitably occur with wind [14]. To explain the high proportion of mixed mating systems in animal-pollinated plants, it has been suggested that mixed strategies are sometimes unavoidable as pollinators can induce geitonogamous reproduction (pollination between flowers of the same individual) in partially self-compatible but otherwise outcrossing plants (incomplete self-incompatibility). Another explanation is that mixed mating systems provide some reproductive assurance to animal-pollinated plants in the case were pollinators are scarce or unpredictable [14]. In general, ecological factors (and not only genetic ones), such as pollinator deficiency, male gamete discounting (missed opportunity for the pollen/sperm to be used for outcrossing because it was used for selfing), sperm/pollen limitation (insufficient amount of male gametes to fertilise the eggs), population size and density (which affect

mate availability), stresses (e.g., pathogens, starvation) and natural enemies can stabilise mixed mating strategies and are likely to play a role in mating system evolution [179].

A number of empirical studies in facultatively sexual species have also shown that the rate of outcrossing *versus* selfing in mixed systems can be condition-dependant [74]. To relate a few animal examples, an empirical study in *C. elegans* showed that starvation induces a shift in mating strategy increasing the proportion of outcrossing [142]. In *Daphnia*, a short photoperiod, food limitation and crowding cues induce sexual reproduction [101]

As mentioned earlier, mixed mating systems are also predicted to be maintained in parasitic species [1, 121]. This is observed in several species of nematodes (detailed in the following section).

1.2 Nematode mating systems and sex determination mechanisms

1.2.1 Reproductive strategies in nematodes

Nematoda (round worms) is a very diverse animal phylum. Species number estimates range between 100,000 and close to 100 million [114, 160]. Of these, more than to 20,000 species have been described [160]. Nematodes are ubiquitous and have very diverse lifestyles. Some are free-living and others are parasites [50, 99, 160] (Figure 1.2). They also display a huge diversity of phenotypic traits such as varying sizes [176], mouths forms [167, 186] and developmental trajectories [200]. In terms of reproductive strategies, they also display a large spectrum, ranging from gonochorism (male and females) to selfing hermaphroditism and parthenogenesis (See [50] for a review, Figure 1.2).

The ancestral and most common mating system of all nematodes is dioecious, with separate males and females [204]. However, selfing hermaphroditism and parthenogenesis have evolved many times independently (Figure 1.2) [50]. Mixed mating strategies, such as androdioecy and trioecy, have also been recorded in this phylum (Figure 1.2). In particular, the model organism *Caenorhabditis elegans*, the first animal to have its genome sequenced, and its satellite model *Pristionchus pacificus* are androdioecious. The populations of these extensively studied nematodes are mostly composed of selfing hermaphrodites, the males representing only a small fraction of the population.

The multiple transitions between mating systems provides an ideal framework to study the origin and maintenance of new reproductive strategies (and more generally the mechanisms at the origin of new traits).

1.2.2 Sex determination mechanisms

Sex in nematodes is usually determined through an XX/XO system [26, 73], where feminine morphs (females or hermaphrodites) possess 2 X chromosomes (XX) and males only have one X (XO) [26, 73]. This genetic sex determination (GSD) is thought to be ancestral, although some lineages, such as mermithid nematodes have switched to an Environmental Sex Determination (ESD) [73] and some species, such as some *Strongyloides* members, have lost their sexual chromosomes [183].

Although the XX/XO system does not present a sex determining chromosome (such as the Y), there exists a number of sex determining genes. These genes and, more broadly, the sex determination mechanisms have been extensively studied in *C. elegans* and, to a lesser degree, in other *Caenorhabditis* and *P. pacificus* (see [73] for a review on the topic).

In most nematodes, the ratio of X chromosomes to autosomes (X:A ratio), i.e., the ploidy, sets the XX and XO individuals on different sexual fates [128, 135, 150]. The subsequent equalisation of expression levels of X-linked genes in both XX and XO animals is done through a dosage compensation mechanism, which represses by half the X genes' expression in XX individuals [128, 135]. This differs from flies (*Drosophila*), which double the X transcription rate in XY males, and mammals, which randomly inactivate one of the X chromosomes in females.

In *C. elegans*, the gene *xol-1* is responsive to the X:A ratio. An X:A ratio (ploidy of X chromosomes over the ploidy of autosomes) of 1, inhibits *xol-1* (XX animals), while a ratio of 0.5 (XO animals) promotes its transcription [135]. The presence or absence of the *xol-1* product is the starting point of a gene regulation cascade leading to the sexual differentiation of the male or female soma and to the production of sperm or oocytes in the germline (Figure 1.3). This cascade ends with the master sex regulation gene *tra-1*. The gene *tra-1* is a transcription factor that is homologous to *Drosophila*'s *Cubitus interruptus* and the human *gli-1* [212]. In *C. elegans*, loss-of-function mutations of *tra-1* lead to the transformation of XX individuals into males (soma and germline) [77]. Likewise, gain-of-function mutations (increasing the level of *tra-1* product)

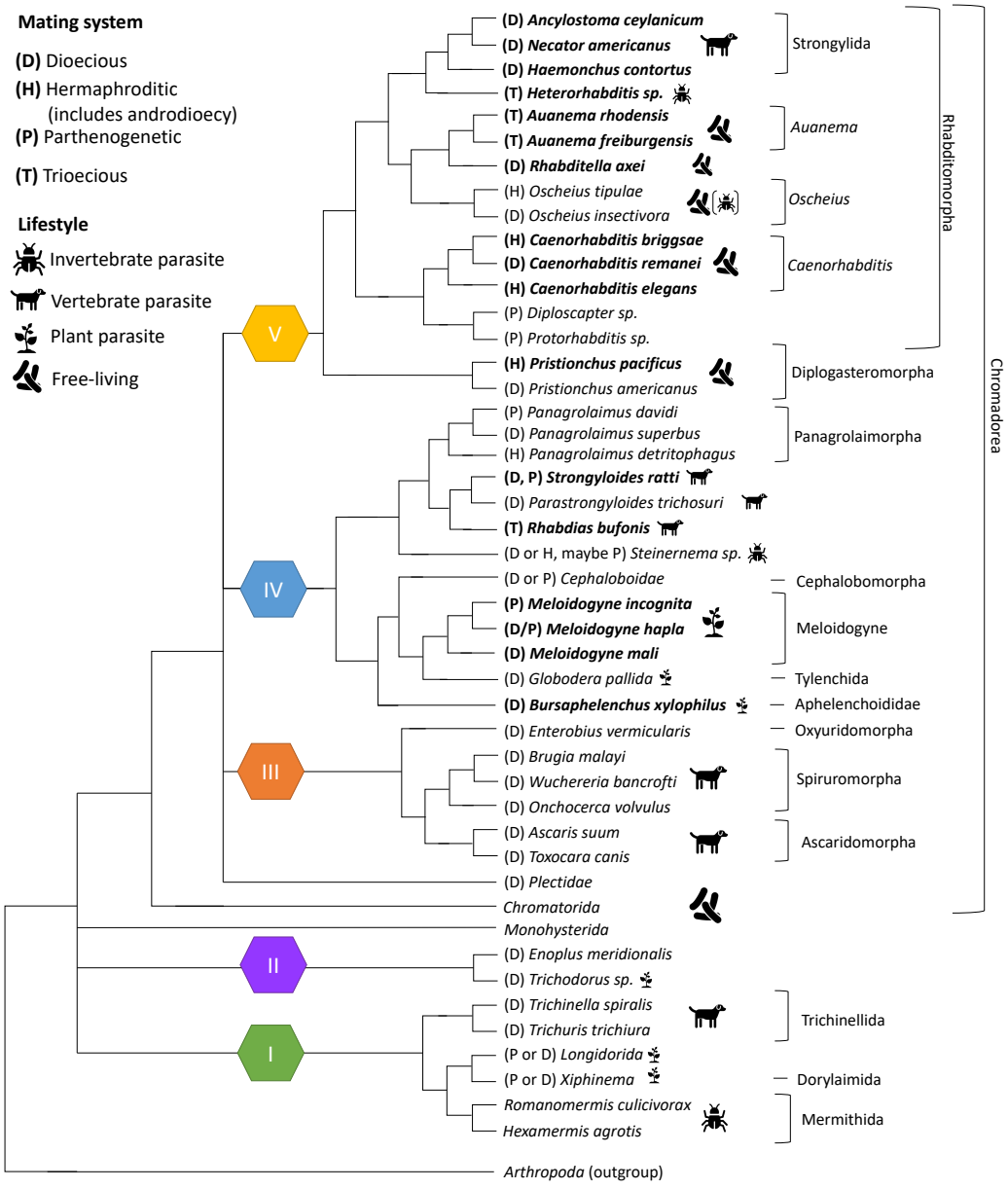


Figure 1.2: **Simplified phylogenetic tree of the phylum Nematoda highlighting the mating system and the lifestyle.** This phylogeny was built using information from various sources (see [3, 18, 42, 50, 58, 95, 99, 115, 138, 160]). The five clades (I-V), highlighted in coloured hexagons, were defined based on 18s RNA gene sequences [18]. Square brackets on the right side on the tree highlight some genera, orders, infra-orders and the class Chromadorea to aid the viewing of the phylogeny. It is to note that the nematode phylogeny still has uncertainties. In bold are the species mentioned in the thesis.

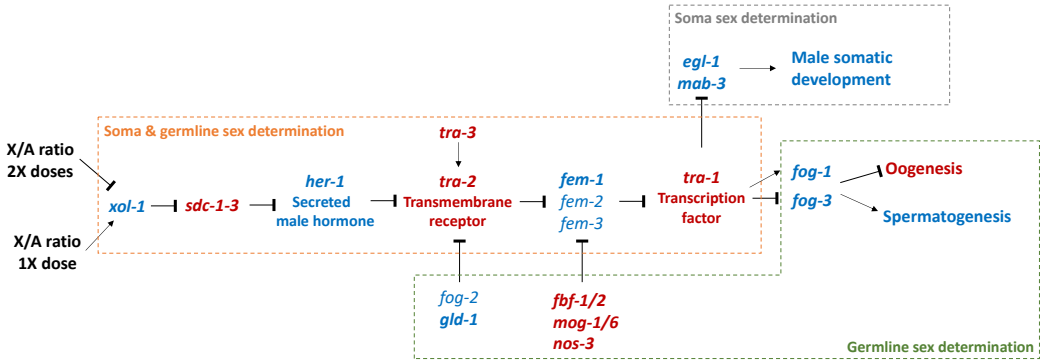


Figure 1.3: **Sex determination pathway in *C. elegans*.** Adapted from [21, 73, 157]. In Blue, are the genes that are present/activated for male sex determination control, in red are those promoting female sex determination. The genes that are not in bold are those that do not have an orthologue in *C. briggsae*.

feminise XX and XO individuals [77]. Mutants in the *tra-1* homologue of *P. pacificus* also showed that *tra-1*'s female-promoting role is conserved [159].

tra-1 regulates many genes (estimated around 364 by genome wide analyses of sex-biased gene expression during *C. elegans* larval development) but only two have been identified as direct targets: *egl-1* and *mab-3* [157].

Interestingly, *mab-3* is a deeply conserved nematode sex determining gene. *mab-3* is a DM (*doublesex/mab-3*) domain transcription regulator that is homologous to the sex determining genes *doublesex* in *Drosophila* and *dmrt1* in humans [73]. Actually, DM domain genes have been found to be expressed in the gonads of almost all animals, vertebrates and invertebrates alike (reviewed in [8]). Of particular interest, a homologous DM domain gene was discovered in corals (Cniderians), which suggests that most metazoans share an ancestral sex determination system via a DM domain regulator [136]. In *C. elegans*, *mab-3* is responsible for specifying the male fate of a limited number of cells (in the male tail).

Besides *tra-1*, *fem-1-3*, *tra-2* (a Patched-like receptor) and *tra-3* are core components of the sex determination pathway of both the soma and the germline. Specifically controlling the germline sex determination (promoting spermatogenesis or oogenesis), are *gld-1, fog-1-3, mog-1-6, fbf-1-2* and *nos-3* (Figure 1.3) [73].

It is out of the scope of this thesis to delve in the details of the sex determination pathway of *C. elegans*. Indeed, sex determination genes evolve extremely fast and even the more conserved players can have divergent roles in closely related species [8]. This is the case of the gene *gld-1*, which promotes male-fate

in *C. elegans* but seems to have the opposite role in *C. briggsae*, a closely related nematode [21, 147]. Furthermore, closely related species sometimes lack orthologues for sex determination genes. For example, no orthologue of *C. elegans*'s *fog-2* gene, necessary for hermaphrodite spermatogenesis, was found in *C. briggsae*, which is also hermaphroditic [21].

This rapid evolution of sex determination players and pathways impacts on the mating system. Indeed, by altering the expression pattern of sex determining genes (especially the ones controlling the germline sexual identity) it is possible to evolve hermaphrodites or parthenogenetic females.

1.2.3 Mating system transitions and mixed breeding strategies

Hermaphroditism in nematodes has evolved multiple times independently through convergent evolution [100, 131]. It is commonly thought that hermaphroditism emerged from a male/female population in which a mutant female, capable of making (and using) her own sperm, appeared.

In accordance with this idea, studies within the *Caenorhabditis* clade have shown that only a few mutations in sex determination genes could lead to a functional hermaphrodite [21]. Comparative studies between the hermaphroditic species *C. elegans* and *C. briggsae* and their dioecious related species *C. remanei* have shown that the gene *fog-3*, having a conserved role in promoting spermatogenesis, is expressed in the germline of *C. elegans* and *C. briggsae* hermaphrodites but is not expressed in *C. remanei* females (although *C. remanei* males express it) [21, 38].

However, hermaphroditism seems to have evolved differently between *C. elegans* and *C. briggsae* and some conserved genes have been co-opted for distinct functions.

In *C. elegans*, for example, the knockdown of any *fem* gene—which acts to repress the female-promoting gene *tra-1*—transforms hermaphrodites into females (soma and germline) [55]. However, in *C. briggsae*, the *fem* genes, although playing a role in somatic sex determination, are not essential for hermaphrodite spermatogenesis (germline) [21]. Similarly, spermatogenesis in *C. elegans* hermaphrodites requires *fog-2* and *gld-1* to downregulate *tra-2* [55]. *C. briggsae* lacks *fog-2* altogether, and *gld-1* seems to have the opposite role (promoting female-fate) [21, 147].

Finally, an elegant study by Baldi *et al.* [12], showed that it is possible to induce functional sperm production in the females of the dioecious nematode *C. remanei* by repressing the genes *tra-2* (female-promoting gene) and *swm-1* (regulator of sperm activation).

Taken together, these results show that the transition to hermaphroditism can be achieved through relatively few steps and can involve the alteration of different components of the sex determination pathway.

The appearance of a mutant female capable of making and using her own sperm (factually a hermaphrodite) in a population would subsequently result in the production of hermaphroditic progeny as they would inherit this selfing ability. Under conditions favouring selfing, this newly emerged hermaphrodite would then outcompete the females as they do not suffer from the cost of males and can reproduce without the need of a mating partner. Over time, the proportion of males would also diminish resulting in an androdioecious population or exclusive hermaphroditism (if the male populations goes extinct).

In this scenario, the initial population would pass through a transient trioecious state with males, females and hermaphrodites. In this scenario, mixed mating systems, especially trioecy, would be evolutionarily unstable.

In accordance with this view, very few trioecious species have been described. However, trioecy and trioecy-like strategies were already reported in nematodes by Maupas in his work entitled 'Modes and forms of reproduction of nematodes', published in 1900 [130]. In his monograph, Maupas writes (page 142 from the translation from French by Marie-Anne Felix): 'This incomplete hermaphroditism [described in the same work for *Rhabditis marioni*, *R. duthiersi*, and *R. viguieri*] expresses itself through the existence of females, among which some are not hermaphrodites at all any more and some are only halfway, one of the ovaries producing both genital elements, whereas the second one produces only female elements.'

More recently another case of animal trioecy was described in the sea anemone *Aiptasia diaphana* [6]. In this species, sexually undifferentiated individuals differentiate, in a first stage, into males and hermaphrodites. In a second stage, the hermaphrodites undergo sex allocation and can differentiate into males, females or hermaphrodites [6].

Returning to the subject of nematodes, a number of species displaying mixed reproductive strategies (combining outcrossing with selfing) have been actively studied (Figure 1.2). Most of these are parasitic, presenting complex life cycles in which the parasitic stage (within the host) alternates with a

free-living stage (outside the host). *Strongyloides* sp. for example, goes through a parasitic stage, characterised by females reproducing parthenogenetically, and a free-living dioecious stage [182, 183, 198, 199]. Species of the Rhabdiasidae family (*Rhabdias* sp.), parasites of amphibians and reptiles, alternate generations of parasitic hermaphrodites and free-living males and females [112]. *Heterorhabditis* spp., a genus of entomopathogenic nematodes (insect parasites), displays a complex life cycle where infective juveniles colonise an insect host and develop as self-reproducing hermaphrodites. Subsequently, males, females and hermaphrodites are produced in the host. As conditions deteriorate (lack of nutrients) more hermaphrodite-fated larvae (infective juveniles) are produced [40, 93, 215]. The evolution of these complex life cycles, intertwined with a parasitic lifestyle, is little studied and therefore, poorly understood.

Many of these species, e.g., *H. bacteriophora* and *Strongyloides*, use an environmental sex determination system together with a genetic sex determination mechanism [93, 199]. Indeed, when conditions are unfavourable (lack of food, crowding), a switch between selfing and outcrossing can be observed [93, 199]. Moreover, outcrossers have the ability to generate selfing individuals, which are adapted to colonise a new host. Not only can the selfing individuals reproduce and generate a new population on their own but, they also go through a non-feeding and dispersive larval stage referred to as the infective larvae (sometimes called the filariform larvae). The infective larvae is putatively homologous to the dauer stage in non-parasitic species, which is a non-feeding, motile and resistant larvae [45]. The molecular basis of the link between the mating strategy and this dauer-like stage has yet to be fully investigated.

1.3 *Auanema rhodensis*, a free-living 3-sex nematode

1.3.1 Isolation, maintenance and handling

Auanema rhodensis (previously referred to as *Rhabditis* sp. SB347) is a trioecious, free-living nematode. It was first isolated from blood engorged deer ticks in Rhode Island (USA) in 2001 (strain SB347). Subsequently, another isolate was discovered in West Virginia in 2012 (strain TMG33) on a dead tiger beetle. Both strains were acclimated to lab conditions and can be cultivated and studied in the same way as *C. elegans*.

A. rhodensis presents many of the advantages found in *C. elegans*: it is easily maintained and handled in the lab, it has an extremely fast life cycle (about 4-5 days). It is also phylogenetically close to *C. elegans*, allowing for comparisons to be made between both species [95, 99]. Additionally, and unlike parasitic species, there is no need to maintain host populations. However, it is closely related to parasites of the genus *Heterorhabditis* and to strongylid nematodes (e.g. *Haemonchus contortus*) [18, 95]. Its study could bring valuable insights to the origin and evolution of mating systems and perhaps on the evolution of parasitism.

1.3.2 Description of the sexes and sex determination

A. rhodensis's populations are composed of co-existing males, females and hermaphrodites [59, 95]. Like other nematodes, the sex determination of males *versus* non-males is chromosomal. Males have only one X (XO) while females and hermaphrodites have two (XX) [170]. The mechanisms controlling the sex determination and differentiation of hermaphrodites and females are unknown.

Adult characteristics

Similarly to *C. elegans*, *A. rhodensis*'s males have a characteristic blunt tail, which serves to locate and transfer the sperm into the vulva of a female or hermaphrodite. They represent only a small percentage of the populations (∼10%) [59]. Male spermatogenesis is atypical and result in the production of predominantly X-bearing sperm [170]. This is due to an asymmetrical division that occurs in spermatocytes, during meiosis II, in which most of the cellular components necessary to produce a functional sperm (e.g. mitochondrias, sperm proteins) locate in the X-bearing spermatids [170]. This is different from *C. elegans* males, which produce 50% X-bearing sperm and 50% nullo-X sperm. The asymmetrical division in *A. rhodensis* likely contributes to the small percentage of males in the population.

Female and males mutually attract each other [36]. Indeed, unlike hermaphrodites, females produce pheromones (ascarosides) that attract males. This attractiveness is lost after mating, but is recovered about 24h after the mating [36]. By ablating the vulva, the females are unable to attract males indicating that the pheromones are secreted through this organ [36]. Likewise (and contrary to hermaphrodites), females are attracted to males, although they lose this attraction after mating [36].

Females and hermaphrodites are phenotypically extremely similar. In fact, adult hermaphrodites and mated females (carrying sperm) cannot be distinguished. The chief difference is that hermaphrodites produce a continuous amount of sperm (typically around 300–400) during their adult life, which are used for self-fertilisation [132]. This is different from *C. elegans*, which produces a limited number of sperm during the L4 stage only [2, 105]. In the strict sense, *A. rhodensis* presents true simultaneous hermaphroditism while *C. elegans* displays sequential protandrous hermaphroditism. Similarly to other hermaphroditic nematodes, the hermaphrodite sperm cannot be transferred to other individuals as hermaphrodites lack the male tail to transfer the sperm (hermaphrodites are essentially females producing sperm).

Female *versus* hermaphrodite developmental differences

One of the main differences between females and hermaphrodites is their distinct larval development (Figure 1.4).

As early as the first larval instar (L1), it is possible to differentiate both sexes, under a microscope, as female-fated larvae present a large gonad primordium whereas hermaphrodite-fated have a smaller one [37].

Hermaphrodites also always pass through an arrested larval stage called the dauer [37]. The dauer is a non-feeding stage, which is usually considered the resistant and dispersive form of many nematode species. In *A. rhodensis*, the dispersive behaviour of the dauer known as nictation, i.e., when the worm stands on its tail and waves its body (also called 'tube waving'), can be observed [59]. The dauer phase is necessary and sufficient to determine the hermaphrodite sex and consequently, females and males never go through a dauer stage [37].

The obligate link between dauer and hermaphroditism, observed in *A. rhodensis*, is reminiscent of the link between infective larvae and selfing individuals in some parasitic species [199, 215]. For example, infective juveniles of *H. bacteriophora* develop as selfing hermaphrodites [215] and infective L3 larvae of *Strongyloides* spp. develop into parthenogenetic females [199].

All things considered, *A. rhodensis* presents an ideal model to (i) understand the origin and stability of alternative mating systems, (ii) explore the mechanisms controlling the sexual fate of females *versus* hermaphrodites, two karyotypically identical sexes, (iii) investigate the molecular basis of the dauer-hermaphroditism link, (iv) explore the possibility that free-living trioecy, displaying the dauer-hermaphroditism link, could have been co-opted in the

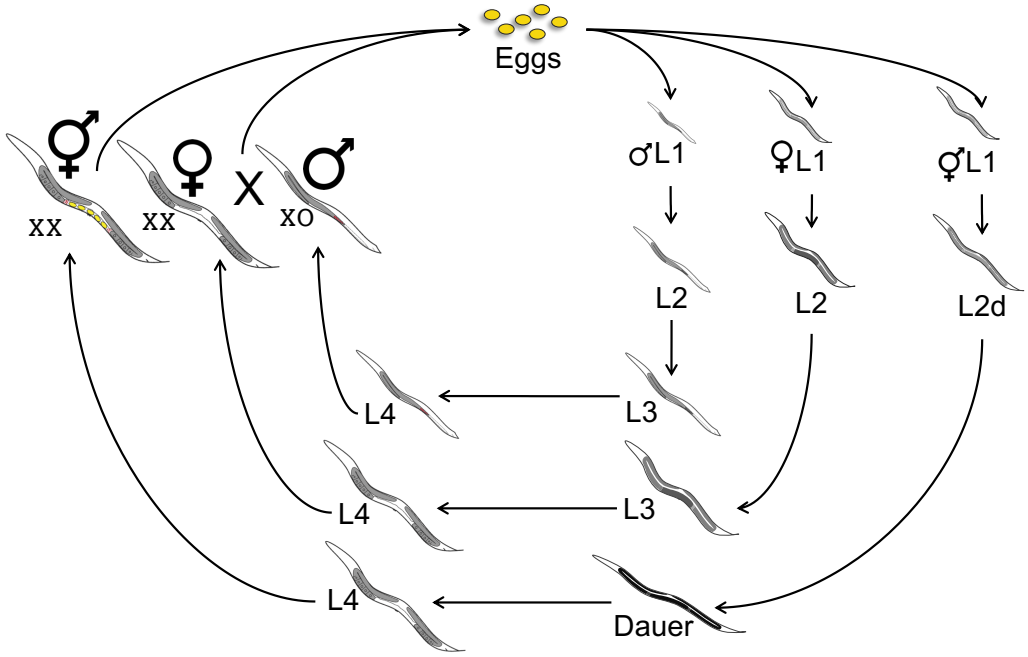


Figure 1.4: **Life cycle of *A. rhodensis*.** Hermaphrodites are self fertile but unable to transmit their sperm to a female. They always pass through an obligate dauer stage (stress resistant larvae). Males and females must cross to reproduce and never pass through the dauer stage. Females and hermaphrodites can be distinguished at the L1 stage, under the microscope, as female-fated L1s have bigger gonad primordia than hermaphrodite-fated L1s. Males are capable of mating with hermaphrodites.

evolution towards parasitism.

1.4 Scope of the thesis

This thesis explores the biology of the three-sex nematode *Auanema rhodensis*. First, I will present empirical data about the population dynamics of *A. rhodensis* and simulations based on the data collected in various population scenarios (alternative mating strategies) (Chapter 2). From this I will discuss the stability of trioecy in *A. rhodensis* and speculate on possible evolutionary explanations for the maintenance of this strategy.

Secondly, I will characterise the genome and genetic map of *A. rhodensis* (Chapter 3). In this chapter, chromosome-wide characteristics will be discussed, especially differences observed between the autosomes and the sex chromo-

some (X). I will also explore chromosomal reorganisations that have occurred in the phylogenetic branch leading to *A. rhodensis*. Additionally, I will present genomic comparisons between *A. rhodensis* and other nematodes with different mating strategies and lifestyle (e.g., parasitic or free-living).

Finally, I will present the atypical inheritance of the X chromosome in *A. rhodensis* (Chapter 4). This chapter continues the investigation of the X chromosome peculiarities, which were already observed in the genomic data (presented in Chapter 3).

Chapter 2

Population dynamics of *A. rhodensis*

Summary

In this chapter, I investigate the intricate life cycle dynamics of *A. rhodensis* and show that hermaphrodite mothers produce more females than female mothers, which essentially produce hermaphrodites. Female progeny are produced early in the brood, suggesting a maternal control of the production of females *versus* hermaphrodites. Simulations of various populations help discuss the maintenance of trioecy and the role of the different sexes.

2.1 Introduction

Trioecy is thought to be an evolutionary unstable mating system, which would only appear as an intermediate stage in the transition from one mating system (e.g., gonochorism) to another (e.g., hermaphroditism) [111]. The different mating system transitions between gonochorism and hermaphroditism are depicted in Figure 2.1. More generally, as we have seen in the introduction, mixed reproductive systems (combining outcrossing and selfing within a same species) have long been considered transient as one strategy (e.g., outcrossing) would eventually be favoured over the other (e.g., selfing) leading to the evolution of one predominant strategy (e.g., outcrossing) [111].

Nematodes are great models to study the evolution of mating systems as many novel mating strategies have emerged, some of them many times independently (Figure 1.2, see [50] for a review). Moreover, they are simple animal models, usually reproducing rapidly and abundantly, which facilitates their study. Of particular interest to us, some species, notably parasitic, such as *Heterorhabditis* spp., *Strongyloides* spp. and *Rhabdias* spp., are stably

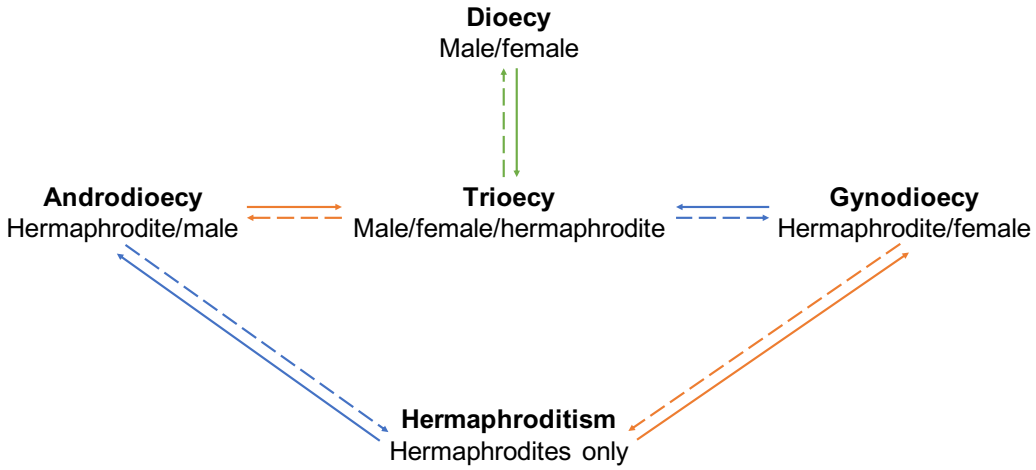


Figure 2.1: **Possible transitions between mating systems.** Blue, orange and green refer to the acquisition or loss of males, females and hermaphrodites, respectively. Dashed lines represent losses, solid lines represent acquisitions.

trioecious or exhibit heterogonic life cycles (alternation of outcrossing and selfing generations) [40, 112, 182, 198, 199, 215]. In these cases, the mating system is tightly linked to the particular lifestyle of the species, especially to the eco-environmental conditions (host colonisation and migration).

A. rhodensis is free-living and all three sexes can be observed simultaneously in its populations [36, 37, 59, 95]. The developmentally arrested and dispersive stage, named dauer, is strictly linked to hermaphroditism, such that all individuals passing through the dauer stage develop as hermaphrodites [36, 37, 189]. This is a common point with some parasitic species with mixed mating systems, such as *Strongyloides* spp. and *Heterorhabditis* spp., in which the infective larvae, which is putatively analogous to the dauer stage of free-living species [45], is strictly associated with the selfing sex [199, 215]. It is therefore an interesting model to investigate if this selfing-dispersive link was co-opted in the evolution towards parasitism (Figure 2.2).

In *A. rhodensis*, females and hermaphrodites are karyotypically identical and assumed to be genetically identical as well [37]. Yet, they present very different developmental trajectories [36, 37, 189]. As we have seen in the introduction, the dauer stage is obligatory and sufficient to hermaphrodite development [36, 37, 189]. Actually, by modulating dauer entry using chemicals, it is possible to convert female-fated individuals to hermaphrodites and vice versa (personal communication Sally Adams, [37]). These observations suggest that an Environmental Sex Determination system (ESD) could be at play.

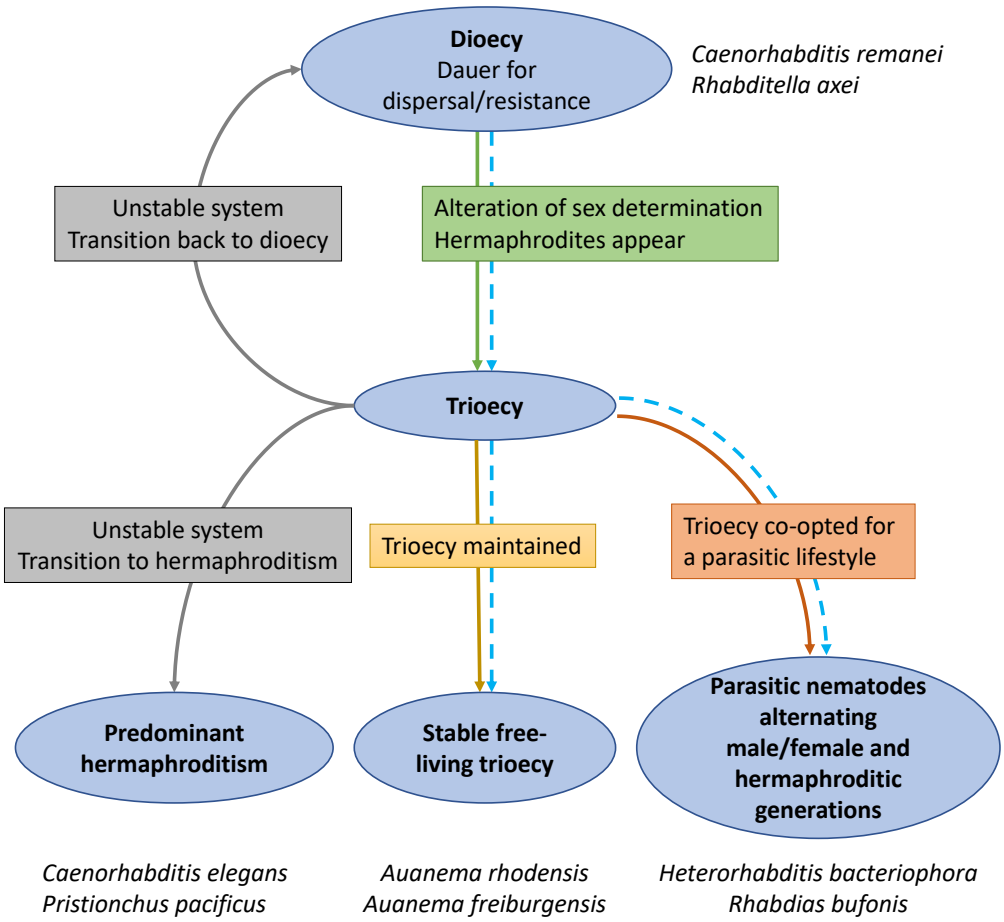


Figure 2.2: **Diagram representing hypothetical evolutionary paths relating dauer formation, trioecy and parasitism.** In orange are the paths linking trioecy to parasitism, in gray are the ones showing trioecy as a transitive mating system. In blue, are paths leading to a stable free-living trioecious system (factors and mechanisms largely unknown). The paths shadowed in dashed blue are those in which the coupling between dauer/infective larvae and sex determination of self reproducing individuals could have originated.

In *A. rhodensis*, sexual fate is decided early on (during embryogenesis) as L1 females present a larger gonad primordium than L1 hermaphrodites [37, 59]. Moreover, in standard, non-stressful and constant laboratory conditions, hermaphrodites are also produced at high levels [37]. Furthermore, it was also shown that hermaphrodite mothers produce most of the female progeny within the first 15 h of their reproductive life [37]. The molecular mechanism controlling this sequential production of females and hermaphrodites is unknown. Because sex is specified so early and sequentially, a maternal sex determination system may also control the female *versus* hermaphrodite sex determination.

A. rhodensis is also interesting to look at from the view of sex allocation and, more generally resource allocation (especially the allocation balance between selfing and outcrossing). Indeed, the nature and mechanisms controlling the outcrossing *versus* selfing are unclear as we do not know if external factors are implicated (as in *Heterorhabditis bacteriophora* [93] and *Strongyloides ratti*) [199] or not (as in *Rhabdias* spp. [112]).

To better understand the dynamics of *A. rhodensis* populations and assess the stability of this three-sex system, I first characterised the F1 progeny of selfing hermaphrodites and outcrossing females during their adult lifespan. Using these empirical data, I simulated the population growth in various settings. I discuss the population dynamics and speculate on the potential advantages and constraints of this trioecious system.

2.2 Materials and Methods

2.2.1 Nematode strain and husbandry

In this chapter, the inbred *A. rhodensis* strain APS4 was used. This strain was derived from the inbreeding of the original strain SB347, isolated from a deer tick (Rhode Island, USA) [59]. Inbreeding was accomplished by performing several rounds of bottlenecking using single selfing hermaphrodite animals. The strain APS4 underwent 50 rounds of bottlenecking. Inbreeding was performed prior to this project by former lab members.

Strains were maintained at 20 °C on plates containing Nematode Growth Medium ($3\,\mathrm{g\,l^{-1}}$ sodium chloride, $2.5\,\mathrm{g\,l^{-1}}$ bacto peptone, $17\,\mathrm{g\,l^{-1}}$ agar, 1 mM magnesium sulfate, $5\,\mathrm{mg\,l^{-1}}$ cholesterol, 1 mM calcium chloride, 25 mM potassium phosphate) [22] seeded with the *Escherichia coli* streptomycin resistant

strain OP50-1. Microbial contamination was prevented by adding 10 µg ml^{-1} of nystatin and 50 µg ml^{-1} of streptomycin to the NGM.

2.2.2 Sex ratios from outcrossing females and selfing hermaphrodites

The entire broods of 20 selfing hermaphrodites and 19 outcrossing females were characterised every 8h of their reproductive life (from the moment they reached adulthood until their death). The characterisation of the hermaphrodite and female broods was not synchronised. All replicates of the female and hermaphrodite brood were performed in parallel. At each time point, the number of males, females and hermaphrodites was counted. It is to note that the number of each sex at each time point is not independent as the same mothers were used throughout the study. We therefore have 20 hermaphrodite and 19 female whole-brood replicates.

To determine the sex ratios the F1s produced from selfing, the self-progeny of 20 hermaphrodites was collected throughout their adult lifespan (∼6 days). The hermaphrodite mothers were selected by first isolating dauers, which in *A. rhodensis* always develop into hermaphrodites [37]. Dauers were placed into single 6 cm agar plates seeded with *E. coli* OP50-1 (streptomycin resistant strain) and left to mature into adults. Mothers were transferred to a new plate every 8 h of their reproductive life until they stopped laying eggs. The sex of the progeny was determined while they were still larvae (two days after hatching, L2/L3 larval stage) to control more precisely for worms that may escape the plate before being scored. I used developmental rates, morphology and colour to distinguish the different sexes. Larvae fated to become hermaphrodites were characterised by being thin and dark (typical characteristics of pre-dauer and dauer stage). Females develop faster (the L3 stage is shorter than the dauer stage), and, in age synchronised plates, are comparatively larger (and whiter) than hermaphrodites. Male larvae have a characteristically blunt tail and a different pattern of pigmentation.

To determine the sex ratios of the F1s produced by outcrossing females, 19 crosses were performed. To ensure that females had been isolated and that they were only crossed having reached adulthood, I waited to observe unfertilised eggs from females prior to crossing them with males. This was done by checking every 2-3 hours female L4s (age inferred by their size) until I observed unfertilised eggs (brownish and disintegrating). The females, which

reached adulthood in the same 2-3 hour period, were crossed with males. Each crossing experiment consisted of one male and one female placed for a few hours (1-5h) in a 6 cm agar plate seeded with *E. coli* OP50-1 (as above). To ensure fertilisation over the entire lifespan of the female, a new male was added for a few hours every day. The sex of the progeny was determined as described above.

Unfortunately, it was not possible to determine the sex ratios of the progeny resulting from the cross between a hermaphrodite and a male. It is known that in androdioecious (hermaphrodite/male) nematode species, such as *C. elegans*, hermaphrodites (that are essentially females able to produce sperm) can successfully cross with males. In *C. elegans*, hermaphrodites produce a finite amount of sperm used for self fertilisation. When they have used all their sperm (sperm-depleted), they are still able to reproduce by crossing with males. By contrast, *A. rhodensis* hermaphrodites cannot be sperm-depleted as they produced sperm continuously [132] and therefore produce progeny during their entire lifespan. As a consequence, the use of sperm-depleted hermaphrodite was not possible to determine the sex ratios from hermaphrodite-male crosses. An alternative strategy is to use a mutant homozygous strain with a very distinct phenotype. In this case, the phenotype (mutant or wild type) of the progeny resulting from a cross between mutant and wild type individuals would indicate if it came from selfing or outcrossing. Unfortunately, at the time of the experiment, a mutant line was not available.

2.2.3 Population dynamic simulations

To assess the stability of the trioecious state and understand the possible role played by each sex, I wrote a Python script (simulator) to simulate a population growth, keeping track of the number and proportion of each sex. Essentially, the simulator receives initial parameters about females and hermaphrodites, such as the rate of birth (number of progeny produced depending on the sex and age of the mother), the proportion of each sex produced, the reproductive lifespan and the larval period. The population is then updated step-wise. An iteration step of the simulator corresponds to 8h during which individuals age and produce offspring according to their sex and age. The simulator outputs, for each iteration step, the number of individuals of each sex (all ages taken together) and their proportions. Alternatively, it can also output the number of dauers present in the population at each step.

This script was used to simulate various populations in different scenarios. The parameters used for the different simulations are summarised in Table 2.1. For the comparisons between the different simulations, I focused on the time interval between 140 h and 210 h (and between 50 h and 260 h for the dauer growth to have a wider perspective) after the colonisation of one dauer in an optimal environment. I chose this interval assuming that *A. rhodensis* has a similar population growth as *C. elegans*. In *C. elegans*, the population can reach a few thousands of individuals before crashing due to lack of food (boom and bust strategy). Another reason to look at this interval, is that in our laboratory conditions, the population reached a very high density (and faced severe lack of food) around 7 days (168 h) after a single dauer was placed in a 6 cm petri dish.

The simulations performed always started with one dauer larva that colonised a habitat favourable for growth.

The script of this simulator with input and output data can be consulted on GitHub: `https://github.com/sophietandonnet/Simulation-SB347` The simulator was written in Python3 with invaluable help from Tássio Naia.

Table 2.1: **Parameters used for the different simulations** 'H', 'F' and 'M' stand for 'Hermaphrodite', 'Female' and 'Male', respectively. The birth rate correspond to the number of progeny produced at each time point of the mother's lifespan. The proportion of H/F/M corresponds to the proportion of each sex produced at a given time point by either the hermaphrodite or female mother. * For this simulation, the female part of the simulator was modified to correspond to hermaphrodites that bypass dauer (fast hermaphrodites).

Simulation	H birth rate	F birth rate	Proportion of H/F/M (by H mothers)	Proportion of H/F/M (by F mothers)	Developmental time
Observed	Observed H birth rate	Observed F birth rate	Observed H Proportions	Observed F proportions	24h delay for H development
Fem_come_later	Observed H birth rate	Observed F birth rate	Reversed observed H proportions	Reversed observed F proportions	24h delay for H development
Rhabdias-like	Observed H birth rate	Observed F birth rate	Only F and M produced	Only H produced	24h delay for H development
Only_herm	Observed H birth rate	Not Applicable	Only H produced	Not Applicable	24h delay for H development
Only_herm_fac_dauer*	Observed H birth rate	Observed F birth rate	H proportion as observed; F as M + F; no M	H proportion as observed; F as M + F; no M	24h delay for slow H development, Fast H as observed F
No_developmental_delay	Observed H birth rate	Observed F birth rate	Observed H Proportions	Observed F proportions	F dev. time for both F and H
Observed_same_rate	Observed H birth rate	Observed H birth rate	Observed H Proportions	Observed F proportions	24h delay for H development

2.3 Results and Discussion

2.3.1 Hermaphrodites and females brood characteristics

Brood size and composition

As a first step to understand the population dynamics of this trioecious nematode, I sexed entire broods of selfing hermaphrodites and outcrossing females. Unfortunately, at the time of this experiment, it was not possible to determine the sex of offspring produced by outcrossing hermaphrodites (see methods).

Both females and hermaphrodites reached their peak offspring production during day 2 and 3 (Figure 2.3). It then declined steadily until their death. On average, selfing hermaphrodites and outcrossing females produced 377 and 296 offspring, respectively. Total brood sizes of females and hermaphrodites differed (t of Student = -2.7988, df = 37, p-value = 0.0081 ; Shapiro Wilk normality test: W = 0.94622, p-value = 0.3401 and homocedasticity F test: F = 0.83999, num df = 18, denom df = 19, p-value = 0.7151). The difference in progeny number between females (296 ± 20 SEM F1s) and selfing hermaphrodites (377 ± 21 SEM F1s) could be due (at least partially) to the waste of the first eggs laid by females (See methods). However, and regardless of the reasons, the brood sizes difference between females and hermaphrodites is minimal compared to the large discrepancy that can be observed in some trioecious species, such as *Rhabdias*. Typically, a *Rhabdias* female produces very few progeny (1–4 F1s), whereas a hermaphrodite produces thousands of F1s [11, 68, 109, 194].

The overall brood compositions of F1s produced by females and hermaphrodites differed ($\chi^2 = 30.118$, df = 2, p-value = 2.884×10^{-07} ; Table 2.2, Figure 2.3). In particular, female mothers produced less female offspring than hermaphrodite mothers (Wilcoxon Mann Whitney test: W = 0, p-value = 9.989e-08) as well as less male offspring (Wilcoxon Mann Whitney test: W = 82.5, p-value = 0.002567). However, it is to note that no difference in the number of hermaphrodites produced by both types of mother was found (W = 167, p-value = 0.5272). An explanation for this is that the variability of the number of hermaphrodite offspring (standard deviation = 85.2 for female mothers and 89.2 for hermaphrodite mothers) was much larger than the variability of the number of female offspring (standard deviations of 8.2 and 34.6 for female and hermaphrodite mothers, respectively) and male progeny (standard deviations

of 5.6 and 3.1 for female and hermaphrodite mothers, respectively).

Table 2.2: **Sex ratios of whole broods of *A. rhodensis* from selfing and crossing parents.** The abbreviations 'Herm.' and 'Fem.' stand for 'Hermaphrodites' and 'Females'. SD stands for Standard Deviation.

Parents	**Mean [SD] (%) F1 Herm.**	**Mean [SD] (%) F1 Fem.**	**Mean [SD] (%) F1 Male**	**Total # F1s**
Selfing Herm.	285.5 [89.28] (75.7)	78.05 [34.68] (20.7)	13.55 [3.17] (3.6)	7543
Female x Male	269.9 [85.22] (91.1)	17.84 [8.20] (6.0)	8.526 [5.68] (2.9)	5642

The mean percentage of male progeny produced over the entire reproductive period is usually below 5% for both selfing and outcrossing parents (Table 2.2). The percentage of male progeny that I observed for selfing hermaphrodite parents is lower than previously reported [59]. However, variations in the degree of inbreeding of the strain, sample size and the methodology used (I counted entire broods and controlled for potential escaping worms) could explain the difference.

Early production of females

In a previous experiment, it was found that hermaphrodite mothers tend to produce more female progeny in the beginning of their adult lives [37]. With this experiment, I was able to confirm this pattern as hermaphrodites produce a higher number of females in the first day of their adult life than on the second day ($\chi^2 = 353.3, P < 0.001$, Figure 2.3A). Furthermore, I could test if a similar trend could be observed in the case of outcrossing female mothers. Indeed, outcrossing females also produced more female progeny in the first day of their adult life than on the second day ($\chi^2 = 381.7, P < 0.001$, Figure 2.3B).

The data of this experiment was included in an article [36] and is available on GitHub at `https://github.com/sophietandonnet/F1-data-of-SB347`

Note: Although all replicates were performed in parallel under the same conditions, and the strain used had been inbred for 50 generations, I observed a lot of variability between the different mothers. This may be due to some stochastic factor or reflect a factor not controlled for. This high variability between individuals was also observed in independent experiments (conducted by other lab members) using *A. rhodensis*.

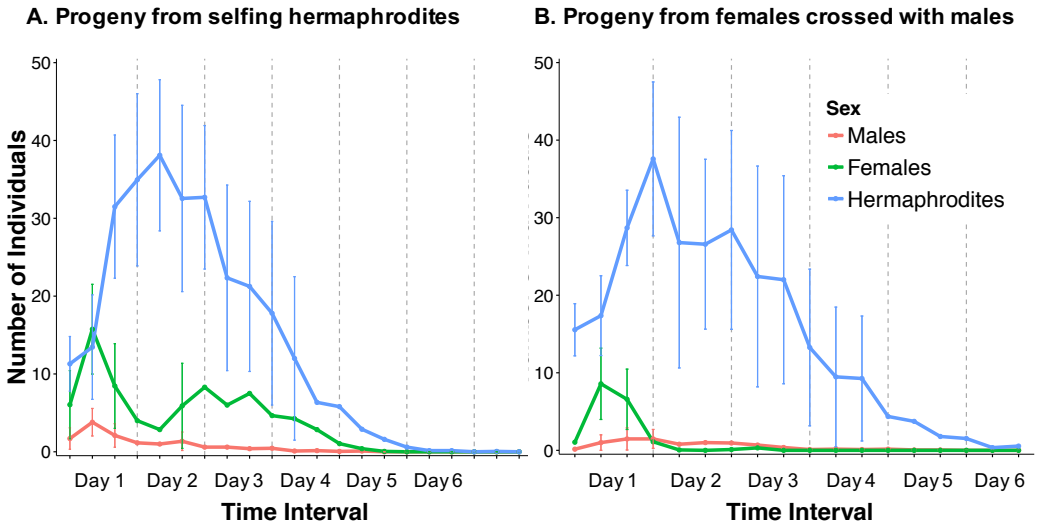


Figure 2.3: **Sex of F1 offspring from selfing or outcrossing parents.** Mean number of males, females and hermaphrodites produced by (A) hermaphrodites (n=20) and (B) females (n=19) each day of their reproductive life. Error bars are standard deviations. The data for each time point is not independent as the same mothers were used throughout the study. Dashed gray lines delimit each 24 h of egg laying. Each x axis tick delimits an 8 h interval of egg laying.

2.3.2 Simulating the population dynamics of *A. rhodensis*

Sex ratio dynamics

I used the empirical data to look into variation in the sex ratios during the population growth originating from a single dauer larvae (Figure 2.4). The sex ratio (taking into account larvae and adults) fluctuates less and less as the population grows and seems to stabilise around 22% females, 74% hermaphrodite and 4% males (Figure 2.4A). The sex ratio dynamics of the larval fraction of the population is very similar to the overall population (Figure 2.4B). However, the sex ratio of the adult fraction of the population fluctuates a lot and, intriguingly, the proportion of adult females and males is much higher (∼40% of females compared to ∼20%). This is due to the faster development of females and males (hermaphrodite development is delayed by 24 h due to the dauer stage). To understand this counter-intuitive result, consider the following simplified mental experiment: a coin is flipped and if it lands on 'heads' we add an individual to the category of males and females, if the result is 'tails' we had an individual to the category of hermaphrodites. If we repeat this experiment many times, the number of hermaphrodites and females will

grow following the distribution of heads and tails obtained from the coin flips. Let's consider now the same experiment but, if the coin lands on 'tails' the hermaphrodite is not counted immediately. This hermaphrodite will be added to the pool of counted hermaphrodites only after 10 coin flips have elapsed (this would represent the delay in development of the hermaphrodites). In this case, the number of hermaphrodites will grow at the same rate as if no delay was applied, however, this growth will be shifted 10 coin flips.

Population growth and survival potential

The script also allowed me to simulate different populations with alternative mating systems (Figure 2.5), the aim being to better understand the trioecious system of *A. rhodensis*. I compared the populations based on the growth rate of the population (Figure 2.5A) and on the number of dauers present (Figure 2.5B). The dauers, being the resistant and dispersive form of the species, were used as an indicator of the survival potential of a population.

I first simulated an '`observed`' population for which I only used data collected empirically (see previous section). In this simulation, mothers produce more females in the beginning of their reproductive lives. I contrasted the results obtained with a population in which mothers produce females late in life ('`females_come_later`' in Figure 2.5). I observed that when females are laid at the end of the reproductive life of their mother the population grows slower than in the '`observed`' population up to around 200 h. From 200 h on the population rate exceeds the one of the '`observed`' population. Females, developing faster and part of the first progeny, could offer an initial population boost. This may be important to quickly take advantage of the resources of the new habitat. The survival potential (number of dauers) was comparable between these two simulations.

To further show the importance of the developmental delay of hermaphrodites, I simulated a population in which hermaphrodites develop as fast as females ('`No_developmental_delay`'). This idealised population grew tremendously fast (the number of individuals of this population was on average 108% more than the number of individuals in the '`observed`' population, within the time interval between 140h and 210h), and produced more dauers sooner. I then simulated populations composed exclusively of hermaphrodites ('`Only_herm`'), in which all individuals (hermaphrodites) pass through dauer. As expected, this population growth is severely slowed down compared to the '`observed`'

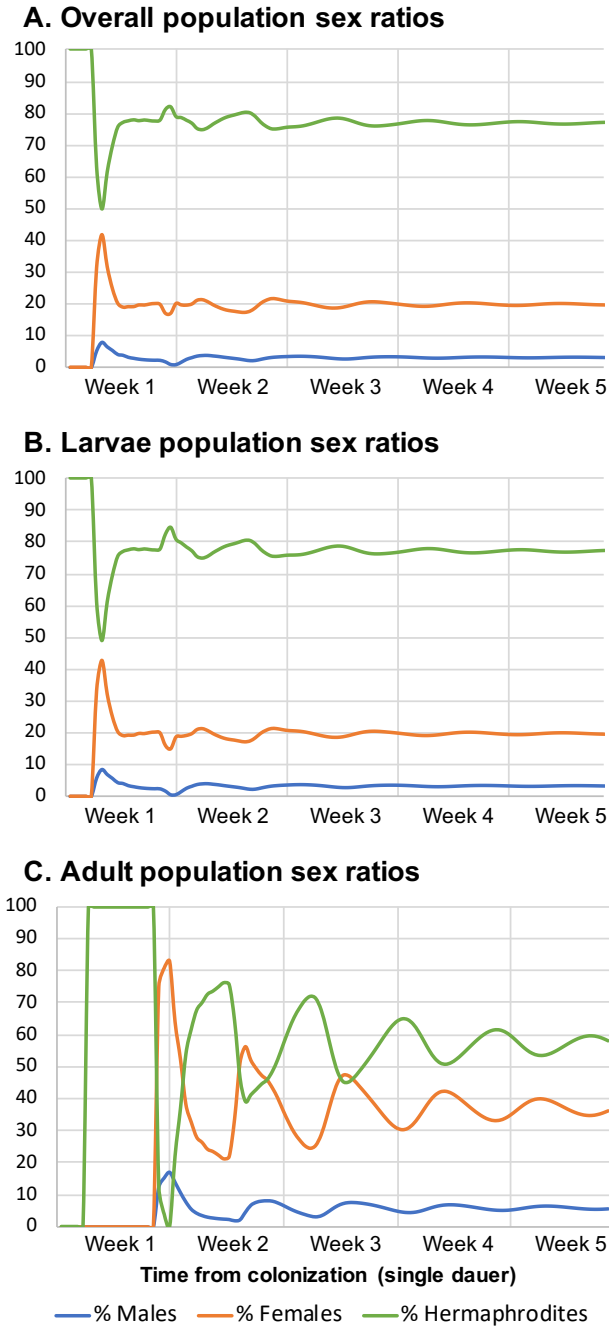


Figure 2.4: **Sex ratios of an expanding population originating from a single dauer hermaphrodite.** (A) Overall population sex ratios (larvae and adults). After some initial fluctuation, the sex ratio stabilises at around 74% hermaphrodites, 22% females and 4% males. (B) Sex ratios of the larval fraction of the growing population. The larvae population sex ratios are very similar to the overall population ratios. (C) Sex ratios of the adult fraction of the growing population. The adult sex ratios differ greatly and fluctuate more than the overall population sex ratios. This is due to the females and males reaching adulthood faster than the hermaphrodites. Hermaphrodite, female and male percentages are depicted by green, orange and blue lines.

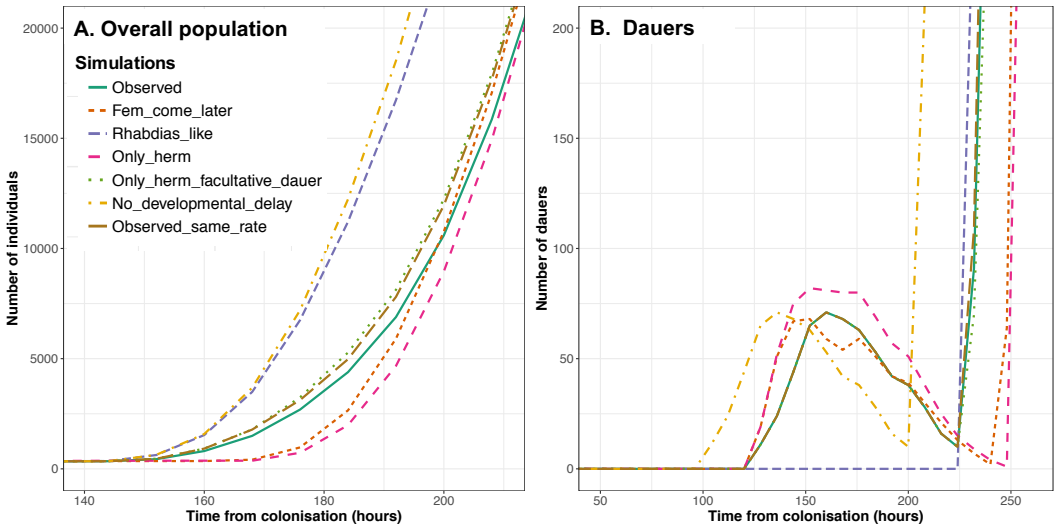


Figure 2.5: **Simulated population growth in various scenarios.** (A) Overall population growth of various simulations from 88 h to 144 h after colonisation of a new habitat from a single dauer. (B) Number of dauers in the different simulated populations.

population (on average, the population had 81% less individuals than the '`observed`' population in the time interval considered) but the number of dauer is slightly higher. This shows that the females and males in *A. rhodensis* could provide a faster growth to the population. This could be important for the population to quickly take advantage of the resources of the habitat to colonise.

We also considered a second and improved hermaphroditic population ('`Only_herm_facultative_dauer`'), where each hermaphrodite either passes through dauer (delayed development) or bypasses this stage (fast development). For this simulation, I chose the proportion of hermaphrodites that bypass dauer to occur in the same proportion as the sum of the observed proportions of females and males in the '`observed`' population. This simulation would be equivalent to the 'observed' population if the proportion of males in the population remained the same. However, we can theorise that in a hermaphroditic population, the proportion of males would be extremely low (here, for simplification, the male proportion was considered to be 0). This simulated population essentially behaves the same as the '`observed`' population, with a slightly higher population growth and the same number of dauers. By removing the obligatory link between hermaphroditism and dauer, the potential advantage of faster development offered by females and males is removed. One possibility is that uncoupling dauer formation and hermaphroditism is not possible because of

developmental constraints. It is also conceivable that outcrossing is preserved to maintain higher levels of genetic variability. Because dauers are produced constitutively and are presumably the dispersive form of the species, gene fluxes between populations may exist. The initial females and males produced by an emigrating dauer in an established population could cross with males and females of that population, potentially giving rise to more adapted genotypes.

Finally, I simulated a '`Rhabdias-like`' population with individuals that cycle between hermaphroditic and gonochoristic generations (as found in parasitic nematodes of the family Rhabdiasidae). This population displays an extremely fast growth rate but dauers only appear 128h after the colonisation event. In the case of *Rhabdias* species, which are parasites of vertebrates, this strategy is very efficient because the nematode populations can grow up to high numbers of individuals. *A. rhodensis* was found on arthropods (beetle and tick), which are much smaller resources. However, it is to note that it is unclear, if *A. rhodensis* completes its life cycle on these species or just uses them as carriers.

The major issue in understanding the population dynamics and assessing the potential of combining outcrossing and selfing is that very little is known of the ecology of *A. rhodensis*. We do not know the maximum size of an average population or if migration fluxes between populations exist. The environmental fluctuations as well as biotic and abiotic stresses are also unknown. Therefore, the discussion on the advantages of sex to maintain genetic diversity and therefore shoulder against random environmental variations remain untested theories.

Furthermore, the population simulations performed here follow a simplistic and deterministic algorithm. The simulator does not take into account hermaphrodite-male crosses, assumes that all females get fertilised by males and considers that all individuals of a same sex/age behave the same. The modelling of the populations' dynamics could be improved by adding a stochastic component to the offspring production, sex determination distribution and developmental duration (particularly dauer duration). A probability could be assigned to the female-male encounters (depending on the proportion of males available, for example). The effect of environmental factors (such as crowding) could also be included in the model. For example, one could imagine a scenario were fewer offspring are produced, the more crowded the population becomes.

2.4 Concluding thoughts

Free-living trioecy is exceedingly rare (perhaps due to our biased knowledge) and it is unclear what are the genetic, physiological and ecological factors stabilising the mixed state (outcrossing and selfing coexisting) in *A. rhodensis*. In laboratory conditions (constant environment), the three sexes of *A. rhodensis* are persistent as multiple generations do not result in the extinction of one or two sexes.

Another observation is that the proportion of males, females and hermaphrodites in *A. rhodensis* is unequal (Figures 2.4, 2.3, Table 2.2). The theory of sex allocation provides a rich background to understanding sex ratios and their deviation from the 1:1 proportion of males and females. Sex allocation is the amount of resources allotted to the male and female functions. In 1930, Fisher developed an explanation for the equal sex allocation between males and females known as Fisher's principle. He argued that any deviations from the 1:1 proportion would provide a selective advantage to the individuals able to produce an increased amount of the rarer sex. As a consequence, the proportion of the rarer sex would increase, eventually restoring the 1:1 initial sex ratio. This idea is valid under the assumption that both sexes are equally costly to produce. If one sex is twice as costly to produce but also twice as efficient, Fisher's principle predicts that the sex ratios would stabilise at 1:2, with a bias towards the less costly sex. Deviations from these 'Fisherian sex ratios' can be observed and the theory of sex allocation has expanded to cover many cases of skewed sex ratios.

However, in our system, hermaphrodites have male function rendering the male *versus* female sex allocation difficult to estimate precisely. For simplicity, one could consider an equal sex allocation between the male and female functions in hermaphrodites. Even considering this assumption, the male *versus* female proportions are still far from equal. Males of *A. rhodensis* are most probably polygynous (a male can mate with multiple females) and sire more progeny than females. As such, if the cost of males is the same as females and hermaphrodites, the population could be at risk of being invaded by males. The cost of producing males compared to females is not easily assessed. However, a ready explanation of how the male proportion is kept low is that males predominantly (if not systematically) produce sperm with one X chromosome as the nullo-X counterpart is discarded during meiosis [170]. This mechanism considerably limits the proportion of males in the population.

In our case, it is also important to, not only, consider the sex allocation but also the selfing *versus* outcrossing resource allocation and the mechanisms balancing this allocation. The link between sex allocation and mating system has been widely studied in plants, in which sex allocation has been used to predict the mating system. Generally speaking, male sex allocation is reduced in selfing species compared to outcrossing species [35]. In a mixed system, the proportion of outcrossing and selfing within a population could reflect certain ecological conditions: for example, in our case, outcrossing may be favoured by the presence of pathogens, whereas selfing (and dispersal) could be promoted by a lack of nutrients or crowding.

The intertwined life cycle of *A. rhodensis* where females produce mostly hermaphrodites and hermaphrodite mothers are largely responsible for the production of females is likely to contribute to the stability of the three-sexed system. At this point it is unclear how the female *versus* hermaphrodite decision is made, although, a maternal effect may play a role as the proportion of each sex depends on the sex and age of the mother.

Additionally, the fact that only hermaphrodites go through a resistant and potentially dispersive stage (the dauer) (this study, [37]) may also contribute to the maintenance of both 'feminine' (female and hermaphrodite) types as, on the one hand, this stage is probably essential for the dispersal and survival of the population but, on the other hand, delays development. As a consequence of this delay, the population growth is slowed down. Theoretically, if hermaphrodites could facultatively go through the dauer stage or bypass it, the advantage of females could be reduced significantly. However, in *A. rhodensis* the hermaphrodite sexual identity and dauer stage are tightly linked (and attempts to separate these traits in the lab have been unsuccessful). One hypothesis is that a developmental constraint exists, linking adult hermaphrodite fate with dauer larva and female fate with absence of dauer during development. The maintenance of hermaphrodites and females (and therefore males) could be enforced if the developmental and sex determination pathways are interdependent at several levels (molecular factors acting on both traits, one pathway activating or repressing the other, physiological consequence of one trait on the other).

Another consideration is that females and males are obligate crossers. As a consequence, the advantages of sex—i.e., increased effective recombination and random assortment of homologous chromosomes, which leads to increased diversity and potentially to new adaptive genotypes—may be preserved in this system. The shuffled material due to sex would subsequently pass into

hermaphroditic bodies (females produced mostly hermaphrodites). The most adaptive combinations gained from sex would then be amplified and dispersed via hermaphrodites.

It is to note that these reflections are speculations and are laid out to help us think about this unusual system and foster new ways of investigating it. At this point, it would be important to improve our knowledge of (i) the ecology of this species (especially the environmental factors shaping the population dynamics and potential migration fluxes) and (ii) the broader evolutionary context of trioecy in nematodes (especially with regard to the hermaphroditism-dauer link). Fortunately, nematode sampling efforts from various labs, have yielded several trioecious strains, which are now available as lab strains. This will enable us to enlarge our knowledge on the biology of trioecious nematodes.

Chapter 3

Genome and genetic map

Summary

In this chapter, I report the genome sequence and genetic map of *A. rhodensis*. With these resources, I obtained the draft sequence of each chromosome. *A. rhodensis* has 7 chromosomes and several chromosomal reorganisations, especially X-autosome translocations and potential chromosome splitting were detected through macrosyntenic comparisons with *C. elegans*, *P. pacificus* and *H. contortus*. Furthermore, compared to autosomes, the X chromosome is about 2.5 times smaller, contains around half the number of genes and is 3 to 4 times more polymorphic.

3.1 Introduction

A. rhodensis is a rare example of a free-living species with coexisting males, females and hermaphrodites. As we have seen in the introduction, mating strategies heavily affect the population genetics and consequently the evolution of a population [34]. Also of interest is the fact that other nematode systems combining outcrossing and selfing in their life cycles are associated with a parasitic lifestyle, contrary to *A. rhodensis*. In Chapter 2, we have observed and discussed various aspects of the population dynamics of *A. rhodensis*. To further our understanding of this system, it was proposed to sequence its genome.

Several nematode species with differing mating strategies and lifestyles have their genomes sequenced. Of particular interest are those with chromosome-long assemblies, such as *C. elegans* (free-living androdioecious species), *P. pacificus* (necromenic androdioecious species), *Haemonchus contortus* (parasitic dioecious species) and *Strongyloides* spp. (parasitic facultative parthenogenetic species). In this chapter, I characterise the genome and genetic map of *A. rhodensis*

and build a first draft of each chromosome. I make whole genome comparison with other available genomes and identify various chromosomal reorganisations which took place in the branch leading to *Auanema*.

Another aspect of the biology of some nematode species is the presence of the bacteria *Wolbachia*. *Wolbachia* is a genus of intra-cellular and maternally inherited α-proteobacteria. There are widespread in arthropods (such as insects, spiders, mites and crustaceans) and filarial nematodes [190, 205]. In arthropods, *Wolbachia* are reproductive parasites and can manipulate their host's biology provoking feminisation of males, male killing, induction of parthenogenesis and cytoplasmic incompatibilities [205]. In nematodes, *Wolbachia* have a mutualistic relationship with the nematode host and are not reproductive parasites but may play a role in modulating embryogenesis and host immune responses [49, 60, 191]. Although *Wolbachia* is almost exclusively found in filarial nematodes (Order Spirurida), it has been reported in the tylenchid plant parasite *Radopholous similis*, a distantly related species to the filarial nematodes [75]. Furthermore, the analysis of the genome of *Dyctiocaulus viviparus*, a species from the Rhabditina group (like *A. rhodensis*), revealed an ancient *Wolbachia* infection in that lineage (*Wolbachia* sequences were found in the nuclear genome of *D. viviparus*) [104]. Additionally, two expressed sequences highly similar to *Wolbachia* have been found in *Ancylostoma caninum* (also from the Rhabditina group), although it is yet to be confirmed if they are indeed *Wolbachia*-derived sequences [104].

The genome and genetic map described here are an addition to the growing database of nematode genomes providing the first genome of a free-living trioecious species. It will help to build a more accurate nematode phylogeny, which is important to provide a strong phylogenetic backbone to understand the evolution of complex traits such as parasitism and mating strategy as well as genome and chromosome evolution. In itself it will be a reference for functional studies (molecular biology and transgenic projects) aiming at understanding better this atypical system.

3.2 Materials and Methods

3.2.1 Strains

Two independent and inbred isolates of *A. rhodensis*, APS4 and APS6, were used for DNA and RNA sequencing. The strain APS4, originally referred to

as SB347 (prior to inbreeding) is the same used in Chapter 2 (Section 2.2.1, on page 25). The strain APS6, originally named TMG33 (prior to inbreeding) was collected from a dead tiger beetle (West Virginia, USA; found in May 2012, GPS 38.230011, -81.762252) (T. Grana, personal communication). Inbred strains were generated as detailed in Section 2.2.1 (on page 25). The strains APS4 and APS6, underwent 50 and 11 generations of inbreeding (rounds of single worm bottlenecking), respectively. Strains were maintained in standard conditions, as detailed in Section 2.2.1 (on page 25).

3.2.2 DNA extraction, sequencing and data pre-processing

Nematode cultures and DNA extractions were performed by Manish Parihar. To extract nematode DNA with minimal bacterial contamination, a split plate method [156] was used. Nematodes were cultured on one of the compartments of a 10 cm, two-compartment plate. The compartment with nematodes contained NGM seeded with *E. coli* OP50-1, and the second compartment contained M9 buffer. As the compartment with nematodes became crowded, dauer larvae migrated to the compartment with M9. The dauers were collected from 10 plates and washed twice with M9 buffer. To do so, the worms were allowed to sink naturally to the bottom of an Eppendorf tube. The M9 was then removed as much as possible without aspirating the worms. These procedures were repeated a second time. The nematode pellet obtained was stored at $-80\,^{\circ}\mathrm{C}$. DNA was extracted and treated with RNAse using the Gentra Puregene Core A Kit (Qiagen) according to the manufacturer's instructions. The DNA was dissolved in nuclease free water for library preparation and sequencing.

Genome assembly was joint work with Georgios Koutsovoulos. The genome was assembled using sequencing data from the APS4 strain. Three Illumina pair-end (PE) libraries with insert sizes of 250 bp, 450 bp and 600 bp were constructed at UT Southwestern (Dallas/TX) on an Illumina HiSeq 2500 sequencer (Table A.1). Unfortunately, estimates of the insert sizes revealed that all pair-end libraries had an insert size of 250 bp (Figure A.2). Two Illumina mate-pair (MP) libraries were then prepared at the GenePool facility at the University of Edinburgh on an Illumina HiSeq 2500 sequencer with desired insert sizes of 3 kb and 5 kb. Insert sizes estimations were of 3 kb and 6 kb, as expected.

The processing of the reads and the assembly of the genome was completed

following the following steps. First, we ran the program `FASTQC` [5] to have an overview of the raw data (quality, possible contamination, GC content). From this overview we were able to conclude that the MP libraries had an extremely good overall quality and a normally distributed GC content (Figure A.1). The PE libraries showed an overall decrease in quality at the end of the reads although the libraries still exhibited high quality (Figure A.1). The GC content curves showed a small bump at a higher GC content (Figure A.1), which we interpreted as possible bacterial contamination (nematodes are fed with *E. coli* but plates can get contaminated by other bacterial strains). We then trimmed the low quality regions of the reads using the program `Skewer` (version 0.2.1, `-Q 20 -l 51 -t 32`) [89].

To check for contamination we ran the Blobology pipeline [107], which outputs Taxon- Annotated-GC-Coverage plots (TAGC plots), also called Blob plots. A TAGC plot represents the different contigs (a preliminary assembly is performed on the data as part of the pipeline), according to their coverage (number of reads aligning to the contig), their GC content and their taxonomic group. This allows us to spatially separate and group contigs showing similar characteristics (for example some bacteria strains will have a higher GC content than the worm sequenced) (see Figure A.3). In our case, we observed that the PE libraries displayed different bacterial contamination whereas the MP libraries were relatively clean (only showing some *E. coli* contamination). We cleaned the libraries by removing the reads that mapped to a database of contaminants using the program `Bowtie2` (see an example of before/after Figure A.3).

The cleaned and trimmed reads were then error corrected using the program `Fiona` (version 0.2.1, `-nt 48 -g 60000000`) [165]. Because all three pair-end libraries displayed about the same insert size (∼250bp), they were concatenated into a same library (i.e. the reads of all 3 libraries were merged into a single file).

An additional pair-end library, from the strain APS6, with insert size of 450 bp (Table A.1), was also sequenced at UT Southwestern (Dallas/TX) using a HiSeq 2500 and was used for calling variants between the strains APS4 and APS6 as well as within the strain APS6. Raw reads were preprocessed using `Skewer` in an identical way as the APS4 libraries [89].

3.2.3 Genome assembly

To estimate some parameters of the genome from the processed reads, we ran the programs `kmc` [51], `PreQC` (a module from the `SGA` program) (version downloaded the 05/03/2014) [173] and `KmerGenie` (version 1.6741) [39] on each library separately. The program `kmc` was used to count the number of kmers, i.e., small sequences of length k present in the files of reads. Counting kmers is a way of estimating the coverage of our genome, i.e., how many times the genome is present in our read data. For example, if most of the kmers appear 50 times then we estimate that the genome is present 50 times in our data (reads). In our case we chose a kmer size of 19 and from the kmer counts, we estimated the coverage of the genome to be around 200 (Figure A.4).

The program `PreQC` [173] reports, among other things, an estimate of the genome size from each library. In our case, *A. rhodensis*'s genome was estimated to be around 75Mbp (Figure A.5). This size is consistent with other nematode genome sizes being a little larger than *Oscheius* and smaller than *C. elegans*.

The program `KmerGenie` (version 1.6741) [39], also a kmer counter, tests different kmer sizes and reports which size is optimal to use for assembling the genome. To do so, it counts, for each kmer size tested, the number of genomic kmers (the 'bump' in the histogram corresponding to the genome). It then plots the number of genomic kmers as a function of the kmer size. The optimal kmer is one that maximises the number of genomic kmers (kmer long enough to make non-random assemblies). However, it is important to note that the kmer length has to be short enough to construct the most complete contigs. In our case, from `KmerGenie`'s report, we chose a kmer size of 71 for our assemblies.

We assembled *A. rhodensis*'s genome by running different programs with different strategies. Because the pair-end reads had a lot of bacterial contamination, we designed non-standard assembling strategies. In the first approach all libraries were used to assemble the contigs and only the MP libraries were used to bridge the contigs into scaffolds. Scaffolds are longer fragments usually presenting gaps (runs of Ns). Our second approach was to use the PE library only for scaffolding (MP libraries were used for contig and scaffold assemblies). The third strategy was to simply not use the PE library, but we quickly realised that the assemblies were not as good.

The different assemblies were cleaned by removing contigs shorter than 500bp (as they could be errors) and by removing contigs likely to be contamination. To find these contigs we mapped the MP reads (non contaminated

libraries) to the genome and removed all the contigs which had a low mapping coverage (<5 MP reads per contig/scaffold). After cleaning the genome assemblies, we attempted to resolve the gaps (stretch of Ns in the scaffolds) by running the program `GapCloser` from the `SOAPdenovo2` package (version r240) [126]. Gaps represent zones of the genome of low coverage or of low complexity (e.g., repeats).

To assess the quality of the assemblies, we ran `CEGMA` (version 2.4) [153] to assess genome completeness. `CEGMA` checks if the assembled genome contained most of the known Core Eukaryotic Genes (essential genes) and counted the number of transcripts with more than 70% length coverage in one contig/scaffold. The `CEGMA` result along with other metrics (N50, span of the runs of N, number of scaffolds) helped us compare the assemblies and chose the best one. The *de novo* genome assembly performed with `SOAPdenovo2` (version r240) [126], `k-mer length=71`), using both the PE and MP libraries for contig assembly and only MP libraries for scaffolding resulted in the best assembly strategy tested.

`Reapr` (version 1.0.17) [80] was run to identify mis-assemblies within the scaffolds (unjustified bridged regions). By mapping the MP libraries separately to the draft genome, `Reapr` identified 42 common gaps in the draft scaffolds (stretches of Ns), that were questionable. We manually inspected these using `Tablet` (version 1.14.11.07) [137] and found 4 scaffolds that contained unjustified gaps. These scaffolds were split manually. Repeats were masked by running `RepeatModeler` [174] and `RepeatMasker` [175].

3.2.4 Genome annotation

Gene prediction was also joint work with Georgios Koutsovoulos. We added structural annotation to the genome by running *ab initio* and evidence-driven gene predictors. *Ab initio* methods predict genes using Hidden Markov Models (HMM). In this case, HMMs define the gene characteristics (for example exon composition, intron-exon boundaries, start and stop codons). These models are then used to decide if the genome sequence processed is likely to be a gene or not. If the genome sequence fits the gene HMM then it will be annotated as a gene. On the other hand, evidence-driven methods make use of existing information indicating the presence of a gene (RNA-seq derived transcripts and essential eukaryotic genes for example). These predictions are more accurate because they make use of real gene clues instead of gene models. Specifically, used the programs `GeneMark` (version 2.3)[192], `SNAP` (release 11/29/2013) [103],

`Maker` (version 2.31) [29] and `Augustus` (version 2.5)[177] to add structural annotation to the genome. Briefly, the outputs from `SNAP`, `GeneMark` and `CEGMA` along with Expressed Sequence Tag (EST) evidence from a set of *A. rhodensis* transcripts previously assembled by `Trinity` [72] and the UniProt database as protein homology evidence were used as inputs for `Maker2`. The output from `Maker2`, along with gene hints directly generated using RNA-seq reads and the set of transcripts, were used as inputs for `Augustus`. I used `Augustus`' final gene predictions for the analyses.

Functional annotation of the protein coding genes was achieved by joining the results from `BLAST`, `InterProScan` and `Blast2GO`. I performed a protein `BLAST` (`-evalue 1e-5 -max_target_seqs 50 -outfmt 5`) against a database of all metazoan sequences available in NCBI (28/08/2015) using `BLAST+` (version 2.2.31+) [28]. `InterProScan` (`-goterms -iprlookup`, version 5.14-53.0) [91] identified protein motifs and signatures. I then used `Blast2GO` (version 3.1) [71] to merge the `InterProScan` and `BLAST` results and add Gene Ontology (GO) terms associated with the `BLAST` hits or the protein domain. I added implicit GO terms to the already existing annotation using `Annex` [144].

I annotated putative functional RNAs (non-coding RNAs) by running the program `Infernal` (INFERence of RNA ALignment, version 1.1.1) [146], which uses the Rfam (RNA family) database. Transfer RNAs were also identified using the program `tRNAscan` (version 1.3.1)[124]. We identified ribosomal RNAs by running `Infernal` (identified 5S rRNAs), `RNAmmer` (version 1.2) (identified 8S RNAs) and `BLASTn` (`BLAST+`) using as a database the partial 18S (accession number EU196004.1) and 28S (accession number EU195960.1) sequences of *A. rhodensis* [98]. We counted unique functional RNA features using `BEDTools intersect` (`-s -c`, version 2.25.0, [161]).

3.2.5 RAD-seq and Genetic Map Construction

A genetic map was constructed using markers obtained from RAD-sequencing (Restriction site-Associated DNA sequencing) of 95 Advanced Intercross Lines (AILs) [162] derived from *A. rhodensis* inbred strains APS4 and APS6 [95]. To generate the AILs, crosses between APS4 females and APS6 males were performed to generate several F1 hermaphrodites, which were allowed to self-reproduce. Each progeny line was established from single F2 hermaphrodite progenitors, which were left to expand for 3 to 10 generations (i.e. each progeny sample comes from a population devired only from one F2 hermaphrodites). We

used the split plate method as described earlier to isolate DNA from the lines. This method relies on the isolation of dauers. Since dauers of *A. rhodensis* always develop into hermaphrodites [37, 59], the DNA isolation was derived only from this sex. Pair-end RAD-seq was then carried out for each of the parental and AILs [9]. The restriction enzyme PstI was used to shear the DNA. The raw RAD-seq reads (RAD-tags) were first demultiplexed (the sequences corresponding to each recombinant line were separated into different files) and low quality regions were removed using the program `Process_RAD_tags` from the `Stacks` package (version 1.35) [32]. We then used the `denovo_map.pl Stacks` pipeline to determine the genotype of each locus (region sequenced adjacent to the PstI cut site) for each progeny sample. Briefly, the RAD-tags were aligned into exactly matching 'stacks'. By comparing, the different stacks it was then possible to build 'loci' (sequences representing specific locations on a genome). The distance allowed for two stacks to be merged into a single locus was 2 nucleotides. This step was performed by running the program `ustacks` [32]. After the loci were built, a catalogue of the parental loci was created, conserving the information of the alleles of each parent. This is important, as the next step involved mapping the progeny 'stacks' to the catalogue to determine which progeny sample has which parental allele.

With this information it is then possible to know which loci are genetically close together. Indeed, in each F2-derived lines, the DNA from the parental lines will have recombined differently. If two loci are genetically close to each other, the likelihood that they would have been be separated due to homologous recombination is low compared to completely unlinked loci (for example, loci on different chromosomes) or loci genetically distant. We therefore expect that genetically close loci will have segregated mostly together, displaying a similar genotype pattern across the progeny samples. The process of grouping the loci (or markers) into different linkage groups and to order them within each group was performed using the R packages `OneMap` (version 2.0-4) [129] and `R/qtl` (version 1.38-4) [24]. With `OneMap`, I did an initial grouping of the markers, expecting to cluster them into chromosome. The grouping of the markers was performed using a LOD (logarithm of odds, a statistical estimate of how near two markers are from each other) value of 20 and a maximum recombination fraction of 0.5.

This preliminary map was then refined using the `R/qtl` package. This package allows a more in-depth analysis of the markers and samples by looking at the pattern of missing data, duplicate samples and markers, problematic

markers and samples and possible genotyping errors. With this analysis, I decided to remove 6 markers, which were present in only ∼50% of the samples. No samples were in duplicate but 225 markers displayed the same genotypes in the same samples. These were considered duplicates and were removed. The removal of duplicate markers is important, as it is impossible to determine the order of these duplicate markers and the programs end up choosing arbitrarily an order. From the relative position of the markers in each linkage group, large gaps around loose markers (markers separated from other markers by a distance 5 times or more than the average distance observed) were studied and, in case of a clear improvement of the likelihood of the map when removed, they were eliminated. Three markers were eliminated this way. After performing these 'clean-up' steps, I re-clustered the markers using `R/qtl`. The resulting groups were the same as for OneMap except for three markers, which `R/qtl` could not associate with a group. These doubtful markers were removed. All the steps were followed according to `R/qtl` tutorial for constructing genetic maps (`http://www.rqtl.org/tutorials/geneticmaps.pdf`). The ordering of the final map was re-estimated using OneMap as this package was faster to run and seem to give a better ordering of the markers. A LOD of 15 and a maximum recombination fraction of 0.5 were used for this second ordering. The final map contained 1052 markers (Figure A.6).

3.2.6 Building a Draft of Each Chromosome

The software `Chromonomer` (version 1.07) [4] was used to anchor the genomic scaffolds to the genetic map to obtain a draft sequence of each chromosome. I first mapped the marker sequences of the genetic map to the genome using `Bowtie2` (version 2.3.3) [113]. From this alignment, the description of genetic map (the order and distance information of the markers along the linkage groups) and the genome itself, `Chromonomer` is able to place and orient the scaffolds along the genetic map. In case of inconsistencies some markers are dropped. I then lifted over the annotation files (protein-coding and non-coding genes), to suit the new integrated genome using a python3 script written by Tássio Naia.

3.2.7 Synteny analyses and identification of the X chromosome

The resulting chromosomal blocks were aligned to the *C. elegans* genome to visualise the macro-synteny between both species. The whole genome alignments were built using the program `PROmer` (version 3.07) [108] with default parameters. `PROmer`'s alignment between genomes is based on the six-frame amino acid translation of the DNA. This increases sensitivity as protein sequences tend to remain more conserved than the nucleotide sequences on which they are based. `PROmer` is especially useful when comparing divergent genomes. This method yielded better results than direct nucleotide alignments. Additionally, potential homologous genes were identified between *C. elegans* and *A. rhodensis* by performing reciprocal `BLAST`s (`BLASTp`, `-evalue 0.01 -max_target_seqs 100 -outfmt 6`). From this, I was able to quantify the proportion of *A. rhodensis* homologous protein-coding genes in *C. elegans* chromosomes and vice versa. The macro-synteny was visualised on a `Circos` plot (version 0.69) [106]. One linkage group (LG5) aligned almost exclusively to the X chromosome of *C. elegans*. The genotyping of 5 polymorphic markers placed along this linkage group in F1 hybrid males (from and APS4 x APS6 cross) confirmed it to be the X chromosome (see Chapter 4, on page 75 and [188]).

3.2.8 Genome characterisation

Gene density was plotted for each chromosome using the R package `karyoploteR` (version 1.5.1) [66]. It has been observed that conserved genes between *C. elegans* and distantly related species (such as *Saccharomyces cerevisiae*) are predominantly found in the centre of the chromosomes [206]. To check if this pattern could be observed in *A. rhodensis* I identified conserved genes between *A. rhodensis* and the fruit fly *Drosophila melanogaster* through best reciprocal `BLAST`s (`BLASTp`, `-evalue 0.01 -max_target_seqs 100 -outfmt 6`). These parameters were chosen as they resulted in a good compromise between output size and accuracy of the results. The localisation of the genes and conserved genes along the chromosomes was also visualised using `karyoploteR`. To test if the proportion of conserved genes on the X chromosome was different from the proportion of conserved genes on the autosomes, I performed a χ-square test, implemented in R (function chisq.test).

Variants (SNPs and InDels) were identified using the three preprocessed (see

method Section 3.2.2, on page 42) pair-end libraries of APS4 and the pair-end library of APS6. Cleaned reads were aligned to the integrated genome using `bwa` (version 0.7.12-r1039) [118] and the resulting SAM alignments were converted to a BAM format and sorted by coordinate using `Picard` (version 2.14) [23] `SortSam`, de-duplicated using `picard MarkDuplicates` and finally the BAM files were indexed using `picard BuildBamIndex`. The three APS4 libraries were merged prior to de-duplication and indexing. Joint Variant Calling was then performed using `Samtools mpileup` (version 1.4) [119] and the raw BCF (Binary Calling Format) output was filtered using `bcftools view` (version 1.4-16-g4dc4cd8) [117] and `vcftools vcf-annotate` (version 0.1.14, `-f +/d= 5/D= 10000/q= 20/Q= 15/w= 20/W= 30/c= 3,10/a= 2/1= 0.0001/2= 0/3= 0/4= 0.0001`) [48]. Intra-strain variants were defined as heterozygous polymorphisms (0/1) occurring within one strain regardless of the other strain's polymorphisms at the same locus. Inter-strain polymorphisms were defined as different genotypes between the two strains at a same locus. Intra- and inter-strain variant density was plotted along each chromosome using `KaryoploteR` [66]. To statistically assess if the X chromosome carried more or less variants (intra- and inter-strain variants) than the autosomes, I performed χ-square tests.

Potential duplicated genes within the *A. rhodensis* genome were identified by running a `blastp` of *A. rhodensis*' protein set against itself. The blast result was filtered according to the following criteria: the E-value was to be smaller or equal to 1×10^{-50}, the percentage of identity of the blast alignment was to be at least 50%, the percentage of the query length involved in the alignment was to be at least 30%. If an alignment satisfied these criteria it was considered a 'good' alignment. For two genes A and B to be considered duplicated, the amino acid sequence of gene A had to align 'good' to the one of gene B and vice versa. The potential duplicated genes identified were then visualised using `KaryoploteR` and intra- as well as inter-chromosome duplicates were counted.

The gene density, variant density and duplicated genes of unanchored scaffolds (not included in a particular chromosome) were not examined.

3.2.9 RNA-extractions and global gene expression

For each chromosome, global gene expression was assessed using RNA-seq data from L2 hermaphrodites, L2 females, males and animals from various stages and sexes (mixed stages). All RNA-seq data were generated from APS4 samples. The nematode cultures and RNA extractions were performed by

Manish Parihar.

To obtain L2 female and L2 hermaphrodite samples, age synchronised populations were created from a pool of hermaphrodite mothers as follows: dauer larvae were picked from a culture plate, isolated on a 6cm plate and left to develop into hermaphrodites. After 12 h of egg laying the hermaphrodites were removed and their progeny were allowed to develop until the L2 stage. Female and hermaphrodite L2 larvae were distinguished at the L2 stages by their developmental rate (females develop faster), size (females are relatively bigger than their age synchronised hermaphrodite siblings) and colour (hermaphrodites are darker) (See Chapter 2). Around 200 L2 individuals were used for each RNA extractions. L2s were placed in an Eppendorf tube containing 200 µl of M9 buffer and washed 2-3 times using M9. Washes consisted in allowing the worms to sink naturally at the bottom of the tube, removing as much M9 as possible before washing them again by adding more M9. After the final wash, most of the M9 was removed (taking care not to aspirate the worms) and 200 µl of Trizol was added to the tube. The tube was immediately frozen at −80 °C (at least overnight).

Male samples were obtained similarly by individually picking about 500 young males into a 1.5 ml Eppendorf tube containing 500 µl of M9 buffer. The worms were washed twice using M9 buffer as detailed above. After the final removal of M9, 500 µl of Trizol was added to the tubes before freezing the samples at −80 °C.

Mixed stages samples were collected from five 6 cm culture plates of healthy worms. Briefly, M9 buffer ($3\,g\,l^{-1}KH_2PO_4$, $6\,g\,l^{-1}Na_2HPO_4$, $0.5\,g\,l^{-1}$ NaCl, $1\,g\,l^{-1}$ NH_4Cl) was gently poured onto the plates, avoiding the OP50-1 bacterial lawn. The worms were allowed to naturally detach from the plate and start swimming in the M9. By not disturbing the bacterial lawn, the bacterial contamination of the samples is greatly reduced [158]. The nematodes were then placed in a centrifuge tube and washed twice with M9 (as detailed above) before being frozen in liquid nitrogen.

The L2 and male samples were then freeze-cracked two to three times using liquid nitrogen (followed by thawing at room temperature). The samples were then shaken on a bead-beater homogeniser using a few sterile 0.5 mm glass beads (3 times 20 s with 30 s intervals). For the mixed stages samples, a probe homogeniser was used to grind the worms (1 min) instead of the bead-beating.

RNA extractions were carried out by performing a standard chloroform method. Specifically, Trizol was added to each tube making a final volume of

Trizol of 500 µl. Under a fume hood, 80 µl of chloroform (1/5 volume of Trizol) was added to the tubes and mixed by vortexing (15 s). Samples were incubated for 5 min at room temperature before being centrifuged at 12 000 g for 15 min at 4 °C. The upper aqueous phase was then transferred into a new tube and 200 µl of 100% isopropanol (1/2 vol of Trizol) was added. The solution was mixed gently and incubated at room temperature for 10-15 minutes before being centrifuged at 12 000 g for 10 min at 4 °C. The supernatant was carefully removed and the RNA pellet was washed with 400 µl of 70% ethanol. After centrifuging the samples at 7 500 g for 5 min at 4 °C, the ethanol was removed and the pelled air-dried for 10 min to remove the residual alcohol. The RNA was then dissolved in DEPC water. DEPC water was obtained by adding 0.1 ml of DEPC (diethylpryrocarbonate, an inhibitor of RNase activity) to 100 ml of deionised water overnight at 37 °C followed by autoclaving the solution the next day. The RNA was then stored at −80 °C until sequencing was performed. Illumina pair-end (PE) RNA-seq libraries were constructed at UT Southwestern (Dallas/TX) using an oligo-d(T) bead capture of the mRNAs. RNA extractions were performed independently in triplicates for each condition (L2 females and L2 hermaphrodites, males and mixed stages).

To determine if the genes on the X chromosome were significantly less expressed than those on the autosomes, I randomly sampled the 600 autosomal genes and 600 X-linked genes, and compared their expressions using a Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney tests between autosome-X pairs. The sampling of the same number of genes is important to avoid biases due to a big difference in sample size. The gene expression across the same chromosome in different replicates of the same condition was confirmed to be similar by performing Kruskal-Wallis tests.

The global gene expression of genes on unanchored scaffolds (not included in a particular chromosome) was not examined.

3.2.10 Chromosomal rearrangements

To study macrosyntenic patterns and chromosomal reorganisations which could have occurred between *A. rhodensis* and other Rhabditid nematodes, I first identified orthologous proteins between *C. elegans*, *P. pacificus*, *H. contortus* and *A. rhodensis* through best hit reciprocal `BLAST` searches (`BLASTp`, `-evalue 0.01 -max_target_seqs 100 -outfmt 6`) [28]. Reciprocal best BLAST hits are defined as two proteins in two different genomes being to each other the best

scoring match. These reciprocal best hits are used here as a proxy for finding orthologous genes. Localisation of the orthologous genes coding for proteins was visualised on Circos plots to determine possible macrosyntenic patterns. The genomes and protein sets came from the versions 'PRJNA13758.WS259' for *C. elegans* and 'El_Paco_v1' for *P. pacificus*. For *H. contortus*, the genome, annotation and protein set was downloaded from the following link `ftp://ftp.sanger.ac.uk/pub/pathogens/sd21/HCON_V4_GENOME/`, kindly provided by Steve Doyle (Sanger Institute).

3.2.11 Gene Ontology (GO) enrichment analyses

Gene ontology (GO) enrichment analyses were performed to examine possible GO terms found over- or under-represented in the X chromosome gene set *versus* the autosomal set. To test which GO categories were possibly enriched or depleted in the X chromosome gene set compared to the autosomal gene set, I used a two-tailed Fisher's exact test (FDR < 0.05) implemented in the program `Blast2GO` (version 4.1.9) [71]. The list of GO terms found enriched or depleted in the X chromosome set was then reduced to the most specific terms.

3.2.12 *Wolbachia* searches

The presence/absence of the intracellular bacterium *Wolbachia* was assessed by aligning the DNA-seq raw reads (prior to contamination removal) to a database of nematode *Wolbachia* genomes (accessions numbers NC_006833.1, NC_018267.1, AE017321.1, HE660029.1 and HG810405.1) using the program `Bowtie2` (version 2.3.3) [113]. `BLAST` (`tblastx -evalue 0.01 -max_target_seqs 100 -outfmt 6`) searches using the *Wolbachia* genome sequences were also conducted against the assembled genome (scaffolds) of *A. rhodensis*.

3.3 Results

3.3.1 Genome characteristics

The assembled genome (`SOAPdenovo2`, see Methods) consists of 440 scaffolds ($>$ 1000bp) and spans 60.6 Mbp. The genome size is smaller than the estimate made by `PreQC` ($\sim$75 Mbp). This could be due to duplicated regions that were collapsed during the assembly. Indeed, assembly programs deal poorly with repetitive and duplicated regions, as they cannot deduce the number of repeats.

Some of those repeats are therefore only present once in the genome. Also, it could be due to an over estimation of the genome by `PreQC`. The gene prediction pipeline yielded 11,570 protein coding genes and 833 unique functional RNAs (non-coding genes) (Tables 3.1 and 3.6). The number of protein-coding genes is lower than previously predicted for other Rhabditine species (e.g. *C. elegans* has 20,082 protein-coding genes). The lower genome size and coding gene content in *A. rhodensis* does not seem to be due to an incomplete assembly or to a large number of genes missed during the annotation process as the current assembly contains 99.19% of the core eukaryotic genes (predicted by CEGMA). Another discrepancy observed is the much lower number of ribosomal RNA genes annotated in *A. rhodensis* (60 rRNAs) compared to the estimated number in *C. elegans* (around 275). This is possibly due to an underestimate of the number of rRNAs in *A. rhodensis* as they are presumably found in a unit tandemly repeated similarly to *C. elegans* [184], which may not have been properly assembled (repeats tend to be collapsed during the genome assembly process as they are perceived by the assembly programs to represent a same region). Functional annotation (`InterProScan`, `BLAST` and `Blast2GO`) of the protein-coding genes revealed that the top `BLAST` hits of *A. rhodensis* proteins were most similar to the Strongylid species *Ancylostoma ceylanicum*, *Haemonchus contortus* and *Necator americanus* than to *C. elegans* (Figure 3.1), consistent with the phylogeny (Figure 1.2, [18, 95]). The sequence similarity between the protein sequences and the `BLAST` hits was around 67% for most sequences (Figure 3.2). In the final annotation, 10,449 (90%) proteins were assigned some kind of annotation (`IntroProScan` signature, GO term, `BLAST` hit) and 8181 (71%) were attributed at least one GO term.

3.3.2 The mitochondrial genome

The complete mitochondrial genome was found in 'scaffold130_61'. Manual inspection using the program `Tablet` (version 1.14.11.07, [137]), revealed an unjustified gap and an artifactual duplicated region in the scaffold. The mitochondrial genome was manually curated and annotated using `Dogma` [210] and `Geneious` (version 6.1, [96]). Both software packages annotated concordantly the 12 mitochondrial genes and the two ribosomal subunits (Figure 3.3). The transfer RNAs (tRNAs) were more ambiguous to annotate and are therefore not included in the current mitochondrial annotation. The corrected mitochondrial genome spans 13,907 bp, which is very similar to the *C. elegans* mitochondrial

Table 3.1: **Basic genome statistics of *A. rhodensis* and *C. elegans*.** *C. elegans* statistics were calculated on the WormBase annotations of the genome version PRJNA13758.WS262. CDS stands for CoDing Sequence. Unplaced scaffolds (unpl. scaf.) information is between square brackets. The column 'scaffolds' refer to the *A. rhodensis*' genome assembly which resulted in scaffolds. The 'Integrated' column refers to the draft sequenced of each linkage group which was produced by anchoring and ordering the scaffolds to the genetic map.

	A. rhodensis (Scaffolds)	*A. rhodensis* (Integrated)	*C. elegans*
# scaffold/chromosomes	636	6 aut. + 1 X [493 unpl. scaf.]	5 aut. + 1 X
Assembly size (Mb)	60.6	57.8 (LGs) [+2.7]	100.2
# scaffolds (> 200 bp)	636	7 [+493]	NA
# scaffolds (> 1,000 bp)	440	7 [+297]	6
N50 (scaffolds > 1,000 bp)	556,081	8,804,062	17,500,000
Longest scaffold (bp)	3,360,731	9,627,060	20,924,180
GC content (%)	32.2	32.2	35.4
Span of runs of Ns (≥ 10 Ns)	915,180	928,780	NA
# genes (protein-coding)	11,570	10,861	23,629
# CDS	135,144	130,644	189,079
CDS span (bp)	16,655,465	15,869,469	39,400,137
CDS mean (SD) (bp)	123 (131)	121 (122)	208 (263)
CDS median (bp)	109	109	146
Min/max CDS length (bp)	3/11,659	3/11,659	1/14,975
# of introns	123,693	119,812	200,020
Intron span (bp)	16,862,509	16,308,478	79,153,867
Intron mean (SD) (bp)	136 (521)	136 (521)	396 (962)
Intron median (bp)	47	47	82
Min/max intron length (bp)	7.0/24,877	7.0/24,877	1/100,912

genome that is 13,794 nucleotides in length [116]. The gene collinearity is conserved between *C. elegans* and *A. rhodensis* (Figure A.7).

3.3.3 Genetic map

95 F2-derived progeny lines were generated from crosses between two polymorphic inbred strains of *A. rhodensis*, APS4 and APS6[1]. From the RAD-tags generated, I identified 1,052 polymorphic RAD-seq markers that clustered in 7 linkage groups (Table 3.2). In accordance with this finding, DAPI stainings confirmed the presence of 7 chromosomes in *A. rhodensis* (Observation by Diane Shakes). This contrasts with most nematodes from the Rhabditina

[1]Crosses and DNA extraction was performed by Manish Parihar.

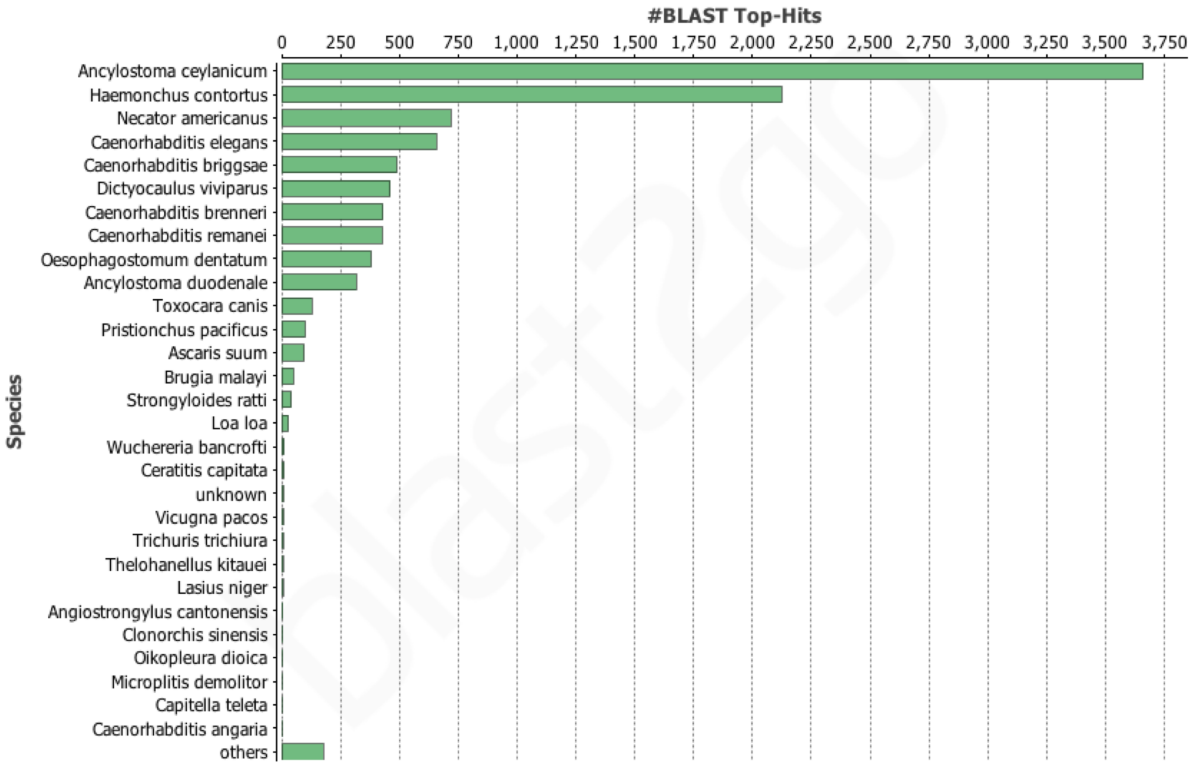


Figure 3.1: **BLAST top hits species distribution.** The species which shared the most similarity with *A. rhodensis* proteins were *Ancylostoma ceylanicum*, *Haemonchus contortus*, *Necator americanus* before the well-annotated *C. elegans*.

group, which usually have 5 or 6 chromosomes [17, 41]. However, we can note that *Heterorhabditis* spp, a genus of trioecious insect parasite species, also has 7 chromosomes [46].

To build an 'integrated genome', I then anchored the genomic scaffolds of *A. rhodensis* to the genetic map to complete the sequence of each linkage group (see Methods). Of the 1,095 markers and 636 scaffolds (> 200 bp), 1,038 markers (∼94%) and 143 scaffolds (∼22%) were retained to build the integrated genome. The loss of scaffolds and markers is presumably due to the imperfect marker ordering, where some markers probably had an inverted order on the map. Nevertheless, the anchored scaffolds represent 93% of the size of the genome (scaffold span) and contain close to 94% of the predicted proteins (10,861/11,570 predicted proteins). The scaffolds that could not be anchored were generally small and probably lacked the presence of at least two markers, which is necessary to place them within a chromosome.

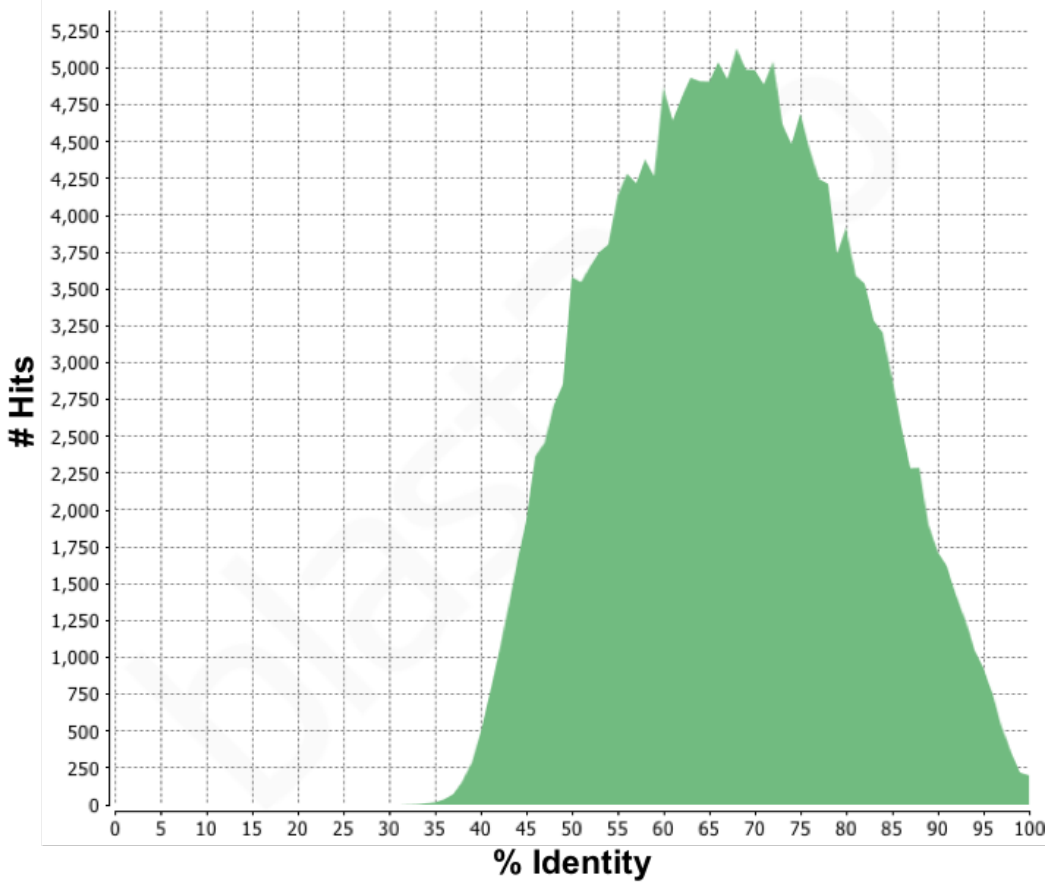


Figure 3.2: **Sequence similarity distribution of the `BLASTP` hits.** Protein `BLAST`s were used to functionally annotate the genome of *A. rhodensis*.

3.3.4 Macrosynteny with *C. elegans* and identification of the X chromosome

I aligned the draft *A. rhodensis* chromosomes to the genome of *C. elegans* to identify potential homologous regions and synteny between these species (Figure 3.4). *A. rhodensis* linkage groups LG1, LG6 and LG7 mapped widely to *C. elegans* chromosomes V, IV and II, respectively (Figure 3.5). In contrast, the mapping of LG2, LG3 and LG4 was split between several *C. elegans* chromosomes (I, III and X) (Figure 3.5). The smallest linkage group, LG5, mapped almost entirely to the X chromosome of *C. elegans* (Figure 3.5 and 3.4). I confirmed LG5 to be the X chromosome by performing male-female crosses between the strains APS4 with APS6. The resulting hybrid males are expected to be heterozygous at all loci differing between APS4 and APS6 except for those on the X chromosome. Indeed, the males carry only one copy

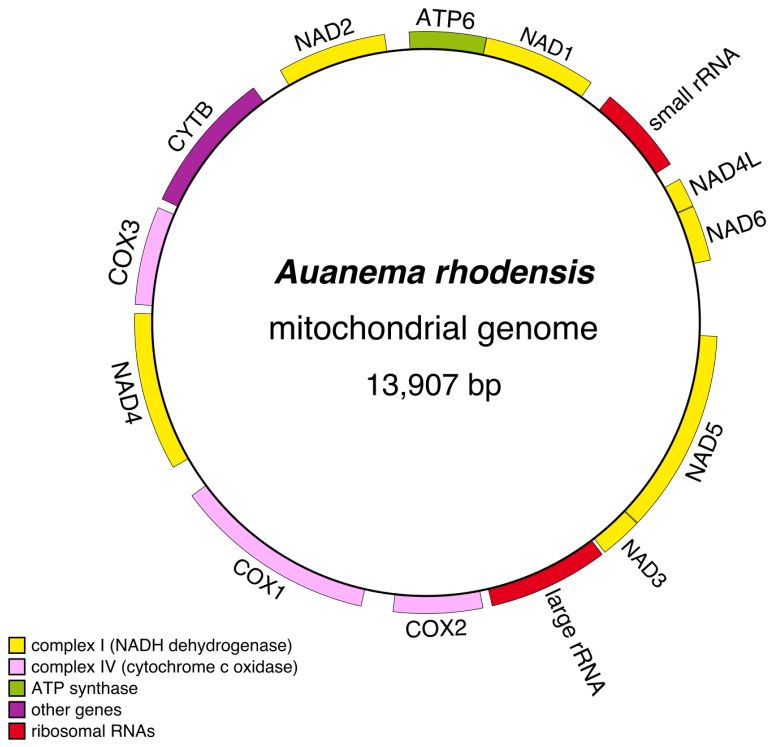


Figure 3.3: **Schematic view of the mitochondrial genome of *A. rhodensis*.**

of the X chromosome which will either have the APS4 or APS6 alleles. By genotyping 40 F1 hybrid APS4/APS6 males at 5 polymorphic loci spread across the chromosome (see next chapter and methodology used in [188]) I found that they were carried either the APS4 or the APS6 alleles (depending on the cross performed), consistent with the hemizygosity of males (see Chapter 4, on page 75 and [188]).

3.3.5 X-autosome translocations and other chromosomal rearrangements

I then examined the X chromosome evolution by comparing the coding sequences of *A. rhodensis*, *P. pacificus*, *C. elegans* and *H. contortus* for which the sequence of each chromosome is known (Figure 3.14). It was previously established that a large translocation from an autosome to the X chromosome occurred in the lineage leading to *C. elegans* [163]. Macrosynteny indicates that *P. pacificus* chromosome I partially corresponds to the *C. elegans* chromosomes V and X

Table 3.2: **Characteristics of *A. rhodensis* genetic map.** * Length after integration map-scaffolds.

	# Markers	Hom. APS4/APS4 freq.	Het. APS4/APS6 freq.	Hom. APS6/APS6 freq.	Approx. length (bp)*	# Protein-coding genes
LG1	109	0.3	0.35	0.35	8,489,927	1,538
LG2	149	0.29	0.37	0.34	9,627,060	1,760
LG3	184	0.21	0.38	0.51	8,741,542	1,748
LG4	143	0.35	0.39	0.36	8,804,062	1,586
LG5	92	0.04	0.88	0.08	3,488,253	604
LG6	185	0.35	0.39	0.26	9,421,540	1,871
LG7	190	0.23	0.42	0.35	9,306,279	1,754
Overall	1052	0.26	0.42	0.32	57,878,663	10,861 (93%)

[163] (Figure A.8, left of the last row). It was proposed that the translocation, i.e., the autosomal region of *P. pacificus* chromosome I that moved to the X chromosome, occurred in the branch of the phylogenetic tree leading to *Caenorhabditis* and strongylid nematodes [163]. *A. rhodensis* is phylogenetically closer to *Caenorhabditis* and strongylids than to *P. pacificus* [95, 98, 99]. Thus, we would expect to see more similarities with *C. elegans* and *H. contortus*, and especially a signature of the autosome-to-X translocation. Contrary to expectation, the chromosome I of *P. pacificus* does not seem contain more X orthologues than the other autosomes (Figure A.9)

Table 3.3: **Chromosome correspondence between *P. pacificus*, *C. elegans*, *H. contortus* and *A. rhodensis*, using *P. pacificus* as the reference.** The chromosomes in bold denote the material that translocated from an autosome (Chromosome I of *P. pacificus*) to the X in *C. elegans* and *H. contortus*, which probably retranslocated to autosomes (LG2 and LG4) in *A. rhodensis*.

P. pacificus	*C. elegans*	*H. contortus*	*A. rhodensis*
I	V and **X**	V and **X**	LG1 and **LG2/LG4**
II	II	II	LG7
III	III	III	LG2/LG3/LG4
IV	IV	IV	LG6
V	I	I	LG3/LG4
X	X	X	X

Through visualisations of the localisation of orthologous proteins, I observed that the X chromosomes of *C. elegans* and *H. contortus* are partially orthologous

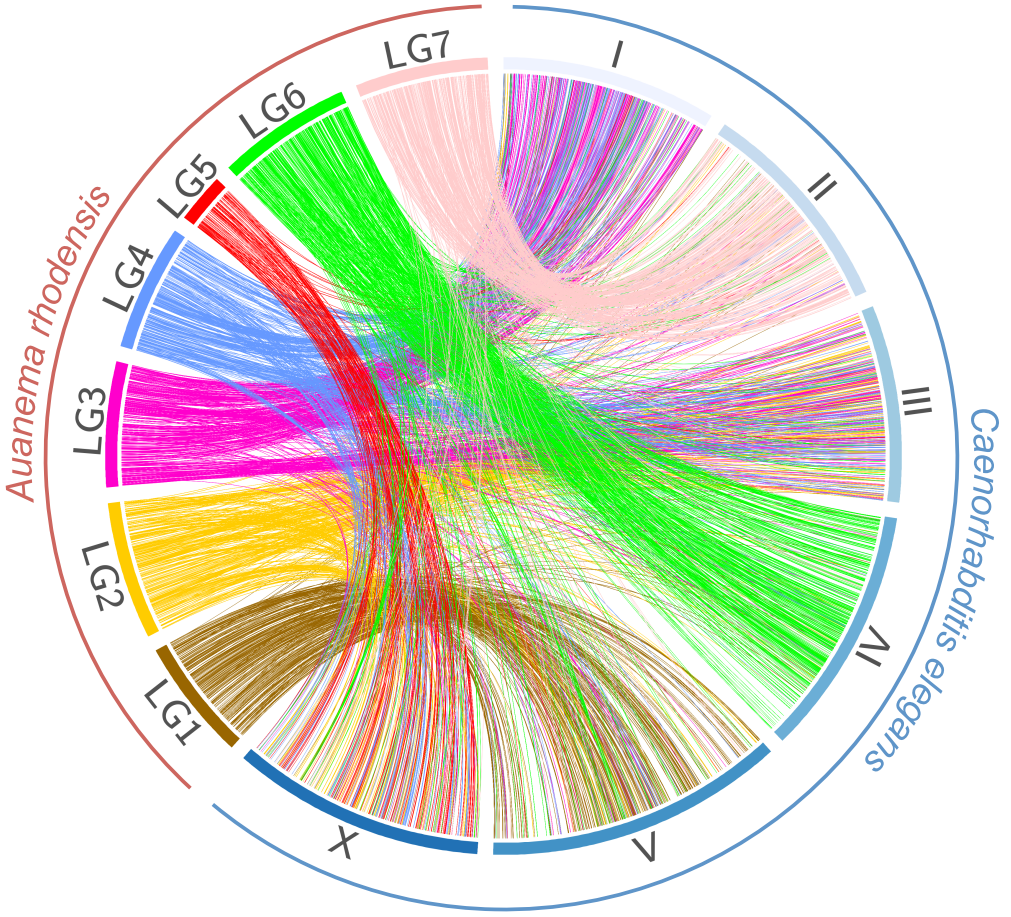


Figure 3.4: **Visualisation of the orthologous sequences between *C. elegans* and *A. rhodensis*.** The circos plot was build using whole genome alignments between *C. elegans* and *A. rhodensis*'s integrated genome (unanchored scaffolds are not shown), obtained by running the program PROmer (version 3.07) [108] with default parameters. Each alignment (hit) between both species is depicted by a line. The lines are coloured according to the *A. rhodensis* chromosomes

to the *A. rhodensis* chromosomes LG2, LG4 and X (LG5) (Figure 3.6, first and second circos plots and Table A.9). We can also note that the part of the *P. pacificus* chromosome I, which underwent the translocation to the X in the lineage leading to *Caenorhabditis*/strongylids, is orthologous to the chromosomes LG2 and LG4 of *A. rhodensis* (Figure 3.6, last circos plot). In all comparisons, the X chromosome of *A. rhodensis* (LG5) largely mapped to the X of *C. elegans*, *H. contortus* and *P. pacificus* (Figure 3.6).

Another pattern observed is that the *A. rhodensis* chromosome LG3 is partially orthologous to *C. elegans* chromosomes I and III (V and III in *P. pacificus* and I and III in *H. contortus*) (Figure 3.7, last line of circos plots and Table 3.3).

		A. rhodensis chromosomes						
		LG1	LG2	LG3	LG4	LG5	LG6	LG7
C. elegans chromosomes	I	4.14	7.66	55.49	33.77	3.70	4.26	5.86
	II	4.53	4.68	2.70	5.73	2.81	4.59	71.10
	III	3.58	42.91	24.46	27.70	1.91	4.70	4.41
	IV	7.06	6.57	3.76	5.37	2.68	73.78	3.51
	V	76.47	5.84	5.42	7.52	2.55	6.19	6.21
	X	4.21	32.33	8.17	19.92	86.35	6.49	8.92
Total number of hits		2848	3288	3487	3856	784	3619	3107

Figure 3.5: **Percentages and heatmap of *A. rhodensis*'s orthologous sequences in each of the *C. elegans*'s chromosomes.** Percentages were calculated on the total number of alignments between each of the *A. rhodensis* and *C. elegans* chromosomes, obtained by PROmer. The lighter the colour of the cell, the higher the proportion of *A. rhodensis*'s orthologous sequences in *C. elegans*. The last line corresponds to the total number of *A. rhodensis*'s sequence hits to *C. elegans*.

Taken together, these observations suggest that various reorganisations have occurred between the chromosomes LG2, LG3 and LG4 of *A. rhodensis*, as they do not correspond to a clear chromosome in either *C. elegans*, *H. contortus* or *P. pacificus*. Another interesting observation is that the orthologous material of *P. pacificus*, *C. elegans* and *H. contortus* is not dispersed in LG2, LG3 and LG4 but rather clustered (Figure 3.7).

3.3.6 Contrasting genomic patterns between autosomes and the X chromosome

Extreme segregation distortion

I found that almost all RAD-seq markers for the X (LG5) chromosome were heterozygous across all 95 samples (Figure 3.8). This surprising result originated a genotyping study of the X chromosome, related in Chapter 4, starting on page 75.

We also observed a high frequency of homozygous markers for the APS6 strain at one end of LG3 (Figure 3.8), perhaps due to the selection of the APS6 alleles on this chromosome or due to the presence of segregation distorters. This bias in LG3 was not further investigated.

Global expression pattern

Global protein-coding gene expression of various stages and sexes (L2 female, L2 hermaphrodite, adult male and mixed stages) was examined separately for

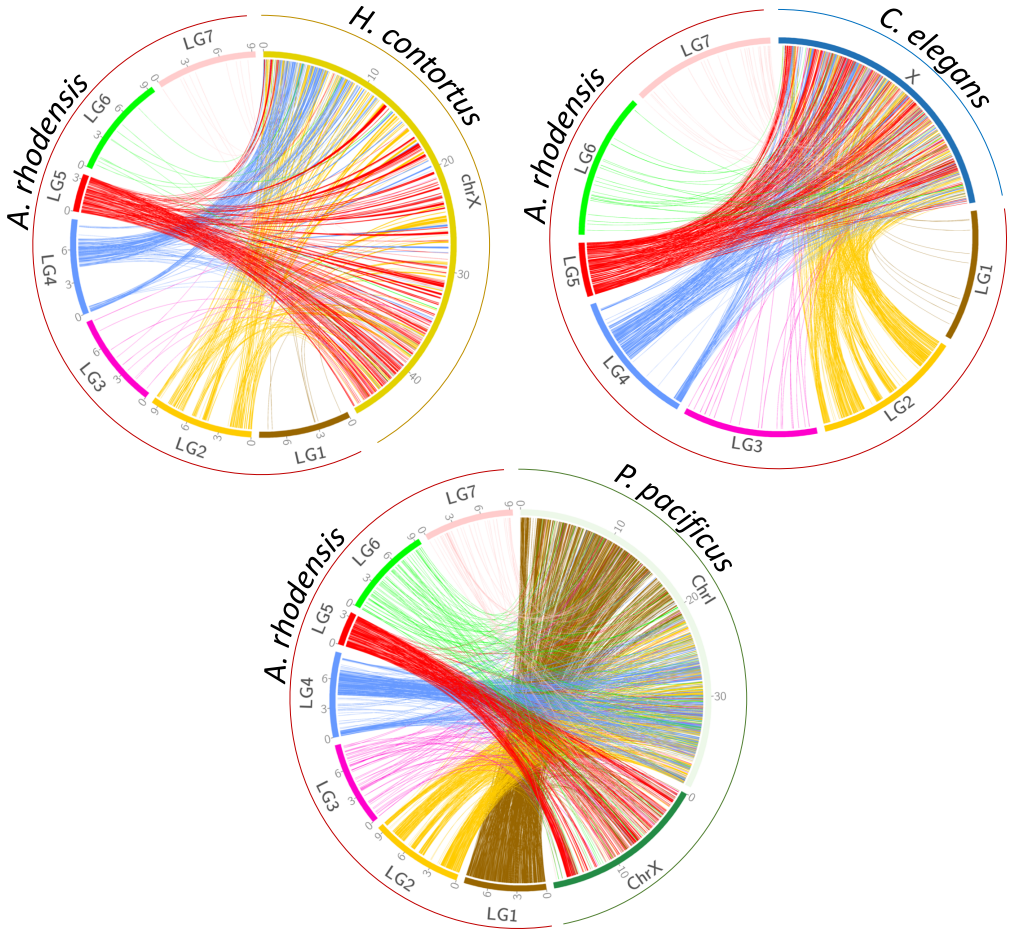


Figure 3.6: **Visualisation of the orthologous coding material of the X chromosomes of *H. contortus*, *C. elegans* and *P. pacificus* in *A. rhodensis*.** In *A. rhodensis*, the chromosome LG5 corresponds to the X.

each chromosome (Figure 3.9). After correction for library size, I found that the X chromosome gene expression was consistently lower than that of the autosomes (Wilcoxon Mann-Whitney, P-value $\leq 1.0 \times 10^{-11}$ in all conditions and replicates). This is also observed for the genes in the X chromosome of *C. elegans*, which are highly repressed in the germline cells [97] and two-fold less expressed in the somatic cells of XX individuals due to the dosage compensation [185].

Intra- and inter-strain diversity

Although the strains used in this study were inbred, we can expect a low level of intra-strain variants due to random mutations and incomplete inbreeding

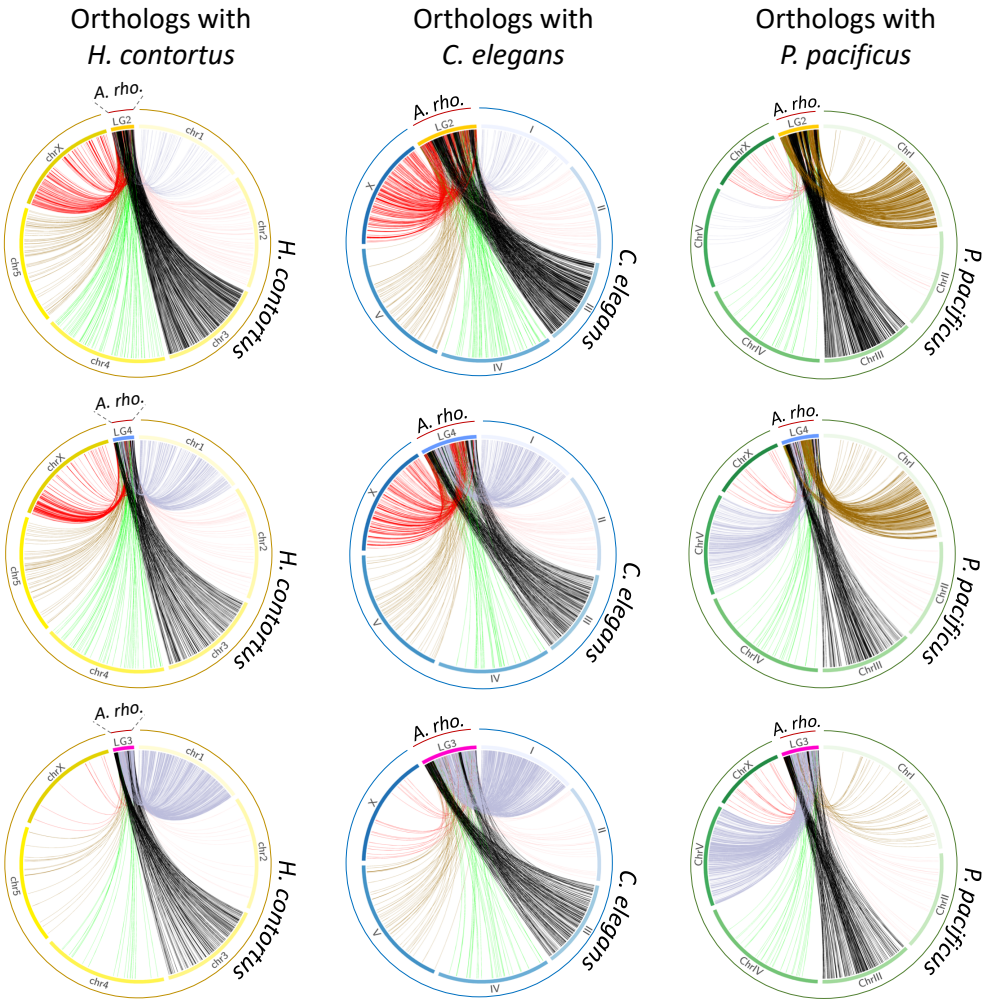


Figure 3.7: **Visualisation of the orthologous coding material of the chromosomes LG2 (top row), LG4 (middle) and LG3 (last row) of *A. rhodensis* in *H. contortus*, *C. elegans* and *P. pacificus*.** In *A. rhodensis*, the chromosome LG5 corresponds to the X.

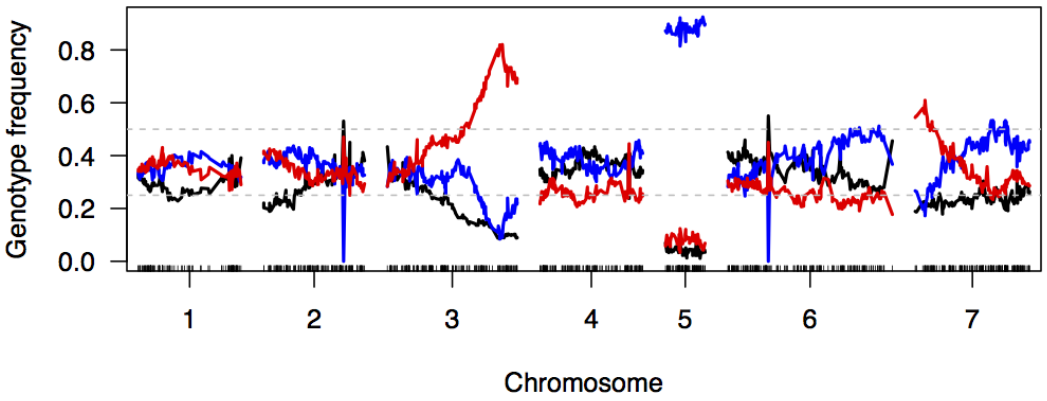


Figure 3.8: **Genotype frequencies across *A. rhodensis* chromosomes, for each marker (Black ticks).** Black and red lines represent the frequencies of homozygous markers for the APS4 or for the APS6 allele, respectively. Blue lines represent heterozygous markers. More than 80% of the progeny samples (n= 95) are heterozygous for the X chromosome (chromosome 5).

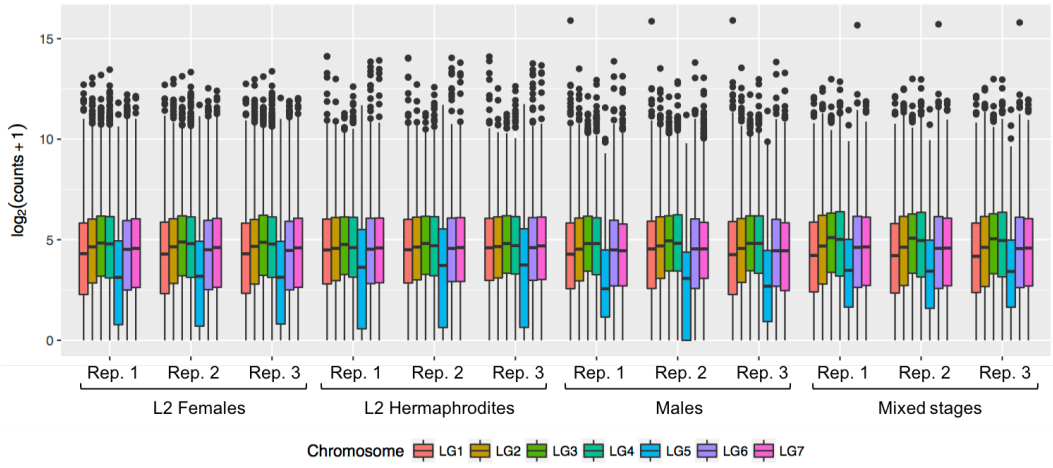


Figure 3.9: **Global gene expression for each chromosome.** Genes located on the X chromosome are generally less expressed than those on autosomes. Boxplots of the log2 normalised expression of the genes corresponding to each chromosome of *A. rhodensis* in different sexes, stages and libraries (biological replicates). The box of the boxplots represents 50% of the data (interquartile range or IQR), where, the upper bound of the box is the 75th percentile (usually referred to as Q3) and the lower bound is the 25th percentile (usually referred to as Q1). The line in the box indicates the median. The lower whisker's bound is calculated using the following formula $Q1 - 1.5 \times IQR$. Similarly, the upper bound of the whisker is calculated using the formula $Q3 + 1.5 \times IQR$. The dots above the whiskers are outliers, i.e. values higher than the whisker's calculated bound. The expression levels were normalised by the library size and log2-transformed for a better visualisation. The chromosome LG5 (in blue) is the X chromosome. 'Rep.' stand for biological replicate. This plot was generated using the R package ggplot2.

(and to sequencing errors). Indeed, I found that the strain APS6 displayed more intra-strain variants than APS4 (9142 for APS6 *versus* 4003 for APS4, $\chi^2 = 2008.3$, df = 1, P-value $< 2.2 \times 10^{-16}$). This result is expected, as APS6 underwent less inbreeding than APS4 (11 rounds of bottlenecking *versus* 50). Although the variant density is different between strains, the pattern of intra-variants seems similar for APS4 and APS6 (Figure 3.10). This reveals potential chromosomal regions prone to preserve higher variability.

The X chromosome displayed more intra-strain variation compared to the autosomes (Figure 3.10, Table A.2). More precisely, the X chromosome had 3.85 times more intra-APS4 variants than the autosomes ($\chi^2 = 1338.520$, P-value < 0.0001). For the intra-APS6 variants, the X chromosome had 3.0 times more variants than the autosomes ($\chi^2 = 1649.312$, P-value < 0.0001). The inter-strain variants do not show major macro-scale patterns, although we can note that LG1 and the X chromosome (LG5) display on average 1.54 and 1.63 times more inter-strain variants, respectively, than the other chromosomes (Figure 3.10, Table A.2, χ^2 P-values < 0.0001 between LG1 and the other chromosomes and between the X chromosome and the autosomes).

Gene density and pattern of conserved genes

In *C. elegans*, conserved genes are more common in the centre of the chromosomes and are rarer in the chromosomal arms (except for the X chromosome) [193, 206]. To visualise if the same pattern could be observed in *A. rhodensis*, I plotted the gene density and localisation of conserved genes between *A. rhodensis* and *Drosophila melanogaster* across chromosomes. The gene and conserved-gene densities were uniform and no particular pattern was observed (Figure 3.11). It is possible that our integrated genome is lacking subtelomeric regions, as these regions could to be of lower complexity. It is therefore possible that they were not properly assembled. The alternative hypothesis is that no pattern of conserved genes exists.

Intriguingly, we found fewer conserved genes on the X chromosome (χ^2 13.224, p-value < 0.001; Figure 3.11, Table 3.4). This may reflect a faster evolution of the X-linked genes. This point is further discussed in the overall conclusion, in light of the findings made in Chapter 4.

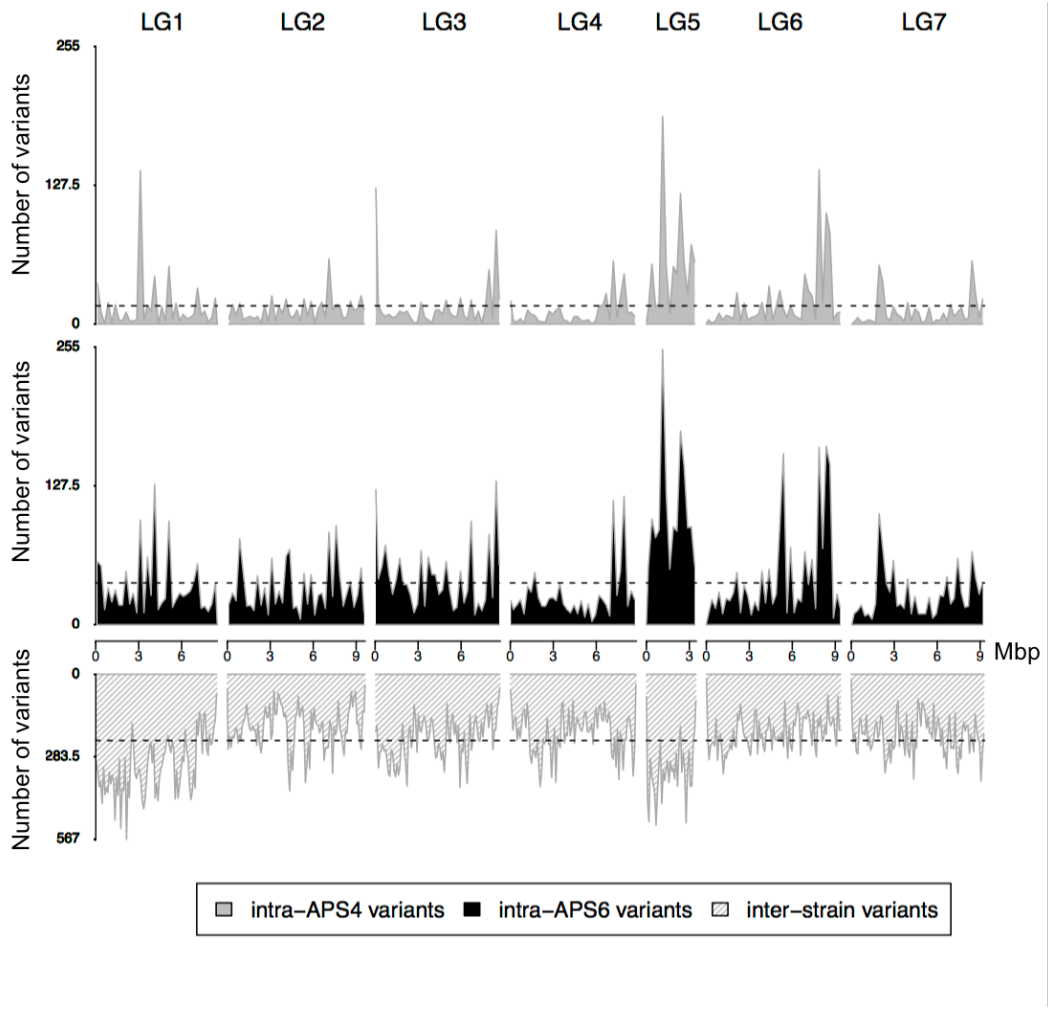


Figure 3.10: **Variant density along each chromosome within a 250,000bp window size for the intra-strain variants (upper panels) and a 100,000bp window size for the inter-strain variants (lower panel).** The dashed lines represent the mean number of variants throughout the genome.

3.3.7 Gene Ontology (GO) analysis contrasting the X and the autosomal gene categorisations

I conducted a gene ontology analysis to discover if the X chromosome genes were associated with over- or under-represented GO terms compared to the autosomal genes (See methods). The process of translation (especially peptide biosynthesis) and the ribosome component were found under-represented in the X chromosome gene set, as well as the functions of binding (particularly nucleic acid binding) and catalytic activity (particularly hydrolase activity) (Table 3.5). The process governing the 'neuropeptide signaling pathway' was found to be enrich in the X chromosome (Table 3.5).

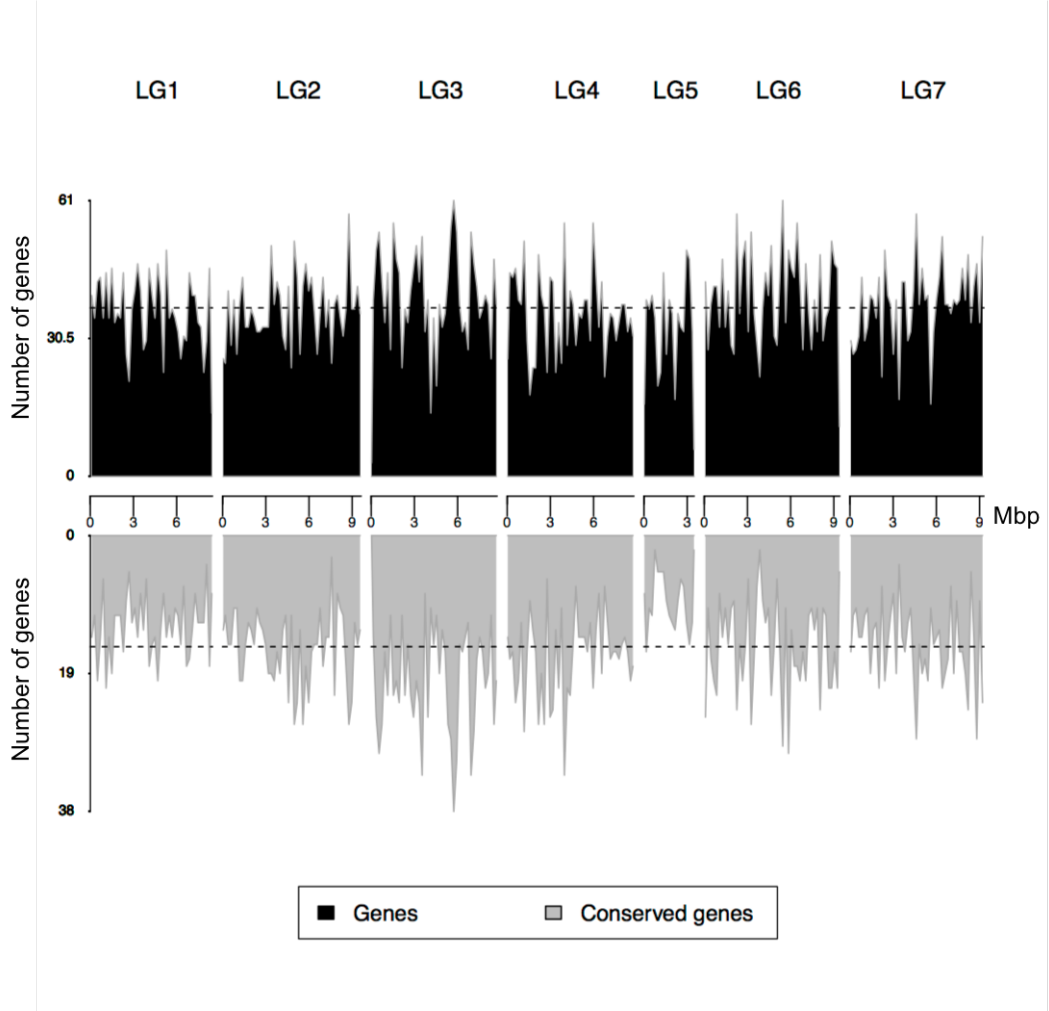


Figure 3.11: **Density of *A. rhodensis* genes (upper panel in black) and conserved genes between *A. rhodensis* and *D. melanogaster* (lower panel, in gray) along each chromosome using a 200,000 bp window size.** Overall, 4,544 conserved genes were identified between *A. rhodensis* and *D. melanogaster*. The dashed lines represent the mean number of genes throughout the genome.

Table 3.4: **Number of conserved genes between *A. rhodensis* and *D. melanogaster* and their proportion compared to non-conserved genes.** The proportion of conserved genes on the X chromosome is significantly smaller than on the autosomes (χ-squared 13.224, p-value < 0.001).

Chrom.	Approximate length	# genes	# conserved genes	Ratio (conserved genes)
LG1	8489927	1,538	526	0.34
LG2	9627060	1,760	745	0.42
LG3	8741542	1,748	866	0.49
LG4	8804062	1,586	735	0.46
LG6	9421540	1,871	714	0.38
LG7	9306279	1,754	692	0.39
LG5 (X)	3488253	604	169	0.27
Autosomes	54390410	10257	4278	0.41

Functional RNAs

Functional RNAs were annotated using Infernal and, to a lesser degree tRNAscan (for transfer RNAs), and RNAmmer (ribosomal RNAs) (see Methods). Small RNAs were identified for all major classes of small RNAs (except interfering RNAs) (Table 3.6). In *C. elegans*, it was found that the X chromosome, although representing only 20% of the genome, contains 44% of the transfer RNAs [206]. When looking at the localisation of small RNAs and, in particular, the transfer RNAs (tRNAs) in *A. rhodensis*, I found that the X chromosome contained no annotated tRNAs (Figure 3.12). These strikingly opposite patterns in *C. elegans* and *A. rhodensis* are difficult to explain (which will not be attempted here). The lack of tRNAs on the X of *A. rhodensis* may be partly due to their imperfect annotation and to the reduction in size of this chromosome. However, these explanations do not fully justify the complete lack of tRNAs on the X.

3.3.8 Other genomic analyses

Intra-genomic duplicates

When looking at potential duplicated genes within *A. rhodensis*, no particular pattern was observed. The X might have slightly more intra-duplicates than autosomes but this was not further investigated (Figure 3.13).

Table 3.5: **Results of the Gene Ontology (GO) analysis comparing the X chromosome GO categories to the autosomal ones.** 'U' and 'O' refer to 'under-representation' and 'Over-representation' of the GO term in the X compared to the autosomes.

U/O	GO ID	GO Name	GO Cat.	FDR	P-Value
U	GO:0006412	translation	BP	0.00537346	1.58E-05
O	GO:0007218	neuropeptide signaling pathway	BP	0.012919665	5.58E-05
U	GO:0005840	ribosome	CC	0.016937127	7.65E-05
U	GO:0003676	nucleic acid binding	MF	0.02400414	1.27E-04
U	GO:0043231	intracellular membrane-bounded organelle	CC	0.024533772	1.35E-04
U	GO:0016787	hydrolase activity	MF	0.03709992	2.26E-04

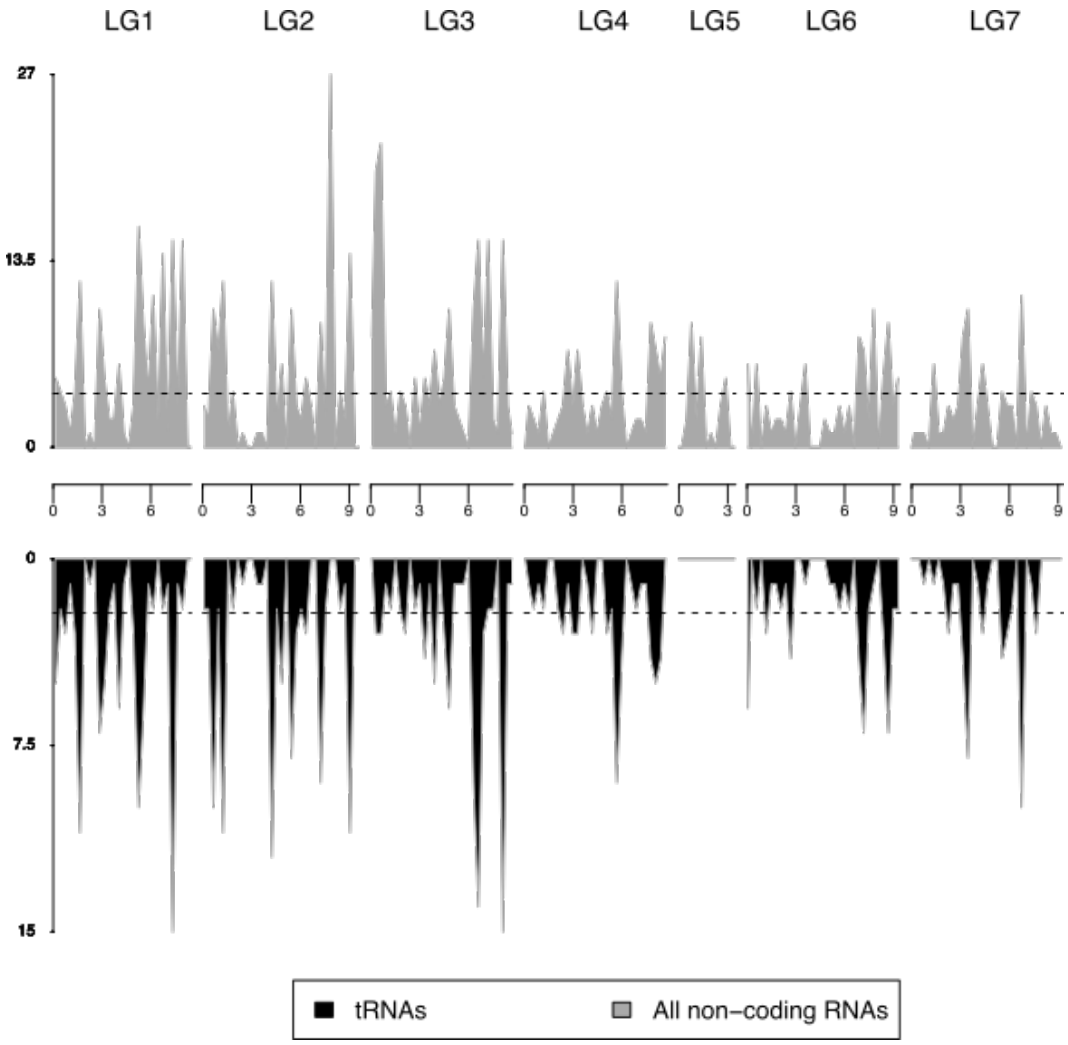
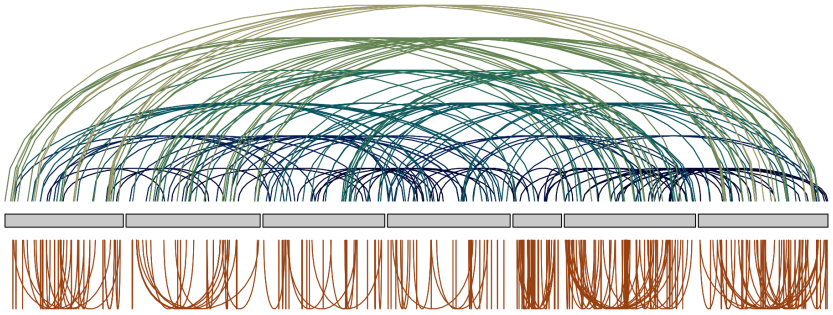


Figure 3.12: **Localisation of the annotated small RNAs and transfer RNAs (tRNAs) of *A. rhodensis*.**

Table 3.6: **Annotated functional RNAs in *A. rhodensis*.** The abbreviations siRNA, tncRNA, snoRNA, snRNA stand for small interfering RNA, tiny noncoding RNA, small nucleolar RNA, small nuclear RNA, respectively.

	A. rhodensis	*C. elegans* estimates [184]
Transfer RNAs (tRNAs)	465 unique (495 non-unique)	569
Ribosomal RNAs	45 (5S), 10 (28S), 5 (18S)	110 (5S), 55 (28S), 55 (18S)
Spliced Leaders	47	30 (10 SL1 and 20 SL2)
Spliceosome snRNAs (U1-6)	59 (19 U1, 9 U2, 4 U3, 14 U4, 7 U5, 6 U6)	72 (12 U1, 19 U2, 5 U4, 13 U5, and 23 U6)
SRP RNA	12	5
Other small RNAs (including microRNAs, snoRNAs, snRNAs)	145 (no siRNAs identified)	∼100 microRNAs, 37 tncRNAs, 25 snoRNAs (∼700 siRNAs)



	LG1	LG2	LG3	LG4	LG5	LG6	LG7	Total
Intra-duplicates	45	31	35	23	46	65	55	300
Inter-duplicates	53	58	64	65	43	83	79	445
Total duplicates	98	89	99	88	89	148	134	745

Figure 3.13: **Visualisation and number of potential duplicated genes in *A. rhodensis*.**

Identification of sex determination genes

Through reciprocal `BLASTp` (see methods), I identified the potential orthologues of the main sex determination genes of *C. elegans* [181], table 3.7). No clear orthologues were found for the sex determination genes sex-1, *xol-1*, *sdc-1*, *sdc-3*, *tra-2*, *fog-1*, *fog-2*, *mag-1*, *fem-3*, *fbf-1*, *fbf-2*, *nos-3* in *A. rhodensis*. This could be due to the fast evolution of sex determination genes [76] or to their absence. The sex determination genes identified in *A. rhodensis* are largely found on

autosomes (only one, *g8527*, was found to be on the X chromosome). This observation is not anomalous as the genetic sex determination mechanism of nematodes does not rely on a sex determinant gene located on a sex chromosome (such as the gene *sry* of the Y chromosome in mammals). The X dosage difference in males (XO) and females or hermaphrodites (XX), triggers a gene cascade leading to either a male or female soma and to spermatogenesis or oogenesis (see section 1.2.2 on page page 12 and [73]). In *C. elegans*, we can also observe that most sex determination genes are not on the X chromosome (Table 3.7)

Following the identification of some key sex determination genes, particularly *tra-1*, *mab-3* and *gld-1*, it will be possible to study their particular role in our 3-sexed system.

A. rhodensis does not harbour *Wolbachia*

Searches for *Wolbachia*-derived sequences in the genome of *A. rhodensis* did not result in any evidence of laterally-transferred *Wolbachia* material (no `BLAST` hits). Likewise, direct searches for the presence of *Wolbachia*, by looking at the raw reads before contamination removal, did not yield any matches with *Wolbachia*, which supports the conclusion that *A. rhodensis* does not harbour *Wolbachia*. This result is not surprising as *Wolbachia* was found absent in multiple non-filarial nematodes [53, 62, 63].

3.4 Discussion

In this chapter, we have examined various characteristics of the genome of *A. rhodensis*, the first free-living trioecious nematode to have its genome sequenced. Besides the assembly and initial characterisation of the genome, a genetic map was also constructed (Figure A.6). The coupling of these resources enabled the construction of an integrated genome (ordering of the genomic scaffolds into chromosomal blocks), which opens the door to genome and chromosome-wide studies. The assembly revealed that *A. rhodensis* genome is small (∼65 Mbp) and contains roughly half the number of genes (11,570) compared to *C. elegans* (∼20,000). It is known that the mating system can influence genome size in nematodes [61, 211]. In the *Caenorhabditis* clade, selfing species have a smaller genome than their outcrossing sister species [61, 211]. The lifestyle (parasitic *versus* free-living) may also have genomic consequences

Table 3.7: **Orthologs of known sex determination genes of *C. elegans* in *A. rhodensis*.** The e-values correspond to the `BLASTp` e-values using either the protein set of *C. elegans* as a dababase (DB) or, for the reciprocal `BLAST`, the one of *A. rhodensis*.

	C. elegans Chrom.	*A. rhodensis* gene	*A. rhodensis* Chrom.	E-value (*C. elegans* DB)	E-value (*A. rhodensis* DB)
fem-1	IV	*g7330*	LG6	9.66E-157	2.87E-144
fem-2	III	*g3906*	LG2	3.69E-74	1.60E-74
fog-3	I	*g2821*	LG4	8.18E-43	3.58E-45
fox-1	X	*g8527*	LG5 (X)	1.42E-60	2.45E-55
gld-1	I	*g5696*	LG1	1.51E-112	1.29E-114
her-1	V	*g4307*	LG1	1.48E-19	6.06E-20
laf-1	III	*g2153*	LG2	0	0
mab-3	II	*g797*	LG7	3.14E-44	3.77E-45
mog-1	III	*g2876*	LG4	0	0
mog-4	II	*g4360*	LG7	0	0
mog-5	II	*g9856*	LG1	0	0
mog-6	II	*g10475*	LG7	0	0
sdc-2	X	*g8906*	LG2	2.46E-135	1.12E-135
tra-1	III	*g4999*	LG2	4.28E-77	2.54E-76
tra-3	IV	*g8312*	LG2	0	0

[81, 82]. However, we lack genomic information on closely related nematodes displaying varied mating strategies and lifestyles to make phylogenetically relevant comparisons.

From our analyses, we observed that the X chromosome of *A. rhodensis* is much smaller than the autosomes (∼3.5Mb), representing only 6% of the genome and containing a little more than 600 genes. The intra-strain genetic diversity was found to be higher on the X chromosome and no transfer RNAs were found on it. Moreover, macro-syntenic patterns between *A. rhodensis*, *P. pacificus*, *H. contortus* and *C. elegans* revealed that a substantial proportion of orthologous X-linked genes in *C. elegans* and *H. contortus* were found on the chromosomes LG2 (32-37%) and LG4 (26-29%) of *A. rhodensis*. These genes largely corresponded to autosomal genes in *P. pacificus* (chromosome I) that underwent a translocation to the X chromosome in the branch leading to *Caenorhabditis* and strongylid nematodes. One working model to explain these reorganisations is that an autosome-to-X translocation happened in the lineage leading to Caenorhabditis/strongylids. This was then followed by an X-to-autosome translocation in the *A. rhodensis* lineage (Figure 3.14). Indeed, the orthologous proteins of the translocated autosomal region of chromosome I of

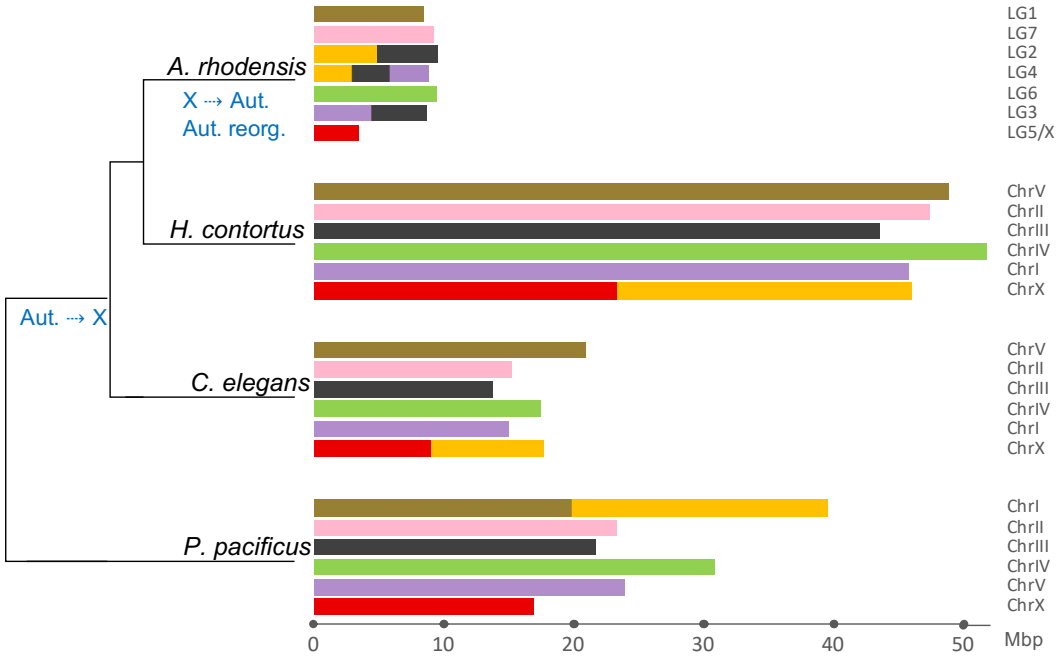


Figure 3.14: **Simplified schematic of the possible chromosomal reorganisations between the Rhabditid nematodes *A. rhodensis*, *C. elegans*, *H. contortus* and *P. pacificus*.** The colours represent largely orthologous chromosomes/chromosomal parts. It is to note however that there is a minor background of between-chromosome translocations (not represented here). Reshuffling within chromosomes is not shown.

P. pacificus are found in the X chromosomes of both *C. elegans* and *H. contortus* (Figure A.8, last line, Table 3.3). This is in accordance with the hypothesis that the autosome-to-X translocation occurred in the branch leading to *A. rhodensis*, *C. elegans* and *H. contortus*, as predicted by [163]. Subsequently, in the branch leading to *A. rhodensis* (phylogenetically closer to *H. contortus* than to *C. elegans*), a relocation of some X material to the autosomes occurred (Figure 3.14). However, it is to note that very few (only five) *A. rhodensis* X-linked genes were found to be orthologous to genes in the hypothesized translocated region of *P. pacificus* chromosome I. One possibility is that, following the large autosome-to-X translocation, the material ancestrally autosomal was preferentially removed from the X in the lineage leading to *A. rhodensis*. This can actually be clearly observed in the circos plot comparing *P. pacificus* and *A. rhodensis* (Figures 3.6 and A.8, top row line, right plot), in which the part of chromosome I in *P. pacificus* (that moved to the X chromosome in *C. elegans* and *H. contortus*) also corresponds to autosomal material in *A. rhodensis*, but not significantly to X material.

Another feature of the genome of *A. rhodensis* is that it has 7 chromosomes instead of 6 (as found in *P. pacificus C. elegans* and most Rhabditids) [17, 41]. One hypothesis is that originally LG2 and LG4 were part of the same ancestral chromosome (which was largely orthologous to the chromosome III in *C. elegans*, *H. contortus* and *P. pacificus*) (Figure 3.7). This chromosome then incorporated material coming from the X, before splitting into separate chromosomes. I speculate that LG3, which also displays a split pattern, was ancestrally orthologous to the chromosomes I in *C. elegans* and *H. contortus* and V in *P. pacificus*. Translocations between the ancestral LG3 and the 'proto-LG2/LG4' or LG2 and, independently LG4 would have occurred resulting in the existing conformation. The orthologous material of *P. pacificus* and *C. elegans* in *A. rhodensis*' chromosomes LG2, LG3 and LG4 was found clustered (Figure 3.7). This suggest that these rearrangements are relatively recent, as the material from the different translocations is not completely shuffled (which is expected to happen over time).

Chapter 4

Atypical inheritance of the X chromosome

Summary

Meiosis is at the core of sexual reproduction and alterations to its programme can have dramatic effects. In this chapter, I investigate the segregation pattern of the X chromosome in *A. rhodensis*. It was previously shown that the X chromosome segregation follows an atypical pattern during male spermatogenesis, resulting in the production of haplo-X sperm. Here, I report that while XX females undergo conventional meiosis to produce mostly haplo-X oocytes, hermaphrodites generate predominantly nullo-X oocytes and diplo-X sperm. Consequences of these meiotic variations are that (i) no recombination occurs between the X homologues in hermaphrodites, (ii) crosses between hermaphrodites and males generate only male progeny and (iii) the X chromosome is transmitted from father to son in an event of a cross. Given the intra-species, intra-individual and intra-gametogenesis variations in the meiotic programme of *A. rhodensis*, I discuss its potential as a model to study the plasticity of meiosis and how it can be modulated.

4.1 Introduction

Meiosis is a conserved process at the core of sexual reproduction, an event which radiated the eukaryote kingdom. It is the process that enables diploid organisms to produce haploid gametes. This is achieved by first doubling the genetic content, followed by two rounds of chromosome segregation. In the first round of reduction, during meiosis I, homologous chromosomes ('homologues') pair and undergo crossing-overs before segregating to opposite poles. In meiosis II, the sister chromatids separate to opposite poles. These meiotic processes not

only ensure correct segregation of the genetic material, but also act to conserve the integrity and stability of the genome as well as providing a mechanism to generate genetic variation through independent assortment and molecular recombination.

More precisely, in the prophase of meiosis I, homologues pair, align and join lengthwise in a process called synapsis (formation of a synaptonemal complex) and undergo crossover (CO) recombination. In *C. elegans*, the activation of pairing centres, i.e., specific regions located at the chromosome extremities, enables homologues to find their homologous partner [164]. At this stage, the sister chromatids of the chromosomes are tightly bound together. Double stranded breaks (DSBs) in the DNA are then initiated along the chromosomes, some of which will mature into crossing-overs, allowing the homologues to undergo a bidirectional exchange of genetic material through the process of recombination. The crossing-overs only occur between non-sister chromatids. The synaptonemal complex, a proteinaceous association, forms to hold the homologues together and assure the close parallel alignment of each pair of homologues from end to end [164, 213]. The crossing-overs are stabilised by the formation of chiasmata, i.e., the physical points of contact between the non-sister chromatids. The chiasmata are critical to hold the homologues together after the dissolution of the synaptonemal complex and to ensure the proper segregation of the homologues to each pole.

The meiotic mechanisms have been widely studied in *C. elegans*. However, it is important to note that major differences exist, even within the nematode phylum, in the dynamics of the meiotic events. For example, in most plants, fungi and animals (including the nematode *P. pacificus*) recombination is essential to initiate homolog pairing and synapsis. In contrast with this mechanism, the model organisms *C. elegans* and *D. melanogaster* use a recombination-independent pairing and synapsis system. Recent studies comparing *C. elegans* and *P. pacificus* are starting to uncover the molecular basis of these differences.

Despite these variations of the meiosis programme, faithful chromosome segregation is usually achieved as mistakes lead to developmental impairments. Errors in homolog pairing and/or recombination result is subsequent meiotic defects, including non-disjunction between homologues and premature separation of sister chromatids during meiosis I [214]. The most common consequence of meiotic errors is aneuploidy, i.e., the abnormal number of chromosomes in the gamete. In humans, aneuploidy is the leading cause for miscarriages and developmental disorders such as Down's (trisomy 21), Klinefelter (XYY) and

Turner (XO) syndromes [145].

The XX/XO sex determination system in *C. elegans* facilitates the study of meiosis because mutants are easy to recognise. Wild type hermaphrodites self-fertilise to give rise to mostly hermaphrodite progeny. In meiotic mutants, however, the X chromosome can fail to segregate properly. A number of genes (notably the *him* genes), if mutated, result in the nondisjunction of the X chromosome homologues [78]. This results in a higher production of gametes with two X chromosomes (2X) or none (nullo-X or 0X). This elevated aneuploidy of the X chromosome in *C. elegans* results in self-progeny with a high-frequency of males due to 0X-1X gamete encounters, triplo-X individuals (2X-1X gamete encounters) and, in some cases a high level of lethality [78, 134]. The transparency of the animal, easy identification of the meiotic events along the gonad, simple karyotype ($2n = 12$), and genetic tools make *C. elegans* a powerful system to study meiosis [164].

A. rhodensis [95] offers much of the same advantages as *C. elegans* for studying meiosis: it has an XX/XO sex determination system, is small ($\sim$1 mm), free-living, has a transparent gonad and can be easily maintained in the laboratory [59, 95, 170]. Furthermore, *C. elegans* provides a good reference for comparative studies for *A. rhodensis*, as they both are members of the Eurhabditis clade [98].

It was previously reported that the *A. rhodensis* sex chromosome displays a non-canonical behaviour during XO male gametogenesis [170, 207]. Indeed, during meiosis I, while the homologous autosomes segregate to each pole, the sister chromatids of the unpaired X chromosome prematurely separate [170]. During meiosis II, following anaphase II, an asymmetric partitioning of the cytoplasm occurs (mechanism unknown): the cellular components necessary for the sperm (e.g. mitochondrias, sperm proteins) are found on the side containing the X chromosome [170, 207]. This results in functional X-bearing sperm and nullo-X residual bodies [170, 207]. This unique meiosis therefore systematically generates X-bearing sperm from XO males.

Through the construction of the genetic map (*confer* Chapter 3), we observed a severe segregation distortion of the markers belonging to the X chromosome (all X-linked markers were heterozygous across most samples). This result implies that the X homologues almost did not recombine during the process of generating the Advanced Intercross Lines (AILs) between the strains APS4 and APS6. To understand this result, I followed the X chromosome dynamics through genetic crosses and genotyping.

In this chapter, I will report the findings of these experiments, which uncovered additional variations in the meiotic X chromosome segregation programme of *A. rhodensis*. These results were published [188] and complemented by cytological studies performed by members of the Shakes lab (College of William and Mary, Williamsburg). To summarise briefly, the genetic experiments (presented here) and cytological studies (See [188]) revealed that the X chromosome segregation pattern differs between sexes (females *versus* hermaphrodites) and gametogenesis type (oogenesis *versus* spermatogenesis) as well as, within the same gametogenesis. The genetic, population-genetic and evolutionary consequences of these modulations are discussed.

4.2 Materials and Methods

4.2.1 Strains

For this study, the experiments were conducted on the inbred strains APS4 and APS6 of *A. rhodensis*, used in Chapter 3 for making the Advanced Intercrossed Lines (AILs). The origin and creation of the inbred strains is detailed in Sections 2.2.1 (page 25) and 3.2.1 (page 41).

4.2.2 Genotyping of chromosomes

To genotype the X chromosome and autosome 4 (LG4), I first identified 5 polymorphic markers (SNPs) between the strains APS4 and APS6, for each chromosome (Table B.1, Figure 4.2). I selected these markers from the genome and strain-specific sequences (RAD-seq markers) (see Chapter 3). The polymorphic markers were chosen based on the presence of a restriction enzyme cutsite specific to one strain but not the other. Genotyping *per se* was performed through PCR amplifications of the region containing the SNP (single-worm PCRs) followed by digestion of the products (Table B.1). For this protocol, it is not necessary to perform a full DNA extraction. For each individual genotyped, one adult worm was picked and placed in a PCR tube (0.2 mL) containing 10 µL of PCR buffer mix (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$). The worm was then freezed-cracked by freezing the tube at -80 °C overnight or until required and then thawing it at room temperature. Following the freeze-cracking, the mixture was incubated at 65 °C for 60 min with 0.5 µl of proteinase K. The enzyme was then inactivated at 95 °C for 15 min. This

worm lysis yields sufficient free DNA to perform PCR amplifications. These were performed in a total volume of 20 µl, using the GoTaq G2 green master mix (Promega), 2 µl of the worm lysis (template) and 5 µmol dm^{-3} of each primer (Table B.1). The cycling conditions of the PCRs were 95 °C for 7 min (initial denaturation), followed by 30-35 cycles of 15 s at 94 °C (denaturation), 30 s at 55 °C (primer annealing), and 1 min at 72 °C (extension). Restriction enzyme digestions of the PCR products were done at 35 °C for one to two h. The genotype of each marker was revealed by gel electrophoresis of the digested products. The markers were confirmed to be X-linked by genotyping APS4/APS6 hybrid[1] F1 males (XO). As expected from hemizygosity in XO animals, F1 males either had the APS4 or the APS6 genotype for X chromosome markers.

4.2.3 Crosses between hermaphrodites and males

To distinguish hermaphrodite self-progeny from cross-progeny, I used morphologically-marked hermaphrodites (*dumpy* phenotype, strain APS19). This strain was created by Talal Al Yazeedi. The *dumpy* phenotype is caused by a recessive mutation (personal communication Talal Al Yazeedi). The *dumpy* phenotype is easily recognisable as the worms are much shorter and, proportionally larger compared to wild type individuals (Figure 4.1). Ten crosses between a *dumpy* hermaphrodite and a wild type APS4 male were performed. The offspring were scored according to their phenotype (*dumpy versus* wild type) and gender at the adult stage. The females and hermaphrodites were not distinguished.

4.3 Results

4.3.1 Tracking the X chromosome dynamics through genotyping

To track the segregation patterns of the X chromosomes from both the maternal and the paternal parents, I identified single nucleotide polymorphisms (SNPs) between two independently isolated strains of *A. rhodensis* (APS4 and APS6). Using 5 polymorphic markers distributed along the length of the X chromosome (Figure 4.2, top), I followed the pattern of inheritance of the X chromosome in F2

[1]Hybrid individuals are the F1s produced by a cross between APS4 and APS6 parents. The'hybrid' F1s are heterozygous at all loci differing between the APS4 and APS6 strains.

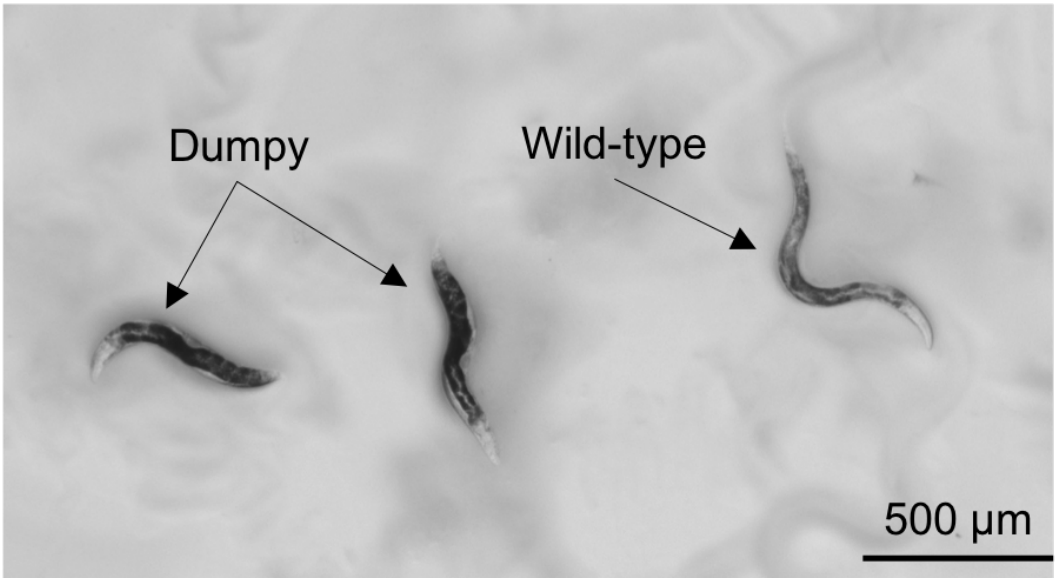


Figure 4.1: ***Dumpy versus* wild-type phenotypes (in XX individuals).**

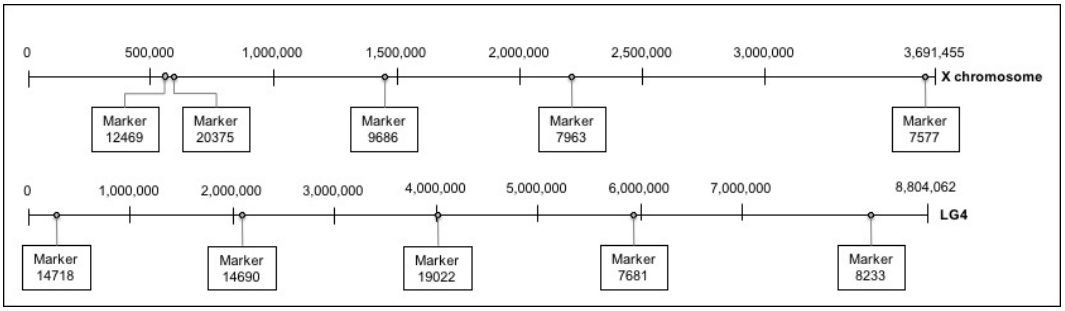


Figure 4.2: **Schematic view of the markers used to genotype the X chromosome (top) and the LG4 autosome (bottom).**

individuals produced by hybrid ($X_{APS4}X_{APS6}$) female or hermaphrodite mothers derived from original crosses between the inbred strains APS4 (carrying X_{APS4}) and APS6 (carrying X_{APS6}) (Figure 4.3). In parallel, the same methodology was used to genotype 5 autosomal markers (on LG4) spread across its length (Figure 4.2, bottom).

X chromosomes in females

Intra-specific hybrid ($X_{APS4}X_{APS6}$) F1 females crossed with males of one of the parental strains (e.g., X_{APS6}) produced F2 XX progeny with the expected 1:1 ratio of homozygous ($X_{APS6}X_{APS6}$) to heterozygous ($X_{APS6}X_{APS4}$) markers in the X chromosome (chi-squared = 3.37, df = 1, p-value = 0.07, Table 4.1). Notably, I also recorded 12 examples of crossing-overs, where the X

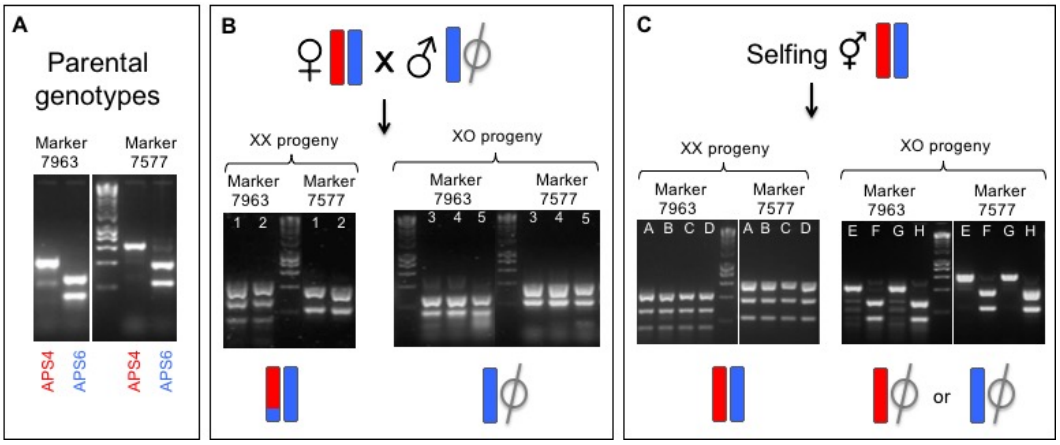


Figure 4.3: **Representative profiles of the X Genotyping Results.** (A) genotyping profile of parental strains. (B) a hybrid APS4/APS6 female crossing with an APS6 male generates XX progeny with both homozygous and heterozygous X markers. Crossovers could be inferred when the X of one individual was part heterozygous, part homozygous, as represented here by individuals 1 and 2. (C) X genotyping of individuals produced by hybrid selfing hermaphrodites reveals that the X chromosome remains heterozygous in XX individuals and hemizygous for each parental strain in males. Numbers in each gel lane represent individual animals.

genotyping showed some markers as heterozygous and others as homozygous in a same individual (Figure 4.3(B), Table 4.1 and Figure B.1). These data suggest a conventional meiotic pairing and segregation of the X chromosome in *A. rhodensis* females.

Table 4.1: **Oocyte meiosis in hybrid females.** Genotype marker counts in F2 XX progeny (females and hermaphrodites) produced by hybrid $X_{APS4}X_{APS6}$ F1 females crossed with either X_{APS4} or X_{APS6} males. The abbreviations 'Fem.' and 'Herm.' stand for 'Females' and 'Hermaphrodites', respectively. Between parentheses are the percentages of homozygous and heterozygous markers and their binomial confidence intervals (calculated for a 95% confidence level). The genotypes (homozygous and heterozygous) refer to the markers (loci) genotyped, not entire chromosomes.

Cross	Progeny	N ind.	Heterozygous	Homozygous	CO
F1 ♀	Fem.	7	8 (29% ± 17.7%)	20 (71% ± 17.7%)	4
×	Herm.	13	29 (60% ± 14.5%)	19 (40% ± 14.5%)	5
APS4 ♂	Total	20	37 (49% ± 11.7%)	39 (51% ± 11.7%)	9
F1 ♀	Fem.	6	17 (89% ± 15.9%)	2 (11% ± 15.9%)	2
×	Herm.	10	21 (62% ± 17.1%)	13 (38% ± 17.1%)	1
APS6 ♂	Total	16	38 (72% ± 12.8%)	15 (28% ± 12.8%)	3

X chromosomes in hermaphrodites

Analysis of F2 XX progeny produced by selfing F1 ($X_{APS4}X_{APS6}$) hermaphrodites revealed a very different pattern. The F2 XX progeny were invariably heterozygous for the X chromosome (Figure 4.3(C), Table 4.2, Figure B.2). For this analysis, I genotyped 82 F2 XX progeny produced by $X_{APS4}X_{APS6}$ F1 hybrid hermaphrodites (generated by either APS4 females crossed with APS6 males or its reciprocal) across the 5 X-linked markers (Figure 4.2, top). Although Mendelian segregation patterns would predict a 1:2:1 ratio of $X_{APS4}X_{APS4}$:$X_{APS4}X_{APS6}$:$X_{APS6}X_{APS6}$ genotypes amongst the XX F2s, all individuals were heterozygous ($X_{APS4}X_{APS6}$) across the 379 markers successfully genotyped (Figure 4.3(C), Table 4.2), which implies that no recombination between the X chromosomes took place.

Table 4.2: **Genotype counts of F2 XX progeny from hybrid ($\mathbf{X_{APS4}X_{APS6}}$) F1 selfing hermaphrodites.** The abbreviations 'N ind.','Het.' and 'Hom.' stand for 'Number of individuals genotyped','Heterozygous' and 'Homozygous', respectively. Between parentheses are the percentages of homozygous and heterozygous markers and their binomial confidence intervals (calculated for a 95% confidence level). The genotypes (homozygous and heterozygous) refer to the markers (loci) genotyped, not entire chromosomes.

Progeny	N ind.	Het.	Hom.	Crossovers
Female	40	183 (100% ± 1%)	0 (0% ± 1%)	0
Hermaphrodite	42	196 (100% ± 0.93%)	0 (0% ± 0.93%)	0
Total	82	379 (100% ± 0.48%)	0 (0% ± 0.48%)	0

Importantly, this behaviour was specific to the X chromosome, as genotyping of the autosome LG4, also across 5 markers (Figures 4.2, bottom and 4.4), yielded a mix of homozygous and heterozygous markers in keeping with Mendelian expectations (24 homozygous and 12 heterozygous markers). In addition, autosomal crossing-overs could be observed, as the genotype was not uniform across all markers for a same individual (see Figure 4.4 for a few examples).

4.3.2 Sex- and gamete-specific variations of the meiotic X chromosome segregation

A. rhodensis male-female crosses result in mostly XX progeny (hermaphrodites or female) (see Chapter 2 and [36]). Additionally, cytological analyses show

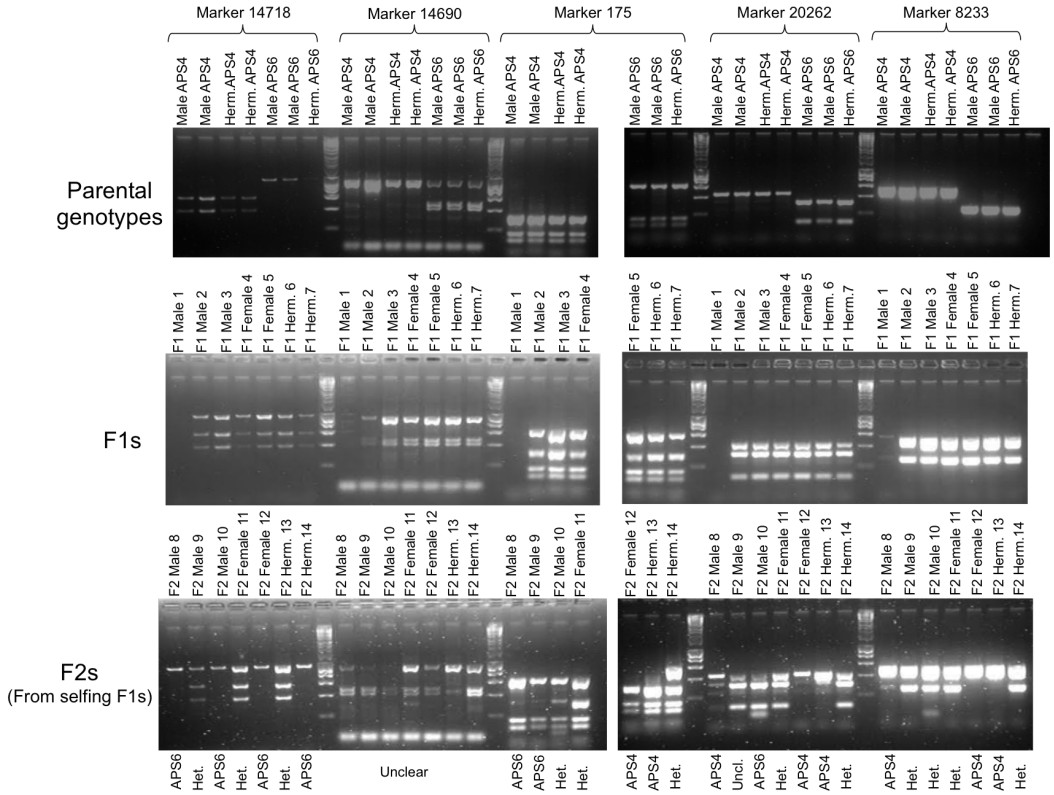


Figure 4.4: **Example of LG4 (autosome) genotyping using amplification, digestion and electrophoresis.** F2 genotypes are reported under the gel pictures. 'Het.' and 'Uncl.' stand for heterozygous and unclear genotypes, respectively. Numbers indicate individual animals. This figure comes from the supplemental information of [188].

that an asymmetrical meiosis in males leads to the production of functional X-bearing sperm and nullo-X polar bodies [170, 207]. *A. rhodensis* XO males routinely produce haplo-X sperm . Without a mutant, it had been impossible to distinguish between self and outcross progeny from hermaphrodite oocytes fertilised by male sperm for the population dynamics study (*confer* Chapter 2). Using a morphologically-marked strain containing a recessive *dumpy* mutation, it was possible to determine the sex of the cross-progeny by crossing *dumpy* hermaphrodites with wild-type males. In such crosses, self-progeny is *dumpy* while cross-progeny is wild-type. Contrary to expectations, all cross-progeny were male (306 normal males scored from 10 hermaphrodite/male crosses).

Since male sperm have a single X [170, 207], this result implies that XX hermaphrodites produce oocytes without an X (nullo-X oocytes) (Figure 4.5(C)). In turn, as self-fertilising hermaphrodites produce 90-95% XX progeny [36,

57, 59], it can be hypothesised that hermaphrodite produce diplo-X sperm (Figure 4.5(D)). The nullo-X oocytes would then be fertilised by hermaphrodite sperm that are predominantly diplo-X. This pattern of diplo-X sperm and nullo-X oocytes was supported by cytological studies, in which (i) during hermaphrodite oogenesis, both X chromosomes appeared to be segregating to the polar body and (ii) during hermaphrodite spermatogenesis, the X chromatids separated prematurely during meiosis I, before partitioning together to the pole fated to become functional sperm.

4.3.3 Father-to-Son X Chromosome Inheritance

The dominant X segregation patterns of female and hermaphrodite meiosis portrayed in Figure 4.5 do not explain how the rare XO males are generated from outcrossing females or from selfing hermaphrodites. To study this, I genotyped the X chromosome of males produced by female/male crosses or by selfing hermaphrodites.

Males Produced by Male/Female Crosses

Males resulting from female/male crosses (either APS4 female crossed with APS6 male or vice versa) always inherited the X markers of their father (∼20 males genotyped across five X chromosome markers for both types of crosses, Table 4.3). This result implies that, at times, the female produces viable nullo-X oocytes as we know that the X chromosome was provided by the father (Table 4.3 and [170]). However, most of the time females produce haplo-X gametes through a conventional meiosis as female-male crosses mostly result in XX progeny (female or hermaphrodite) as we have seen in Chapter 2.

Males Produced by Selfing Hermaphrodites

Males produced by selfing $X_{APS4}X_{APS6}$ hermaphrodites either carried X_{APS4} or X_{APS6} (Figure 4.3(C)). No crossing-overs could be inferred (100 genotypes from 21 males genotyped; Figure 4.3(C)) and it is, therefore, possible that no recombination between the X homologues occurred. This result implies that, at times, the hermaphrodite must produce haplo-X gametes, possibly through an unconventional meiosis as no crossing-overs were recorded. As no XX progeny was observed from the cross between hermaphrodite and male, one hypothesis

is that males (generated from selfing) come from haplo-X sperm fertilising nullo-X oocytes.

Table 4.3: **X chromosome genotyping of F1 males resulting from crosses between the APS4 and APS6 parental strains.** Between parentheses are the percentages of homozygous and heterozygous markers and their binomial confidence intervals (calculated for a 95% confidence level).

	APS4 ♀ × APS6 ♂		**APS6 ♀ × APS4 ♂**	
F1 ♂ genotype	**APS4**	**APS6**	**APS4**	**APS6**
Marker 9686	0	21	18	0
Marker 12469	0	23	18	0
Marker 20375	0	20	20	0
Marker 7963	0	20	15	0
Marker 7577	0	18	19	0

These observations indicate that the meiosis programme is actively modulated within the same type of gametogenesis, generating a flexible system where the proportion of male offspring could be adjusted through regulation of the X chromosome segregation in both female and hermaphrodite mothers. The factors controlling this regulation, and thus the XO:XX sex ratio, could be environmental, and may reflect adaptation to the colonisation ecology of *A. rhodensis*.

4.4 Discussion

4.4.1 Variations of the X chromosome segregation differ between sex and gametogenesis type

In this chapter, we have reviewed the molecular data supporting the conclusion that meiosis of the sex chromosome can be modulated within a species in a same genetic context. *A. rhodensis* is unusual in having three sexes: male, female and hermaphrodite. The meiosis programme governing X chromosome inheritance in this nematode varies with the sex of the parent, the gametogenesis type and even within the same gametogenesis. These data, combined with cytological studies aimed at visualising the X chromosome dynamics (see [188]), enabled us to create a model of the X chromosome dynamics (Figure 4.5). Female oogenesis displays a classical meiosis with recombination and Mendelian segregation of the X chromosomes, generating (most of the time) the expected

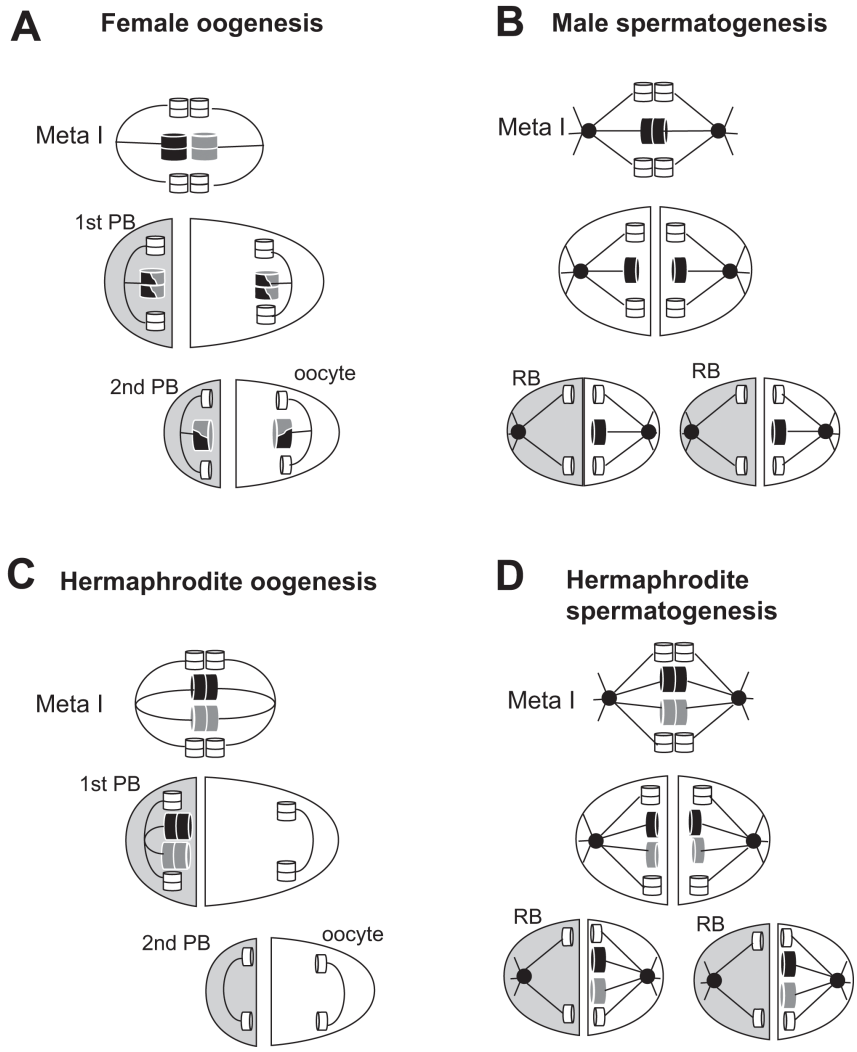


Figure 4.5: **Simplified Model of the X Chromosome Segregation Mechanism in *A. rhodensis*.** (A) In females, autosomes (white cylinders) and X chromosomes (darker and larger cylinders) dynamics follow the canonical segregation pattern, with pairing and crossing-over (most of the time). At times, females produce functional nullo-X oocytes (not represented here). Shaded cells are polar bodies (PBs). Lines represent microtubules. (B) In XO males, the homologous autosomes segregate to different daughter cells in meiosis I, and sister chromatids separate in meiosis II (conventional meiosis). For the unpaired X chromosome, however, sister chromatids separate in meiosis I. In meiosis II, the X chromatids co-segregate with one autosome set to the functional sperm, whereas the other set of autosomes is discarded into a residual body (RB; shaded in gray). Black circles represent centrioles. (C) Hermaphrodite oogenesis generates functional nullo-X oocytes. During meiosis I, the homologous X chromosomes are unpaired at the metaphase plate and, during anaphase I, all X chromatids segregate to the first polar body. (D) Hermaphrodite spermatogenesis generates diplo-X sperm. During meiosis I, the homologous X chromosomes are unpaired at the metaphase plate and separate into sister chromatids. During meiosis II, both X chromatids (non-sisters) segregate to the functional sperm. It is hypothesised that at times hermaphrodites produce haplo-X sperm (not represented here). This figure comes from the article [188].

haplo-X gametes (Figure 4.5 (A)). In both hermaphrodite oogenesis and spermatogenesis, the X chromosomes fail to pair. In hermaphrodite oogenesis, the X chromosomes segregated to the same pole (first polar body), yielding nullo-X oocytes (Figure 4.5 (C)). During hermaphrodite spermatogenesis, a precocious sister chromatid separation of the X chromosomes during meiosis I followed by a partitioning of the X chromatids to the functional sperm during meiosis II, led to the production of diplo-X sperm similarly of the dynamics observed during male spermatogenesis (Figure 4.5 (B)). One consequence of this division is that the two X chromatids contained in the hermaphrodite sperm are homologues and not sisters.

X chromosome fates in hermaphrodite oogenesis and spermatogenesis balance each other, as diplo-X sperm fertilise nullo-X oocytes to create XX zygotes (most of the time). A direct outcome of this atypical system is that it maintains the heterozygosity of X chromosomes transmitted during hermaphrodite reproduction, as crossing-overs and thus recombination between the X homologues never occurs. In this manner, X chromosome segregation differs between gametogenesis in hermaphrodites compared to females, and between spermatogenesis and oogenesis within the same hermaphrodite. As genetically identical X chromosomes can be segregated differentially within each individual, control of the atypical meiosis process observed in hermaphrodite gametogenesis cannot lie in the X chromosome sequence *per se*.

4.4.2 Molecular mechanisms possibly modified to explain the X chromosome atypical segregation

Even though the molecular mechanisms mediating the recognition of homologues are still not fully understood, chromosomal regions named pairing centres (PCs) play a major role in the pairing, synapsis, crossover and disjunction of homologues in *C. elegans* [127, 164]. The recruitment of various proteins including ZIMs/HIM-8, PLK-2, SUN-1, and ZYG-12 at the site of the PCs is required to promote and stabilise homolog pairing and to link the PCs to microtubules (reviewed in [164]). The zinc-finger protein HIM-8 specifically targets the X chromosome PCs of *C. elegans* and is required for proper X chromosome segregation [155]. In *him-8* mutants, the X chromosome homologues fail to pair and synapse, which results in a high rate of X non-disjunction. In *A. rhodensis*, where there is a lack of pairing between the X homologues during hermaphrodite oogenesis, it may be that trans-acting factors specific to X

chromosome meiosis, such as HIM-8, are differentially regulated between female and hermaphrodite oogenesis. As observed in *C. elegans* [44], the resulting X univalents would be preferentially placed in the first polar body and thus eliminated. However, no *him-8* ortholog was identified in *A. rhodensis*.

In *C. elegans*, the cis-acting *me8* mutation directly alters the PCs and results in a lack of crossing-overs and disjunction of the X chromosome [197]. In *A. rhodensis*, because the modulations of X chromosome segregation are independent of the sequence of the chromosome, this genetic mechanism cannot be the cause of the variant segregation behaviours. However, the X chromosome (particularly the PC region) could be subject to differential chromatin modification that affects homologous pairing or recombination. Heteromorphic sex chromosomes have been shown to undergo more condensation than autosomes. This is thought to prevent harmful non-homologous recombination between heterogametic chromosomes [133]. In accord with these findings, the X chromosome of *C. elegans* appears highly condensed and repressed during male meiosis [97]. Despite the fact that the X chromosomes of hermaphrodites are, surprisingly, also found compacted during meiosis, the pairing and recombination between X homologues follows a different pattern than that of the autosomes [97], suggesting the presence of an X-specific meiotic machinery.

The premature separation of sister chromatids (PSSC) of the X chromosomes observed during the spermatogenesis of *A. rhodensis* hermaphrodites (see cytological evidence in [188]) is reminiscent of the atypical X chromatid separation occurring during meiosis I of male spermatogenesis [170, 207]. The X PSSC in both male and hermaphrodite spermatogenesis could be governed by the same mechanism. The cohesin complexes, and particularly the kleisin subunits REC-8 and COH-3/4, necessary to hold the sister chromatids together, could be involved as they have been shown to play an important role in the correct segregation of homologues (reduction of ploidy) and chromatids in *C. elegans* [154, 169]. Indeed, REC-8 and COH-3/4 cohesins are crucial for the efficient formation of crossing-overs and correct assembly of the synaptonemal complex between homologues [168, 169]. REC-8 in particular helps the co-orientation of the sister chromatids towards the same pole [168, 169]. Mutations of the *rec-8* gene induce premature separation of the sister chromatids and lack of connection between homologues [154]. In *A. rhodensis* it is possible that cohesin complexes, required to tether the sister chromatids during meiosis I, follow a different dynamic, allowing the precocious separation of the X chromatids during spermatogenesis diakinesis.

4.4.3 X chromosome transmission through sperm

In male and hermaphrodite spermatogenesis ([170, 207], this study), the X chromosomes segregate to the pole fated to become functional sperm. A remarkable consequence of this system is that the X chromosome is inherited through the sperm-producing germline and from father to son in the event of a cross. This is the only example of a complete X chromosome transmission through the male lineage (but normal autosomal transmission) in a sexually reproducing context that could be found in the literature. This finding also implies that, during female meiosis, unusual meiotic divisions must sometimes generate nullo-X oocytes, presumably in a manner mechanistically similar to the routine production of nullo-X oocytes in hermaphrodites.

The atypical male-to-male transmission of the X chromosome in *A. rhodensis* is reminiscent of androgenesis, a type of reproduction that occurs in a conifer, a few ants and stick insects, and clams of the genus *Corbicula* (reviewed by [166]). In these systems, the male inherits the genome solely from his father. As a consequence, this may lead to the genetic divergence of the female and male lineages over time [64]. However, in *A. rhodensis*, the father-to-son genetic inheritance is limited to the X chromosome, which is transmitted to all sexual morphs and has a chance to recombine in females, thus preventing the genetic divergence of the X between XO males and XX individuals.

One evolutionary consequence of this observation is that any beneficial mutations on the X will spread quickly through the population, as male carriers will transmit it to all their offspring, including their sons, which will, in turn, systematically pass it on. Additionally, no crossing-overs occur between the X chromosomes during hermaphrodite meiosis, which means that the *A. rhodensis* X chromosome has a very different recombinational and evolutionary trajectory from the *C. elegans* X. If X-linked genes control traits subject to selection, the maintenance of diversity in X chromosomes in XX nematode offspring could allow for an enhanced adaptability and therefore promote a more successful colonisation of a new habitat from a single hermaphrodite nematode.

To explain the occurrence of male offspring from selfing hermaphrodites, it is possible that hermaphrodite spermatocytes sometimes divide to generate haplo-X rather than diplo-X sperm (although the genetic data suggest that no recombination between the X chromosome seems to occur even in such events). Interestingly, selfing hermaphrodites tend to produce more males early in their reproductive life (Chapter 2, [36]). This could signify that the type of gamete

produced is developmentally regulated. Additionally, it was shown that sperm, within the hermaphrodite germline, are produced in spermatogonial clusters [132]. One hypothesis is that different clusters produce sperm with different X chromosome complements.

4.4.4 Perspectives of the findings

The modulation of the meiosis programme, occurring within an identical genetic context, make *A. rhodensis* an ideal model to study how the meiosis process is regulated. Particularly, this study opens the door to investigate the mechanisms that control the X chromosome segregation. It is to note that developmental context (hermaphrodite *versus* female) plays an important role in the modulation of meiotic processes affecting the X. For instance, XX animals that develop through a dauer larva stage always become hermaphrodites ([37], introduction), whereas larvae that bypass this stage become females. What triggers this differential development (discussed in the next chapter) and how it links with the meiotic process are still open questions. More broadly, these findings will encourage further exploration of the evolutionary and population genetic consequences of the singular pattern of X chromosome inheritance.

Chapter 5

Conclusions

Auanema rhodensis, displaying three sexual morphs within a same population and genome, is an ideal model to study the origin and maintenance of an alternative mating system, and more broadly, the mechanisms leading to the evolution of novel traits (such as hermaphroditism and perhaps parasitism). In this thesis, we have explored the biology of this unusual system and examined its genome and transcriptome.

From these investigations, we have found that *A. rhodensis* has an intricate life cycle. Perhaps an interesting way to think about this system is that the three sexes are in large part dependent on each other in a sort of untidy 'rock-paper-scissors' manner. Indeed, females are predominantly produced by hermaphrodites; males depend on nullo-X gametes provided by XX animals; and females produce almost exclusively hermaphrodites. This intertwined system may help stabilise the trioecious state of the population. Moreover, the sexual part of the system (females and males) may confer a more rapid growth of the population in addition to maintaining the advantages of sex, while the selfing part (hermaphrodites) would allow for an efficient dispersal and colonisation. A finer modelling of the population is planned and will hopefully allow us to better assess how the trioecious state can be evolutionarily stable. However, a more complete knowledge of the ecology of *A. rhodensis* (and other free-living trioecious species) is necessary to understand the ecological factors (if any) contributing to the maintenance of the three sexes. All in all, *A. rhodensis* is a natural system in which it is possible to study trioecy and its evolutionary path. With the advancement of genome editing techniques and other methods to control gene expression (e.g., RNAi), it will become feasible to untangle and tease apart the different components of the life cycle and perhaps conduct some experimental evolutionary studies. In *C. elegans*, experimental trioecious populations have been created, by employing feminised mutant hermaphrodites (functional females). Evolutionary studies on these

artificial three-sexed populations have shown that the trioecious state could not be maintained and that the hermaphrodites always invaded the population [47, 180]. However, these trioecious systems were artificially created and *C. elegans* has evolved to be a predominantly hermaphroditic species. As such males may not be as efficient as those of dioecious species, for example. In accordance with this, Maupas observed that 'incomplete hermaphroditic species' (which encompasses trioecious species) not only had a higher proportion of males compared to hermaphroditic species but that the males seemed to have 'better conserved their sexual faculties and their reproductive sense' [130]. Testing the stability of trioecy in a natural system, by promoting dioecy or hermaphroditism in *A. rhodensis* for example, would provide an alternative perspective and a better understanding of mixed mating systems.

The factors, molecular players and mechanisms controlling the balance between selfing (hermaphrodite production) and outcrossing (male and female production) are still largely unknown. In the nematode *Auanema freiburgensis*, a closely related free-living trioecious species, it is known that the hermaphrodite mother can perceive environmental cues (crowding cues) and transmit the information to the gonad, inducing the production of hermaphrodites [216]. In the absence of these cues, only females and males are produced. This soma to germline signal transduction of the environmental information requires amphid neurons (which, simply put, play the role of the nose of the nematode). Ablation of these neurons annihilates the ability of the mother to respond to the crowding cue, and she consequently does not produce hermaphrodite offspring. In *A. freiburgensis*, the sex determination and mating system (outcrossing versus selfing) is controlled by the mother and depends on environmental factors.

Similarly, although in a less clear pattern, a maternal component seems to control the sex determination in *A. rhodensis*, as the sex and age of the mother affect the sex of the offspring. One line of research would be to investigate the basis of the potential maternal regulation of the sex determination. One hypothesis is that small RNAs or proteins provided by the mother are at the origin of the female-hermaphrodite decision. An exploratory study, comparing the mRNA transcriptome and small-RNA transcriptome of young and old hermaphrodites (producing different amount of females *versus* hermaphrodites), would help identify potential candidates.

The sex specification in *A. rhodensis* does not seem to depend on crowding cues as in *A. freiburgensis*, as even in the absence of other nematodes and

abundance of food, a hermaphrodite mother will always produce some hermaphrodites. This constitutive production of hermaphrodites might reflect, to some extent, the ecology of the species. Indeed, if *A. rhodensis* is usually exposed to ephemeral and highly variable habitats, the constant production of a certain amount of hermaphrodites may enhance the population's ability to survive. Alternatively, *A. rhodensis* may lack the molecular mechanisms necessary to pass on the environmental information to its progeny or may respond to other factors than population density.

Beyond the initial sexual decision between females and hermaphrodites, it is now established that the feminine individuals have different developments (Chapter 2 and [36, 37]) and gametogeneses (Chapter 4 and [171, 188]). Those that pass through dauer develop into hermaphrodites that undergo an atypical meiosis in which the X chromosomes do not recombine. Females on the other hand, never go through dauer and produce gametes via a conventional meiosis. More precisely, no recombination of the X is observed during the hermaphrodite oogenesis (production of oocytes with no X) and spermatogenesis (production of sperm with 2X). However, the same X chromosomes do pair and recombine during female oogenesis (predominant production of recombined 1X oocytes). These results complement the previous findings in XO males, which produce exclusively 1X sperm due to an asymmetric meiotic division [170]. It is not clear how the developmental trajectory, sexual fate, and X segregation pattern are linked and if these traits could be uncoupled.

Taken together, the unusual inheritance patterns of the X chromosome offer an ideal *in vivo* system to investigate the mechanisms regulating the meiosis process and to understand how the division pattern can be altered. During hermaphrodite and male spermatogenesis, the X sister chromatids prematurely separate during the first meiotic division (Chapter 4, [170, 188]). In the case of XO males (producing exclusively 1X sperm), an asymmetric partitioning of the cytoplasm occurs in the spermatocytes (during meiosis II), in which the cellular components necessary to make a functional sperm (e.g., mitochondrias) migrate to the pole containing the X chromosome. One possibility is that similar molecular and cellular mechanisms act during the spermatogeneses of both hermaphrodites and males, controlling the premature separation of X chromatids during meiosis I and the asymmetric division of meiosis II. The genetic regulation of these processes is unknown. One hypothesis however is that the X acts as a signal to recruit essential components to the pole fated to become functional sperm. Ongoing research is currently investigating this

hypothesis by use of XX *tra-1* mutants. The gene *tra-1* promotes female sex determination and loss-of-function mutations lead to the transformation of XX animals into males. A fertile *A. rhodensis tra-1* mutant was obtained and observations of its spermatogenesis point towards symmetric and asymmetric meiotic divisions (personal communication Talal Al Yazeedi and André Pires da Silva).

One consequence of the atypical meiotic divisions observed in *A. rhodensis* is that, in the event of a cross, the X chromosome is always transmitted from father to son. This observation, along with the lack of recombination between the X homologues in hermaphrodites, indicates that (i) the genes on the X are more exposed to deleterious events and (ii) genetic diversity on the X could be maintained for longer periods of time.

It is possible that the increased 'vulnerability' of the genes on the X lead to the enduring emigration of essential genes onto the autosomes. Consistent with this idea, the genomic analysis revealed that the X chromosome is very small and contains much fewer genes than the autosomes (Chapter 3). Moreover, the macrosyntenic comparisons between *A. rhodensis*, *C. elegans* and *H. contortus* show that many ancestral X genes of *A. rhodensis* are now located on autosomes LG2 and LG4. Interestingly, these X-to-autosome translocations somewhat restore the ancestral conformation of *P. pacificus*, as the autosomal material that moved to the X in the branch leading to strongylids and *C. elegans*, was preferentially placed back in autosomes in *A. rhodensis* (Chapter 3).

Interestingly, the X chromosome of *C. elegans* was found to be depleted of essential genes (genes that, when knocked-down, have a lethal or sterile phenotype) [94]. This could be explained (at least partially) by the fact that the X chromosome is transcriptionally repressed in the germline [97]. Therefore, it is expected that genes necessary for 'basic cellular processes' - which, if mutated, have a non-viable or sterile phenotype - would be absent from the X chromosome [94]. Additionally, the X is present in only one copy in the males and is, therefore, more vulnerable to deleterious mutations. These observations are somewhat in contradiction with the large autosome-to-X translocation, as one could presume that this ancient autosomal region contained essential genes. One speculation is that the detrimental effect of the presence of these genes on the X was not strong enough to cause their emigration to the autosomes in *C. elegans* and *H. contortus*. However, in *A. rhodensis*, it is even more crucial to reduce the preponderance of essential genes on the X chromosome as any deleterious mutations on the X are hard to remove (lower recombination

rates as the X does not recombine in hermaphrodites and males) and has the potential to spread more quickly than an autosomal mutation (through the father-to-son X transmission). Following this reasoning, translocations from the X to the autosomes, especially of the material containing essential or otherwise critical genes (presumably more present in the old autosomal material), would be beneficial.

The atypical segregation pattern of the X chromosome is reminiscent of other (non-nematode) species such as the pea aphid. The pea aphid, like *A. rhodensis*, uses an XX/XO sex determination system and its life cycle combines sexual (males and females) and asexual (apomictic parthenogenetic females) reproduction [85, 86, 140]. Moreover, it has an unusual X chromosome inheritance: asexual females eliminate one X to produce XO males and, similarly to *A. rhodensis*, during male spermatogenesis, only X-bearing sperm are produced and the nullo-X counterpart is discarded [85]. Asexual females and sexual females are clones of one another as the sexual females are produced by parthenogenetic asexual females [85]. The sexual and asexual morphs therefore share the same genome. Similarly, females and hermaphrodites of *A. rhodensis* are believed to be genetically identical. In this system, theoretical and transcriptomic studies have shown that the X chromosome of pea aphids is masculinised (accumulation of male-beneficial sexually antagonistic genes on the X) [85]. It would be interesting to see if the same can be observed for *A. rhodensis*.

Another consideration about the X chromosome of *A. rhodensis* is that it presents a number of similarities with the Y chromosome of some XY organisms: first, it could be considered as somewhat degenerate (very small, few genes, less expressed), it has a lower recombination rate (no recombination occurs in hermaphrodites, the majoritary sex, or in males) and it is transmitted from father-to-son (and more generally, through the sperm-producing germline). The Y chromosome, notably of mammals and *D. melanogaster*, evolved from an autosome ancestor, which then degenerated (massive gene loss) through successive recombinational arrests (the progressive suppression of recombination between the Y and the X chromosomes) [7]. The Y is also restricted to the male sex (it never passes through female bodies). The recombinational arrest in the Y chromosome is useful to keep the male beneficial genes in males only. This is a way to resolve sexual antagonistic gene conflicts. However, the lack of recombination leads to a decrease of the efficacy of selection [7]. This is because deleterious mutations cannot be purged without recombination

and therefore accumulate through Muller's ratchet and genetic hitchhiking (undesired selection of deleterious mutations through the selection of a strongly beneficial allele) [7]. Poor selection efficacy due to lack of recombination also happens when a weakly beneficial mutation is lost because it is linked to a deleterious allele ('ruby in the rubbish') [7, 151]. These consequences of the lack of recombination drive, at least partially, the Y to degenerate [7, 151]. By contrast, *A. rhodensis*'s X is present in two copies in the females and the hermaphrodites and does recombine in females, providing an opportunity to uncouple beneficial alleles from deleterious ones. Although transmitted from father-to-son, it is not limited to the males, making its role as a carrier of male-benefical genes indeterminate. It therefore has composite X and Y characteristics. At this point it is unclear what selective forces are acting resulting in *A. rhodensis*'s X chromosomal peculiarities, what evolutionary trajectory the *A. rhodensis*'s X is following and what consequences these peculiarities have on the populations' evolution.

The genomic resources (genome, genes and proteins) of *A. rhodensis* explored in this study are blueprints for future investigations aiming at deepening our understanding of trioecy and mating system evolution as well as, sex determination mechanisms and genome evolution. Towards this aim, the sequencing projects of *Rhabditella axei*, the closest known dioecious species to *Auanema* (currently performed by Erich Anderson, personal communication) as well as the free-living trioecious nematodes *Auanema sp.* strain JU1783 (in collaboration with Theresa Grana) and *Auanema freiburgensis*, (performed by Jun Kim and Junho Lee, collaboration) will allow finer genomic comparisons and shed light on the origin and maintenance of trioecy in *Auanema*.

Furthermore, the genus *Auanema* is phylogenetically close to the well-studied *Caenorhabditis* clade, enabling us to make use of the vast knowledge gathered from *C. elegans* and its relatives. Although free-living, *A. rhodensis* has a mixed mating system which has been regularly recorded in parasitic species. Moreover, it is phylogenetically close to the parasitic strongylid nematodes (*H. contortus, A. ceylanicum, Necator americanus*) and to the trioecious insect-parasites of the genus *Heterorhabditis*. These observations raise the question of trioecy being coopted towards the evolution of parasitism. This question can now start to be addressed on several fronts. First, the availability of additional genomes will allow phylogenetically-aware comparisons and second, with a more complete sampling of *Auanema* and closely related species, we will gain a better understanding of their general ecology.

Figure 5.1: **Map of the origin of the known trioecious strains currently maintained.**

In this line, we now have a maintained collection of various *Auanema* strains from around the world (see Figure 5.1 and Table C.1), kindly donated by various researchers (see Table C.1 for the full list). In an ongoing project, we aim to determine, which strains can intercross in both directions (same species) and sequence the regions of the 18S, 28S and RNA polymerase II for phylogenetic inferences. Of interest, two *Auanema* strains are viviparous (EJR89 and NKZ329), i.e., the eggs hatch and develop in the mother leading to her death, and, based on the 18S and 28S sequences, are closest to *A. rhodensis* (Figure C.1).

Finally, it is noteworthy to say that studying non-model organisms is extremely important as it challenges current dogmas. For example, the father-to-son X transmission of *A. rhodensis*, or the soma-to-germline inheritance seen in *A. freiburgensis* defy common views. This can lead to new discoveries and widen our horizons.

Appendix A

Genome assembly and annotation (Chp. 3)

Table A.1: ***A. rhodensis*** **genomic and transcriptomic libraries used in the project.**

Type	Strain	Sample	Library type	Raw reads	Trimmed reads	% kept
Genomic	APS4	APS4_250bp	PE	60231762	60231660	99.99
Genomic	APS4	APS4_450bp	PE	89511646	85720848	95.76
Genomic	APS4	APS4_600bp	PE	96260562	91844696	95.41
Genomic	APS6	APS6_450bp	PE	48438048	46337014	95.66
Genomic	APS4	APS4_3kb	MP	283309872	199,259,314	70.33
Genomic	APS4	APS4_5kb	MP	217952054	166,489,564	76.38
Genomic (RAD-seq)	APS4/APS6	2014132_MBlib1 (24 samples)	PE	88126890	85596887	97.12
Genomic (RAD-seq)	APS4/APS6	2014132_MBlib2 (24 samples)	PE	94768310	91946441	97.02
Genomic (RAD-seq)	APS4/APS6	2014132_MBlib3 (24 samples)	PE	97810228	94787418	96.90
Genomic (RAD-seq)	APS4/APS6	2014132_MBlib4 (25 samples)	PE	79485616	76968975	96.83
Transcriptomic	APS4	L2_fem_lib1	PE	34221432	28622834	83.64
Transcriptomic	APS4	L2_fem_lib2	PE	33259222	28038800	84.30
Transcriptomic	APS4	L2_fem_lib3	PE	34758124	28996354	83.42
Transcriptomic	APS4	L2_herm_lib1	PE	31125954	25674986	82.48
Transcriptomic	APS4	L2_herm_lib2	PE	32156848	27189294	84.55
Transcriptomic	APS4	L2_herm_lib3	PE	32852836	27233046	82.89
Transcriptomic	APS4	L2_Conv_fem_lib1	PE	37072152	31139910	83.99
Transcriptomic	APS4	L2_Conv_fem_lib2	PE	34340210	28873512	84.08
Transcriptomic	APS4	L2_Conv_fem_lib3	PE	33924804	28605552	84.32
Transcriptomic	APS4	Males_lib1	PE	32683570	28991154	88.70
Transcriptomic	APS4	Males_lib2	PE	33521364	29646798	88.44
Transcriptomic	APS4	Males_lib3	PE	27249638	24155594	88.64
Transcriptomic	APS4	Mixed_stages_lib1	PE	48380894	43055270	88.99
Transcriptomic	APS4	Mixed_stages_lib2	PE	44500630	39952382	89.77
Transcriptomic	APS4	Mixed_stages_lib3	PE	67438256	60351402	89.4

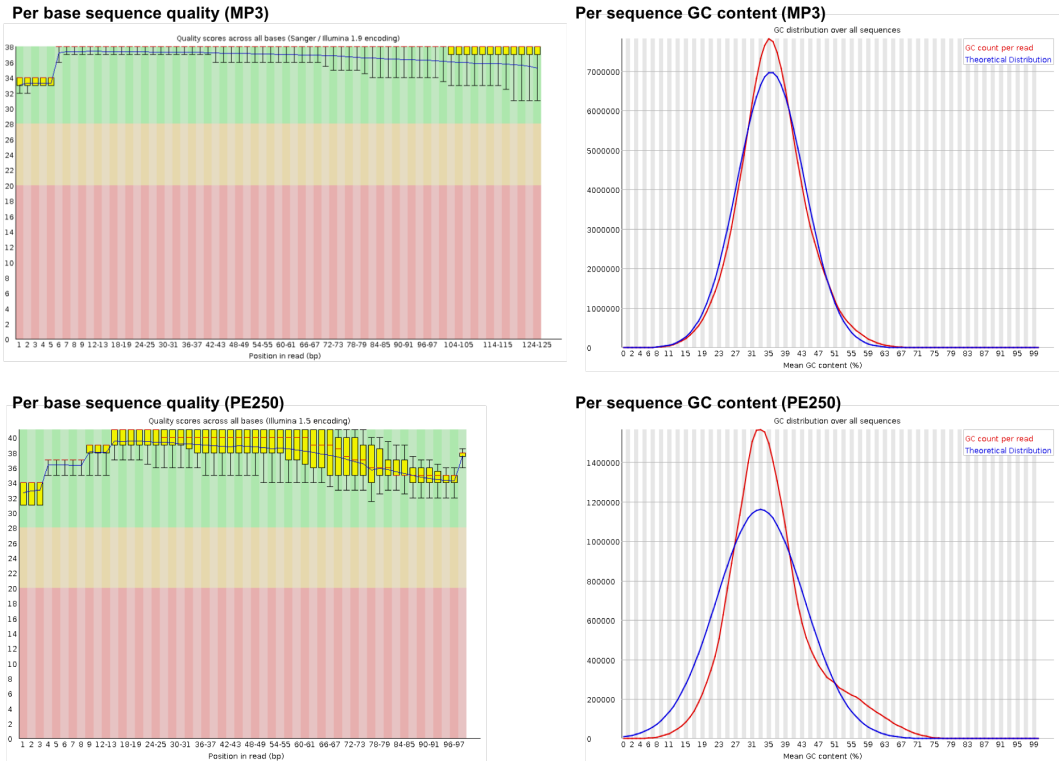


Figure A.1: **Representative example of quality (left column) and GC content (right column) of the raw sequencing data of the mate-pair libraries (here MP3) and pair-end libraries (here PE250).** MP3 and PE250 refer, respectively, to the left reads of the mate-pair library with insert size 3kbp and to the left reads of the pair-end library with insert size 250bp. The small bump of higher GC content which can be observed in the PE libraries is probably due to microbial contamination.

Table A.2: **Number of intra- and inter-strain variants per chromosome and their density (variants/bp).**

Chrom.	Approx. length	Intra APS4	Intra APS6	Inter strains	Intra-APS4 variant density	Intra-APS6 variant density	Inter-strain variant density
LG1	8489927	546	1185	26913	6.43E-05	1.40E-04	3.17E-03
LG2	9627060	507	1281	17869	5.27E-05	1.33E-04	1.86E-03
LG3	8741542	593	1571	19614	6.78E-05	1.80E-04	2.24E-03
LG4	8804062	381	955	17894	4.33E-05	1.08E-04	2.03E-03
LG6	9421540	764	1653	19149	8.11E-05	1.75E-04	2.03E-03
LG7	9306279	419	1022	19909	4.50E-05	1.10E-04	2.14E-03
LG5 (X)	3488253	793	1475	11734	2.27E-04	4.23E-04	3.36E-03
Aut.	54390410	3210	7667	121348	5.90E-05	1.41E-04	2.23E-03

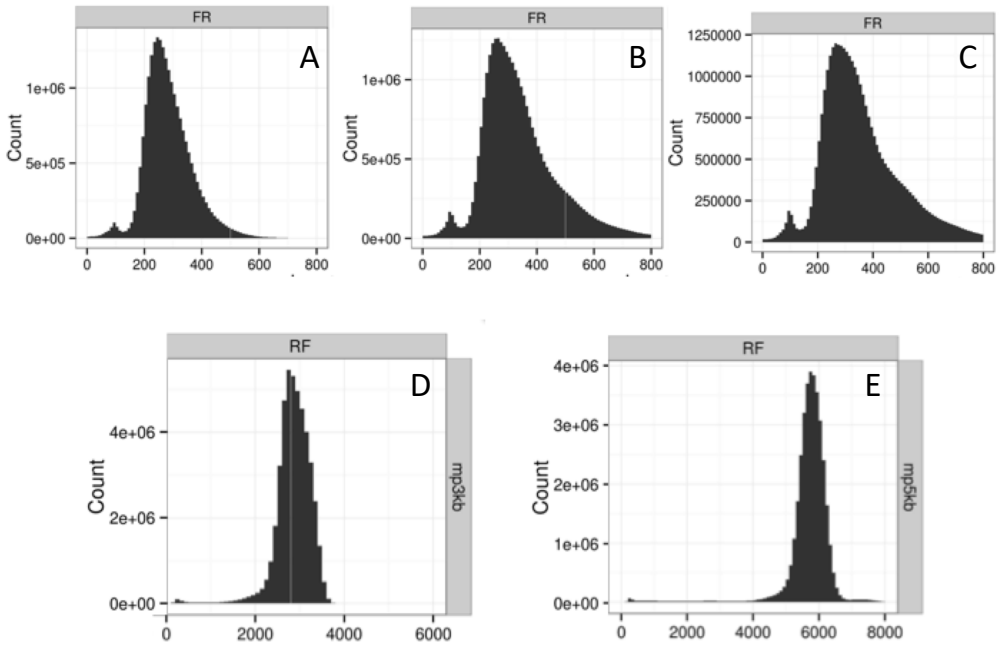


Figure A.2: **Insert sizes estimates of the pair-end (A-C) and mate-pair (D and E) libraries used for the genome assembly.** (A-C) Pair-end libraries with requested insert sizes 250bp, 450bp and 600 bp respectively. (D and E) Mate-pair libraries with requested insert sizes 3kb and 5kb. We can note that the PE libraries have forward-reverse pairs (FR) of reads whereas the MP libraries have reverse-forward (RF) pairs.

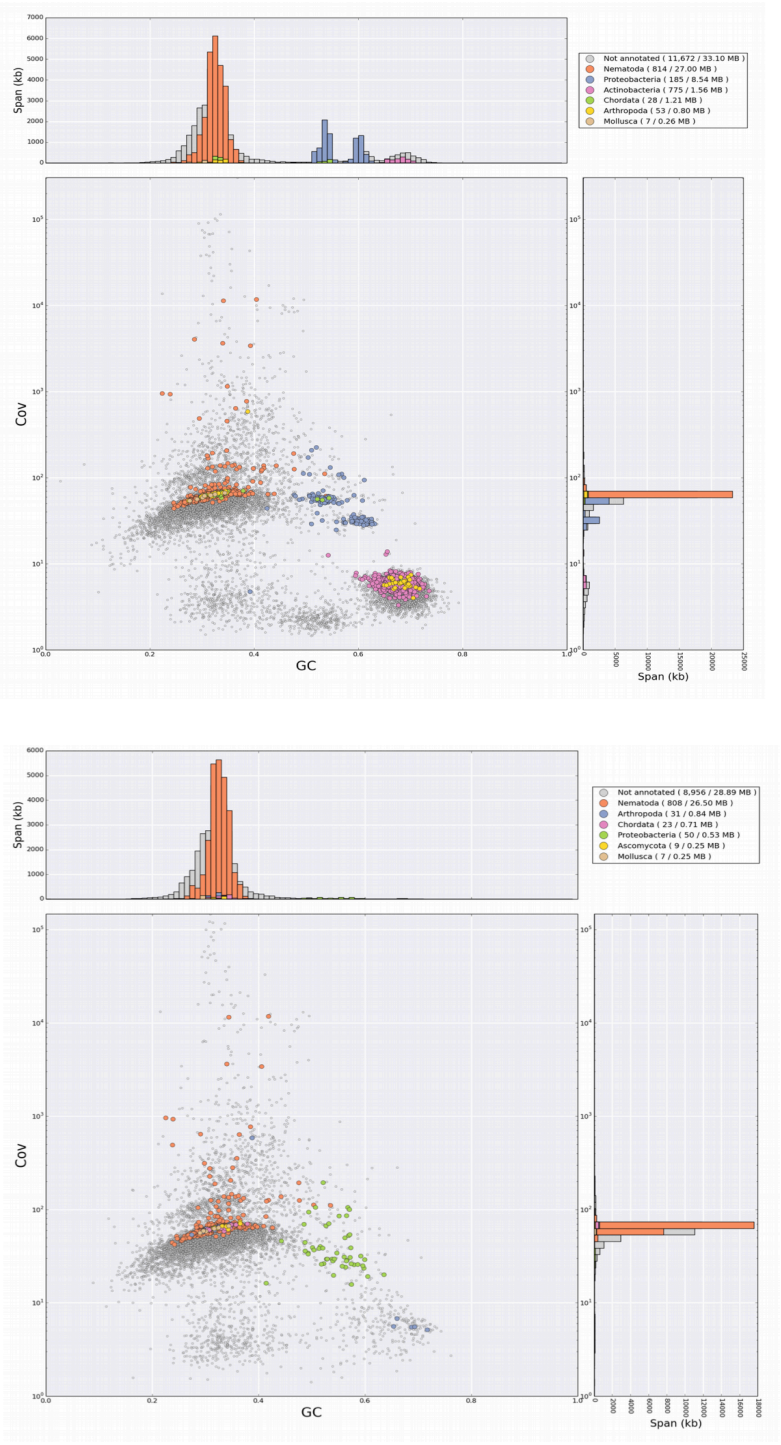


Figure A.3: **Examples of TAGC plots (or Blobplots) of the PE library before (above) and after (below) contamination removal.**

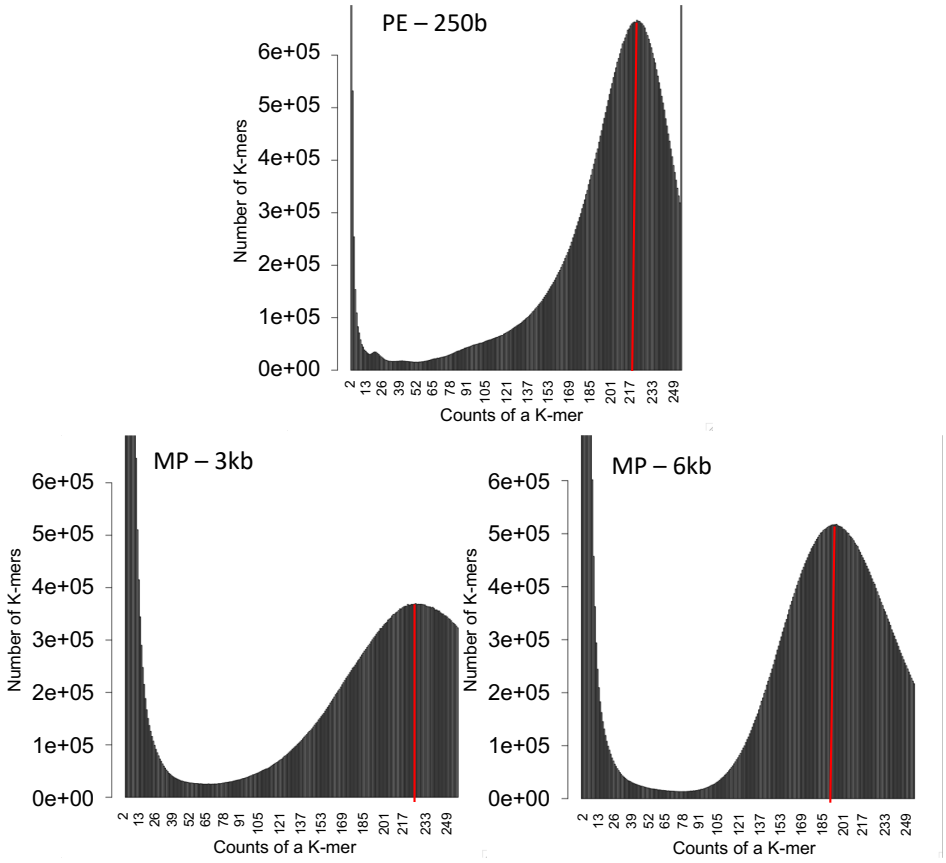


Figure A.4: **Histograms of the counts of 19-mers on the three libraries used for the genome assembly: pair-end 250b, mate pair 3kb and mate pair 6kb.** The red line indicates the coverage of *A. rhodensis*'s genome (number of times most kmers appeared), which is around 200 times for all three libraries.

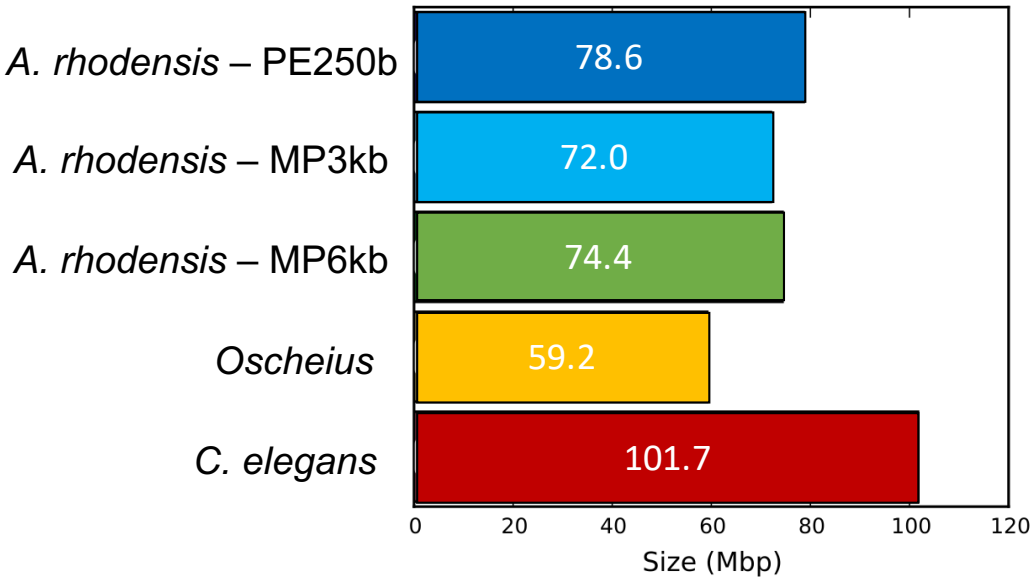


Figure A.5: **Estimates of *A. rhodensis*'s genome size from the three libraries using the program PreQC.** *A. rhodensis*'s genome seems to be bigger than the one of *Oscheius* sp. but smaller than *C. elegans*'.

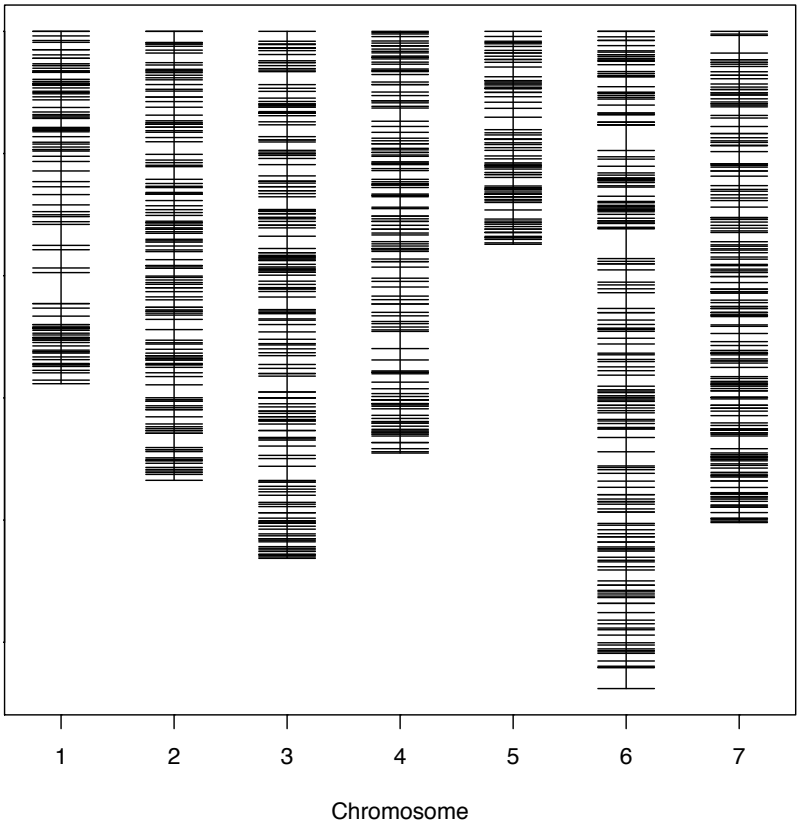


Figure A.6: **Schematic of the final genetic map of *A. rhodensis*.** Each horizontal bar represents a marker. There are 1052 markers in the final map.

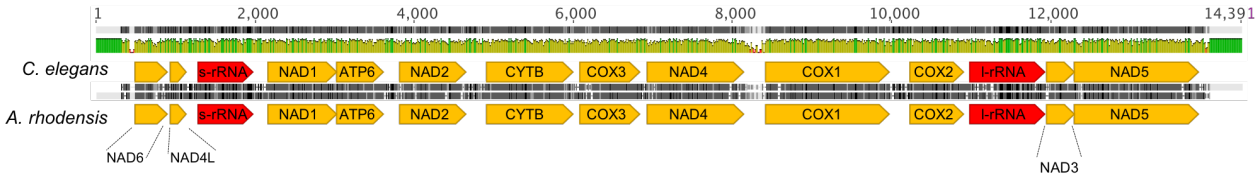


Figure A.7: **Mitochondrial genome alignment between *A. rhodensis* and *C. elegans*.** The gene and strand collinearity of the 12 protein coding genes and the two ribosomal RNA genes is conserved between both species. 's-rRNA' and 'l-rRNA' stand for small ribosomal subunit and large ribosomal subunit, respectively. The histogram above the aligned chromosomal blocks show the identity between *A. rhodensis* and *C. elegans* (the greener and higher the bar, the higher the identity between the two sequenced). The alignment was built using Geneious [96] with default settings.

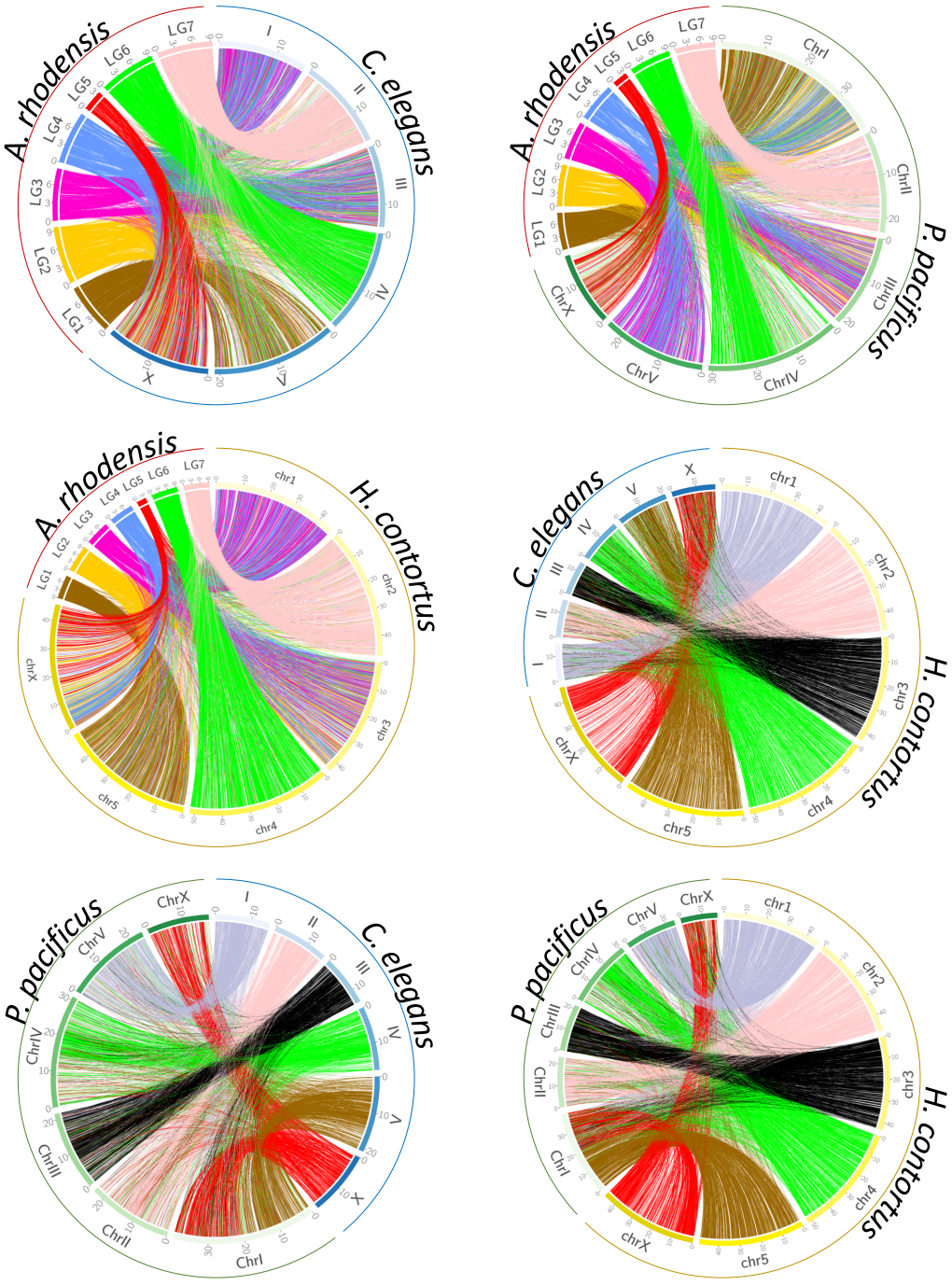


Figure A.8: **Orthologous coding genes between *A. rhodensis*, *C. elegans*, *P. pacificus* and *H. contortus*.**

		A. rhodensis linkage groups						
		LG1	LG2	LG3	LG4	LG5	LG6	LG7
C. elegans chromosomes	I	1.86	5.19	60.15	28.34	0.27	2.26	1.93
	II	1.81	4.05	2.10	3.47	0.51	4.05	84.02
	III	1.36	42.79	24.15	25.37	0.34	3.61	2.38
	IV	2.24	4.63	2.10	2.46	0.22	86.18	2.17
	V	77.07	5.10	3.55	4.14	0.67	5.25	4.22
	X	0.81	37.66	1.79	26.03	28.89	2.15	2.68

		A. rhodensis linkage groups						
		LG1	LG2	LG3	LG4	LG5	LG6	LG7
	I	2.42	5.92	65.27	34.69	1.14	2.38	2.16
	II	2.16	4.25	2.09	3.91	1.99	3.92	86.52
	III	1.73	47.76	25.63	30.37	1.42	3.71	2.61
	IV	2.68	4.86	2.09	2.77	0.85	83.34	2.23
	V	90.22	5.24	3.47	4.56	2.56	4.97	4.24
	X	0.78	31.97	1.44	23.70	92.02	1.68	2.23

		LG1	LG2	LG3	LG4	LG5	LG6	LG7
P. pacificus chromosomes	I	44.3	28	2.01	19.7	0.75	2.93	2.24
	II	2.05	1.64	2.36	2.46	0.31	2.46	88.7
	III	1.06	43.9	27.8	24	0.48	1.45	1.25
	IV	2.93	2.72	2.32	2.62	0.91	85.4	3.13
	V	0.73	1.46	65.6	28.5	0.63	1.77	1.36
	X	6.31	4.55	5.05	4.04	67.7	8.59	3.79

		LG1	LG2	LG3	LG4	LG5	LG6	LG7
	I	89.4	47.9	3.44	36.8	4.28	5.17	4
	II	2.31	1.57	2.26	2.58	0.99	2.43	88.6
	III	1.27	44.7	28.3	26.7	1.64	1.52	1.33
	IV	3.36	2.65	2.26	2.79	2.96	85.7	3.18
	V	0.81	1.38	61.8	29.3	1.97	1.72	1.33
	X	2.89	1.77	1.97	1.72	88.2	3.44	1.54

		LG1	LG2	LG3	LG4	LG5	LG6	LG7
H. contortus chromosomes	I	1.18	4.67	61	29.2	1.31	1.25	1.38
	II	1.01	5.87	0.87	3.12	1.09	2.54	85.5
	III	0.54	45.5	25	25.3	1.08	1.76	0.81
	IV	1.03	5.06	1.3	3.69	1.44	85.7	1.78
	V	83.4	5.58	1.62	3.96	0.89	1.54	2.99
	X	1.05	33.4	1.35	29	32.5	1.35	1.35

		LG1	LG2	LG3	LG4	LG5	LG6	LG7
	I	1.65	5.97	68.4	38.4	6.69	1.4	1.63
	II	1.28	6.81	0.88	3.72	5.02	2.57	91.8
	III	0.73	56.5	27.2	32.3	5.35	1.91	0.93
	IV	1.37	6.22	1.4	4.67	7.02	92.1	2.02
	V	94.3	5.8	1.47	4.24	3.68	1.4	2.88
	X	0.64	18.7	0.66	16.7	72.2	0.66	0.7

Figure A.9: **Percentages and heatmaps of orthologous protein-coding genes between the *A. rhodensis*' chromosomes and other Rhabditid nematodes.** The left side heatmaps presents the percentages of orthologous genes of *C. elegans* (first row), *P. pacificus* (second row) and *H. contortus* (last row) in each of *A. rhodensis*'s chromosomes. The right side heatmaps present the percentages of *A. rhodensis*'s orthologous genes in *C. elegans* (first row), *P. pacificus* (second row) and *H. contortus* (last row). Percentages were calculated on the total number of orthologues between each of the *A. rhodensis* chromosomes and other Rhabditids chromosomes. The lighter the colour of the cell, the higher the proportion of orthologues.

Appendix B

X inheritance (Chp. 4)

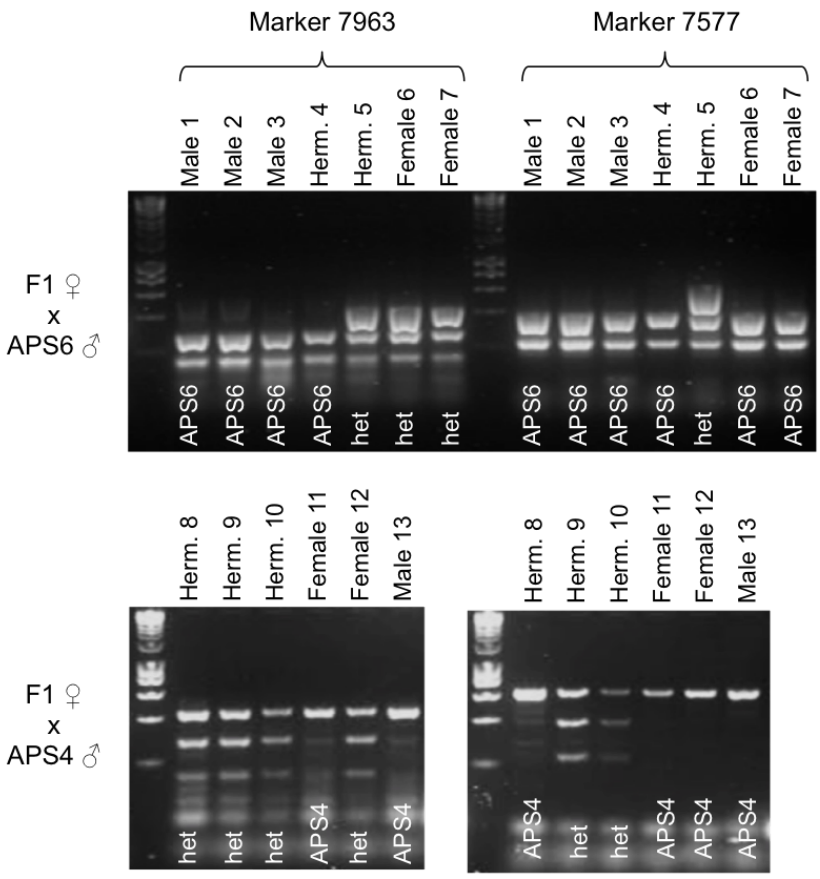


Figure B.1: **Example of X chromosome genotyping of F2s generated from crosses between F1 hybrid $X_{APS4}X_{APS6}$ females and either APS6 (upper panel) or APS4 (lower panel) males.** From this genotyping, we can infer that some crossovers have occurred during female oogenesis as some F2 XX individuals do not display the same genotype across all the markers genotyped. The gel depicts only the two rightmost X markers (see Figure 4.2) as the crossovers were frequently observed between these markers (probably due to the subtelomeric position of the marker 7577). Genotypes are reported under the gel pictures. Numbers indicate individual animals. This figure comes from the supplemental information of [188].

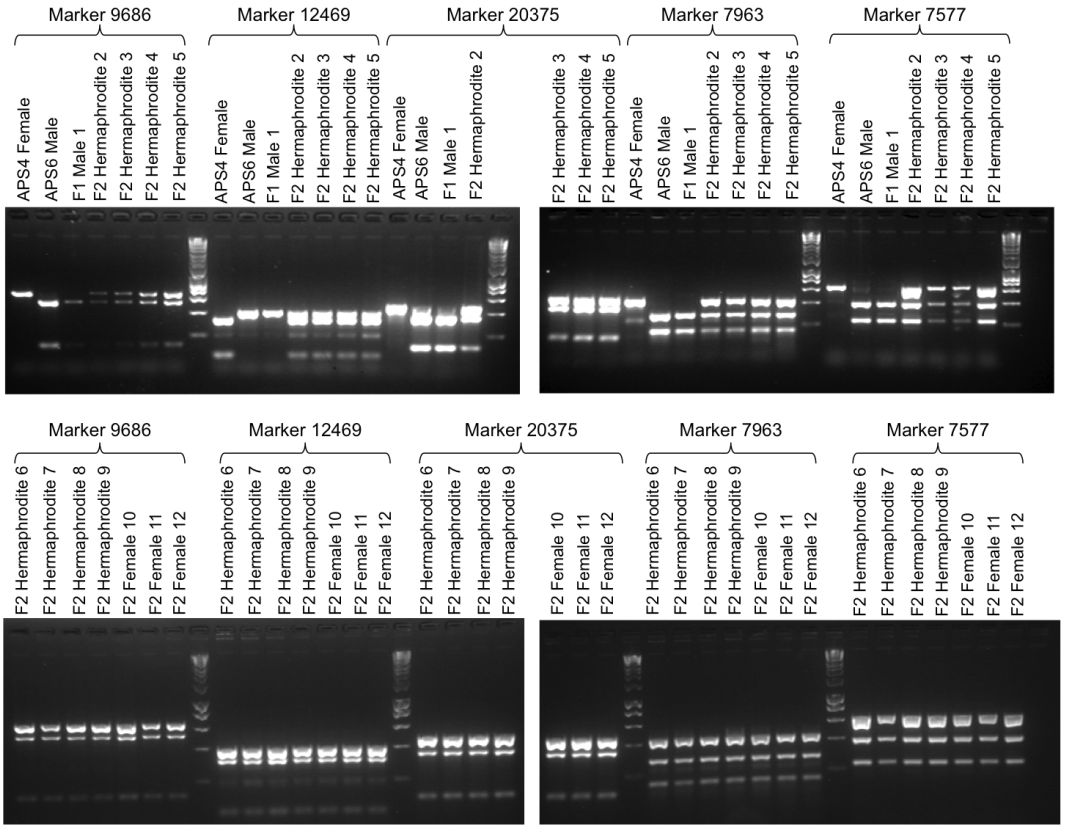


Figure B.2: **Example of X chromosome genotyping using amplification, digestion and electrophoresis of parental individuals and F2 females and hermaphrodites produced by selfing F1 hybrid hermaphrodites.** F2 XX progeny produced by hybrid F1 hermaphrodites are systematically heterozygous across the 5 X markers genotyped. Numbers indicate individual animals. This figure comes from the supplemental information of [188].

Table B.1: **Information on the markers used to genotype the X and LG4 chromosomes.**

Chromosome	Marker	APS4 SNP	APS6 SNP	Restriction enzyme	Strain cut	Forward primer	Reverse primer	Undigested product size (bp)	Digested product sizes (bp)
X (LG5)	12469	T	C	RsaI	APS4	5′-TGCAAGGCAGAC GTCCCTTG-3′	5′-CCAATTCTTCGC TTATTGCCCG-3′	400	327 / 73
X (LG5)	20375	A	T	ScaI	APS6	5′-ACCCTGCTGATC CTCGACTCG-3′	5′-AGGAGTCCCCAA ACACCCCA-3′	481	360 / 121
X (LG5)	9686	G	T	NdeI	APS6	5′-TGTCCTGACCCG CGTGTTGA-3′	5′-AACTGAGTTTGC AGCCCTGT-3′	666	534 / 132
X (LG5)	7963	A	G	HaeIII	APS6	5′-TGGTGGGGCTTG GAGTTCGA-3′	5′-ACGGCTGATGTT GACGCTCC-3′	450	290 / 160
X (LG5)	7577	A	G	EcoRV	APS6	5′-GTTGCACAAGCC CACACTGG-3′	5′-CGACCTTTCTCT TCCAGACATTGC-3′	642	235 / 407
LG4	14718	C	T	NdeI	APS4	5′-CCGAAGCCACTT GGTGCTGT-3′	5′-CGTTCGAGCTGG GCGTGTAA-3′	941	380 / 561
LG4	14690	T	A	NdeI	APS6	5′-CTGCAGCTCGTT TTGGCCGT-3′	5′-GGCACATAAGGG GGAGGCCA-3′	914	488 / 426
LG4	175	C	G	HinfI	APS4	5′-GCTTCGTCAGCG CACTGTCT-3′	5′-GTCGGCTGTTGC TTCTTCGGT-3′	936	639 / 297
LG4	20262	T	C	HinfI	APS6	5′-GGTTTCGAGATT ACCCGACGACG-3′	5′-CCAGCTGTCTTA AGATCCTACAGG-3′	443	121 / 322
LG4	8233	A	T	RsaI	APS6	5′-TGCCGTAAAACC TGCATCCCC-3′	5′-TCGAGCCAACTC TTCCTCCTGT-3′	538	280 / 258

Appendix C

Perspectives (Chp. 6)

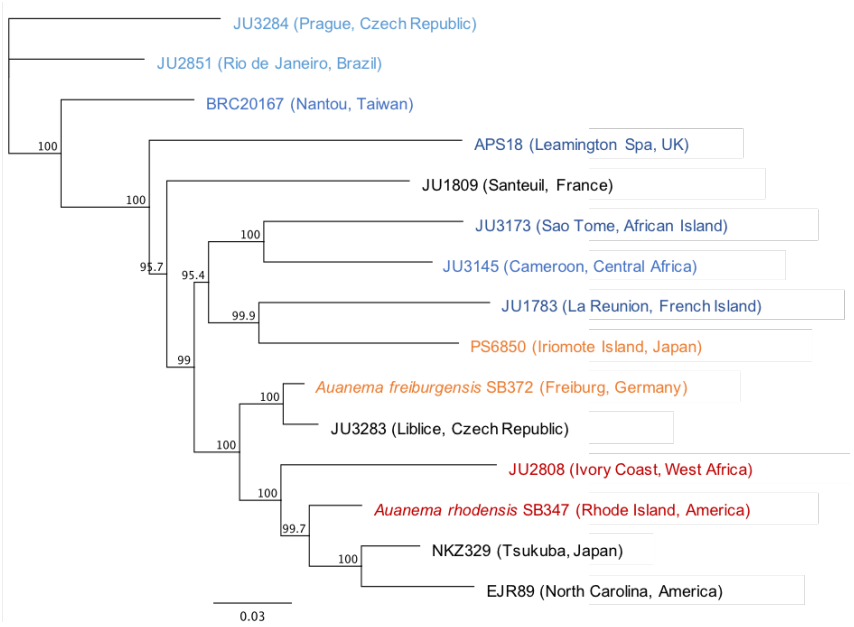


Figure C.1: **Phylogenetic tree of some trioecious nematode strains currently available, based on the partial sequence of the 18S gene.** The tree was built using the Neighbour-joining method, based on the Tamura-Nei genetic distance model. Numbers at the nodes denote support values based on bootstrap resampling of 1000 repeats. The strains are coloured based on successful or partially successful intercrosses. The viviparous strains are 'NKZ329' and 'EJR89'. This figure was made by Sandhya Dhawan and comes from her master's thesis.

Table C.1: **Collection of trioecious strains.** These strains are currently maintained in André Pires da Silva's lab and frozen stocks were made. C,P,NC and N stand for Complete, Partial, Near Complete and None, respectively. The persons collecting or isolating the samples are abbreviated as follows: MAF (Marie-Anne Felix), WS (Walter Sudhaus), RS (Ryoji Shinya), EZ(Elyes Zhioua), IN (Isabelle Nuez), AH (Andrew Hall), CLR (Cris Ledón-Rettig), ER (Eric Ragdale), NK (Natsumi Kansaki), APS (André Pires da Silva), GDJ (Gaby Bodero-Jimenez), JW (John Wang), AA (Alper Akay), TG (Theresa Grana), JD (Jean David).

Strain	Locality	Date	Condition	Collector	18S	28S	RNAPII
SB372	Freiburg, Germany	Aug-03	horse dung pile	WS	C	C	C
SB347	Kingston (University of Rhode Island), Rhode Island, United States	Sep-01	blood-engorged deer ticks (*Ixodes scapularis*) that were used as bait for nematodes. The ticks were placed in the upper layer of the soil	EZ / WS / MAF	C	C	C
TMG33	Steep hill behind 1462 Creekstone Ridge, S. Charleston WV 25309, West Virginia, United States (GPS 38.230011, GPS2 -81.762252)	18-May-12	dead tiger beetle on an AVT trail	TG	N	N	N
JU1782	Ivry (Val-de-Marne), France	24-Sep-09	rotting Petasites stem	MAF	N	P	N
JU1783	Melissa domain, St Benoist, La Reunion	21-Sep-09	star fruit	IN / MAF	C	NC	N
JU1809	Woods near Santeuil (Val d'Oise), France	20-Oct-09	rotting stem of *Symphytum officinale*	MAF	C	C	N
PS6850	Iriomote island in Japan	01-June-14	rotting pandanus fruit (*Pandanus odoratissimus*)	RS	C	NC	N
BRC20167	Parking space (1963m) in Nantou, Taiwan (GPS 23.53346 (Latitude), 120.908896 (Longitude), 1938.4646 (Altitude))	15-June-14	Rotting Flower	JW	P	P	N
JU2851	Serra dos Orgaos National Park, Teresopolis, Rio de Janeiro, Brasil (GPS -22.478844, -43.055788)	28-Mar-15	rotting fruit sample	AA / MAF	C	C	N
JU2808	Near Assinie, Ivory Coast (GPS 5.150393,-3.413095)	Sep-14	soil sample	AH / MAF	C	C	P
JU3145	Oku Mountain, Cameroon	May-16	rotting stems	JD / MAF	C	C	N
JU3173	Botanical Garden of Bom Successo, Sao Tome (GPS 0.289, 6.612.)	23-Sep-2016	compost	MAF	C	C	N
JU3283	Liblice, Czech Republic (GPS 50.31265, 14.5873.)	22-Sep-17	rotting walnuts	MAF	C	C	N
JU3284	Petrin Gardens, Prague, Czech Republic (GPS 50.085, 14.399)	23-Sep-17	rotting plums	MAF	C	NC	N
APS18	Jephson Gardens, Leamington Spa, UK (GPS 52° 17'18.0"N 1° 31'52.0"W)	09-May-17	soil and decaying plant matter sample	GBJ / APS	C	C	N
EJR89	Hillsborough, North Carolina	Aug-17	adult of *Onthophagus taurus*	CLR / ER	C	C	N
NKZ329	Forestry and Forest Products Research Institute campus, in Tsukuba, Japan (36.00'25.21" N, 140.07'41.90" E)	23-june-14	From *Onthophagus* sp. on rotten mushroom (*Amanita* sp.)	NK	C	NC	N

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