Genetics: modes of reproduction and genetic analysis

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SUMMARY

Classical and reverse genetics remain invaluable tools for the scientific investigation of model organisms. Genetic analysis of endoparasites is generally difficult because the sexual adults required for crossing and other manipulations are usually hidden within their host. *Strongyloides* spp. and *Parastrongyloides* spp. are notable exceptions to this and their free-living adults offer unique opportunities to manipulate these parasites experimentally. Here I review the modes of inheritance in the two generations of *Strongyloides*/*Parastrongyloides* and I discuss the opportunities and the limitations of the currently available methodology for the genetic analysis of these two genera.

Key words: *Strongyloides* spp., genetics, parthenogenesis, sexual reproduction, sex determination.

INTRODUCTION

The term 'Genetics' in biology is used for at least two related fields. First it is the science of inheritance and as such investigates the rules and mechanisms of how individuals pass on heritable information to the next generation. Second, genetics denotes a particular approach to studying the functions of genes in an organism. This is the approach of creating or collecting individuals with altered genetic information (mutation or addition of genetic information) and then analysing the differences between the carriers of the new version (mutants) and the unaltered, wild-type individuals. In this paper, I shall discuss both these aspects of 'genetics' for *Strongyloides* and the closely related sister genus *Parastrongyloides*. A further type of genetics, population genetics, which is concerned with natural genetic variation within a species, is not a subject of this review. In this paper, I refer to the genetic properties of the nuclear genome only and not the ones of the mitochondrial genome. For a general introduction, including the life cycle, I refer the reader to the introductory chapter by M. Viney in this special issue.

METHODS EMPLOYED TO STUDY THE INHERITANCE AND GENE FUNCTION IN *STRONGYLOIDES* SPP.

Most of the classical analyses about the modes of reproduction in *Strongyloides* spp. were based on cytological observations (see, for examples, Nigon and Roman, 1952; Zaffagnini, 1973; Triantaphyllou and Moncol, 1977; Albertson *et al*. 1979; Hammond and Robinson, 1994). Owing to the methodology available at the time, these studies were done on fixed specimens, which make elucidating the dynamics of the processes difficult.

Studying certain traits (e.g. the ratios between male and female progeny or between homogonic and heterogonic development) over time and varying culture regimes provided hints about the modes of inheritance. Prominent among these are very extensive studies by Graham, for which he maintained *Strongyloides ratti* for many generations exclusively through the homogonic or the heterogonic cycle, even as successive single worm infections (Graham, 1936, 1938, 1939*a*, *b*, 1940*a*, *b*). In agreement with Sandground (1926), who had done similar but less extensive studies on *Strongyloides papillosus* and *S. ratti*, Graham (1939*b*) noticed a 'remarkable constancy of characteristics' when *Strongyloides* spp. reproduced through the homogonic cycle while variability arose in cultures derived from the heterogonic cycle. This indicated that in the progeny of the free-living generation but not the parasitic generation new genetic combinations are created through recombination associated with sexual reproduction.

Later, with the emergence of PCR and relatively inexpensive sequencing, molecular markers including first micorsatellites and later single copy loci provided tools to study the passage of genetic information in mass matings of males and females of different strains or in single male – female crosses (examples of this type of study are Viney *et al*. 1993; Viney, 1994; Harvey and Viney, 2001; Grant *et al*. 2006; Eberhardt *et al*. 2007; Nemetschke *et al*. 2010*b*).

Unfortunately, as far as studying gene function in *Strongyloides* is concerned, minimal success can be reported at this time. DNA and RNA sequencing efforts have provided probably close to complete gene lists for multiple species of *Strongyloides*

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(Hunt *et al*. 2016) and a number of microarray analyses (Evans *et al*. 2008; Thompson *et al*. 2008, 2009; O'Meara *et al*. 2010; Ramanathan *et al*. 2011) and quantitative RNA sequencing experiments (Yoshida *et al*. 2011; Marcilla *et al*. 2012; Ahmed *et al*. 2013; Nagayasu *et al*. 2013) using RNA extracted from different developmental stages and proteome analyses (Marcilla *et al*. 2010; Soblik *et al*. 2011; Younis *et al*. 2011) provided a comparative overview of gene expression patterns and protein secretion. However, I would argue that so far such omics approaches have provided and can provide only very limited information about the functions of individual genes (see also Viney, 2014).

The possible functions of selected genes, normally selected because of the presence of homologous genes with known functions in other systems, were approached through detailed molecular characterization of the genes and their products including their temporal expression and localization (Tazir *et al*. 2009; Peeters *et al*. 2011; Younis *et al*. 2011; Biewener *et al*. 2012). Thanks to the recent addition of transgenic techniques to the available tool kit for *Strongyloides* spp. research (Lok, 2013), these approaches have been complemented with the use of reporter constructs (e.g. Yuan *et al*. 2014*a*, *b*).

In order to study gene function in model organisms, mutations in genes obtained either by random mutagenesis followed by screening for phenotypes of interest (forward genetics) or by targeted knock out of molecularly known genes (reverse genetics) have been and still are tremendously useful tools for the investigation of gene function (Hodgkin, 2005; Kutscher and Shaham, 2014). However, as yet attempts at both forward and reverse genetics in *Strongyloides* spp. have been unsuccessful.

Isolating mutations for genetic analysis of endoparasitic organisms is usually difficult because the adults, which are required for mutagenesis and later crossing, are located within the host. *Strongyloides*/*Parastrongyloides* with the free-living adult generations is an exception to this and appears much more suitable for this kind of approach; nevertheless, no success can be reported at this time. The only two reports of successful mutagenesis in *Strongyloides* spp. come from *S. ratti*, demonstrating that mutagenesis and screening for phenotypes of interest is possible (Viney *et al*. 2002; Guo *et al*. 2015). However, the main problem that has not yet been solved is the isolation of the molecular mutation causing this phenotype and with it the identification of the corresponding gene. In the model nematode *Caenorhabditis elegans* mutated genes have traditionally been identified by a process called positional cloning (Hodgkin, 1999; Fay, 2006). For this strategy one needs a dense, high-quality genetic map for precise genetic mapping and a reliable physical map (ideally a full genome sequence) that is highly interlinked with the genetic map. In addition, transgenic technology is normally employed for gene verification after tentative identification. Positional cloning has so far been used successfully only in two nematodes other than *C. elegans*, namely in *Caenorhabditis briggsae* (Koboldt *et al*. 2010) and in *Pristionchus pacificus* (Zheng *et al*. 2005; Dieterich *et al*. 2006). Although a genetic map for *S. ratti* (Nemetschke *et al*. 2010*b*), a high-quality genome sequence (Hunt *et al*. 2016) and transgenic technology (Shao *et al*. 2012) have been established recently, positional cloning will probably not be the method of choice for identifying mutations in *Strongyloides* spp. in the future. More likely this will rely on modern sequencing approaches (see conclusions and outlook).

Given the lack of success using forward or reverse genetic approaches for obtaining mutations in known genes in *Strongyloides* spp., attempts have been made to inactivate genes at least temporarily in order to study their functions. Double-stranded RNA interference (Fire *et al*. 1998), which was employed with great success in a number of organisms, unfortunately appears not to work for *Strongyloides* spp., as is the case for many other animal parasitic nematodes (Viney and Thompson, 2008). So far, two approaches to manipulate the function of genes were employed successfully in *Stronglyoides* spp. The first one is modulating the activity of proteins and pathways pharmacologically through the addition of certain chemicals whose activities had been characterized in other systems (Ogawa *et al*. 2009; Wang *et al*. 2009, 2015; Stoltzfus *et al*. 2012*a*, 2014). Second, the recently established methods for transgenesis (Shao *et al*. 2012) allowed the expression of mutant proteins with expected properties, like mimicking or preventing phosphorylation or acting as dominant negatives (Castelletto *et al*. 2009). Inherently, both these approaches are limited to highly conserved proteins such that it can be assumed that the effects of the chemical compounds are the same in *Strongyloides* as they are in the organisms in which they had been previously analysed.

An approach that is occasionally used to characterize genes of parasitic nematodes, among them *Strongyloides* spp. is to test if the parasite gene can rescue the corresponding mutation in the heterologous system *C. elegans* (Massey *et al*. 2006; Crook *et al*. 2010; Hu *et al*. 2010). Normally only the coding region is taken from the parasite because the promoters do not function properly across species. Although useful, great caution must be exercised in order not to over interpret such experiments. If a protein derived from *Strongyloides* spp. can replace the endogenous one in *C. elegans* this only means that the biochemical properties of this protein are similar enough that the *Strongyloides* protein can perform the task of the *C. elegans* protein in *C. elegans*. However, this finding is completely

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uninformative about the function of this gene in *Strongyloides* spp.

MODES OF INHERITANCE IN THE PARASITIC AND THE FREE-LIVING GENERATIONS

Although in the literature various modes of reproduction had been postulated for the parasitic generations of *Strongyloides* sp. (Streit, 2008) there is now wide agreement that at least in the relatively well-studied species of *Strongyloides*, reproduction is by mitotic parthenogenesis such that the progeny of a parasitic female are genetically identical with the mother. Ignoring new mutations, the only exception to this is the elimination of one copy of the X-chromosome or of the X-derived portion of a chromosome, in order to make males (see below). The arguments for this are summarized below and are reviewed in more detail in Streit (2008).

With the exception of two reports from the 1930s (Kreis, 1932; Faust, 1933) describing the same isolates of *Strongyloides* spp. originally from various primates and dogs (presumably *Strongyloides stercoralis* and/or *S. fuellebornei*) all authors agree that no parasitic males exist in any species of *Strongyloides* analysed so far. Indeed, the presence of parasitic males was one of the decisive criteria for installing the new genus *Parastrongyloides* (Mackerras, 1959). In *S. ratti*, at least, individual infective larvae (L3i) frequently lead to productive infections clearly demonstrating that males are not required for reproduction (Graham, 1936, 1938; Viney *et al*. 1992; Viney, 1994). After Sandground (1926) proposed self-fertilization as a mode of reproduction, numerous authors working on multiple species of *Strongyloides* argued for mitotic parthenogenesis based on (i) cytological observations (Chitwood and Graham, 1940; Nigon and Roman, 1952; Zaffagnini, 1973; Triantaphyllou and Moncol, 1977); (ii) the observation that heritable traits remain rather stable through rounds of homogonic reproduction (Graham, 1939*b*), and (iii) molecular genetic observations (Viney, 1994; Nemetschke *et al*. 2010*a*). Interestingly, contrary to *Strongyloides* sp., *Parastrongyloides trichosuri* parasitic adults do reproduce sexually (Mackerras, 1959; Grant *et al*. 2006; Kulkarni *et al*. 2013).

Older literature on the mode of reproduction in the free-living generation is contradictory (Streit, 2008). Although a few authors suggested that under certain circumstances free-living *Strongyloides* spp. females may reproduce in the absence of males (Sandground, 1926; Zaffagnini, 1973), there is wide agreement that males are present and necessary in the free-living generations of all species of *Strongyloides* tested (Beach, 1936; Premvati, 1958*b*; Triantaphyllou and Moncol, 1977; Eberhardt *et al*. 2007). However, most cytological studies on various species of *Strongyloides* concluded that males do not contribute genetically to the progeny but that reproduction occurs by sperm-dependent parthenogenesis (pseudogamy) (Nigon and Roman, 1952; Bolla and Roberts, 1968; Triantaphyllou and Moncol, 1977; Hammond and Robinson, 1994). Contrary to this, Graham (1939*b*) noticed that heritable traits tended to be more variable in cultures maintained through the heterogonic cycle when compared with cultures passaged exclusively through the homogonic cycle, indicating that in contrast to the parasitic generation, recombination of genetic material does occur in the free-living generation. Furthermore, recent molecular genetic work argued clearly for sexual reproduction at least in *S. ratti* (Viney *et al*. 1993; Harvey and Viney, 2001; Nemetschke *et al*. 2010*b*), *S. papillosus* (Eberhardt *et al*. 2007) and *Strongyloides vituli* (Kulkarni *et al*. 2013).

Remark: Rather frequently, I meet colleagues who remember from their textbooks that free-living *Strongyloides* spp. are supposed to be diploid, while parasitic females are triploid. This information originated from a single reference (Chang and Graham, 1957), in which the authors claimed that the sperm of free-living *S. papillosus* males contributes one set of chromosomes to the diploid egg produced by the females, leading to triploid individuals destined to become parasitic. Parasitic females, in turn, were proposed to produce triploid and diploid offspring forming the parasitic and free-living progeny, respectively. For several reasons, I believe that Chang and Graham (1957) should be disregarded. First, this reference is a meeting abstract, which does not contain any detailed description of data and the authors never published these findings in a full publication. Second, multiple authors, based on cytological observations like those by Chang and Graham (1957), concluded that the free-living and the parasitic females of *S. papillosus* (Zaffagnini, 1973; Triantaphyllou and Moncol, 1977; Albertson *et al*. 1979), *S. stercoralis* (Hammond and Robinson, 1994) and *S. ratti* (Nigon and Roman, 1952) have equal numbers of chromosomes. Third, genetic experiments demonstrated that the progeny of free-living *S. papillosus* and *S. ratti* are diploid (Viney *et al*. 1993; Viney, 1994; Eberhardt *et al*. 2007). Fourth, many authors found that female larvae of several *Strongyloides* species produced by parasitic females definitely commit to either parasitic or free-living live only after they became first-stage larvae (Streit, 2008; Viney and Lok, 2015). It is hard to imagine that the larvae going on to become L3i change their ploidy at this stage of development.

SEX DETERMINATION

A puzzling aspect of the life cycle of *Strongyloides* spp. is that parthenogenetic parasitic females

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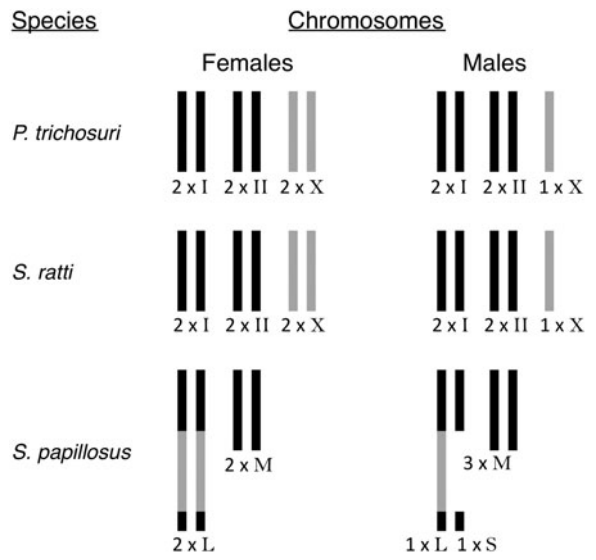

Fig. 1. Chromosomes in *P. trichosuri, S. ratti* and *S. papillosus*. Chromosomes and chromosomal regions present in two copies in both sexes are in black, Chromosomes and regions present in two copies in females but only one copy in males are in grey. I: autosome number 1; II: autosome number 2; X: X-chromosome; L: large chromosome; M: medium-sized chromosome; S: small chromosome. For detailed explanation and references see text.

produce two sexes while, with very few exceptions (Streit, 2008), the progeny of the sexually reproducing free-living generation is exclusively female. For all species of *Strongyloides* where it has been studied it was found that different isolates produce very different sex ratios in the progeny of the parasitic generation, indicating that there exists a genetic pre-disposition for more or fewer males (Sandground, 1926; Graham, 1939*b*; Viney *et al*. 1992; Viney, 1996). However, there is clearly also an environmental effect on the sex ratio produced (Moncol and Triantaphyllou, 1978; Gemmill *et al*. 1997; Harvey *et al*. 2000; Crook and Viney, 2005). In all cases studied, the stronger is the immune response of the host, the more males are produced. At the same time the females are pre-disposed but not fixed for the heterogonic cycle. Further, it has been noted that living in a permissive but suboptimal host species alters the sex ratio in the progeny of the parasitic generation, in most (Brumpt, 1921; Sandground, 1926; Matoff, 1936; Triantaphyllou and Moncol, 1977) but not all (Crook and Viney, 2005) cases towards more males. Among the different species of *Strongyloides* two different numbers of chromosomes have been found (Fig. 1). In *S. ratti* (Nigon and Roman, 1952; Bolla and Roberts, 1968) and *S. stercoralis* (Hammond and Robinson, 1994), the haploid chromosome number is three, namely two autosomes and one X chromosome ($n = 3$) and all chromosomes are roughly of equal size. In these species (diploid), females have two X chromosomes along with two pairs of autosomes ($2n = 6$) and males have only one X ($2n = 5$) resulting in an environmentally influenced XX/XO sex determining system (Harvey and Viney, 2001). In females of *S. papillosus* (Triantaphyllou and Moncol, 1977; Albertson *et al*. 1979), *Stronglyoides ransomi* (Triantaphyllou and Moncol, 1977), *Strongyloides venezuelensis* (Hino *et al*. 2014) and *S. vituli* (Kulkarni *et al*. 2013) the diploid chromosome number is only four ($2n = 4$) and one pair of chromosomes is about twice the size of the other. Correspondingly, females have two large and two medium-sized chromosomes (2L2M). Based on molecular genetic experiments and whole-genome sequencing, it became clear that in these species (strictly shown for *S. papillosus* and *S. venezuelensis*) the genomic regions corresponding to the *S. ratti* chromosomes I and X are combined in the larger chromosome (Nemetschke *et al*. 2010*a*; Hunt *et al*. 2016). Sex determination in this group of species has been best characterized in *S. papillosus*. Triantaphyllou and Moncol (1977) concluded, based on cytological observations that in *S. papillosus* and *S. ransomi* males do not differ karyotypically from females. However, later authors described, based on cytology and molecular genetic evidence, that in *S. papillosus* a male-specific chromatin diminution event takes place in the mitotic oocyte maturation division (Albertson *et al*. 1979; Nemetschke *et al*. 2010*a*). In the process, the genomic region corresponding to the X chromosome in *S. ratti* is eliminated from one of the two long (L) homologous chromosomes (Albertson *et al*. 1979; Nemetschke *et al*. 2010*a*). This leads to individuals with two copies of the regions of the genome that correspond to *S. ratti* autosomes but only one copy of the genomic region corresponding to the *S. ratti* X. Because the eliminated portion is flanked by retained regions, which are not joined together upon chromatin diminution, the diploid chromosome number in males is five, namely one long (not diminished X-I fusion chromosome), three medium-sized (the pair M, like in females and one end of the diminished chromosome which is roughly equal in size) and one small (S, the other end of the diminished chromosome) leading to a 1L3M1S chromosomal configuration. *Parastrongyloides trichosuri* reproduces sexually in both generations and employs XX/XO sex determination with $2n = 6$ in females, suggesting that within the genus *Strongyloides* the mode of sex determination in *S. ratti* is ancestral (Mackerras, 1959; Grant *et al*. 2006; Kulkarni *et al*. 2013; Streit, 2014).

At times when it was assumed that the free-living generation reproduced by pseudogamy the all-female progeny was easily explained because in such a scenario all progeny are genetically identical with the mother and therefore karyotypically

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female. In the case of sexual reproduction, as it was shown to occur in at least three species of *Strongyloides* (Viney *et al*. 1993; Harvey and Viney, 2001; Eberhardt *et al*. 2007; Nemetschke *et al*. 2010*b*; Kulkarni *et al*. 2013), this is more difficult to achieve. Several non-mutually exclusive mechanisms are imaginable. Genetically male (XO) embryos might be nonviable. Alternatively, sperm without an X chromosome might be inefficient or even incapable of fertilizing eggs. Alternatively, such sperm might never be formed in the first place. Two lines of evidence suggest that in *S. papillosus* mature male-determining sperm are never made. (i) For markers that are very closely linked with the region undergoing male-specific chromatin diminution only the allele present on the complete homologue of the autosome-X fusion chromosome and never the one present on the remnants of the diminished chromosome is present in mature sperm (Nemetschke *et al*. 2010*a*). (ii) Quantitative DNA sequencing revealed that autosomal and X-derived chromosomal regions are present in equal amounts in mature *S. papillosus* sperm (Kulkarni *et al*. 2016). Contrary to this, when DNA isolated from mature *S. ratti* sperm was quantitatively sequenced, X-derived sequences were present in lower amounts than autosomal sequences, indicating that not all sperm contain an X chromosome (Kulkarni *et al*. 2016). Dying early embryos were observed consistently in *S. ratti* but not *S. papillosus*. However, the number of these dying embryos was lower than what would have been expected based on the number of nullo-X sperm suggested by the sequencing experiments (Kulkarni *et al*. 2016). This might indicate that in addition, nullo-X sperm fertilize eggs less efficiently than X-bearing sperm.

HOMOGONIC–HETEROGONIC SWITCH

The switch between homogonic and heterogonic development is the most extensively studied process in basic *Strongyloides* biology. First, the analysis of various isofemale/inbred lines of *S. ratti* and *P. trichosuri* demonstrated that there is a heritable component to this switch such that some isolates/lines are much more prone to heterogonic development than others (Viney *et al*. 1992; Stasiuk *et al*. 2012). It was also shown that lines with more or less heterogonic development can be selected from a genetically heterogeneous population (Viney, 1996; Guo *et al*. 2015). However, environmental factors, in particular the immune status of the host, the population density and the temperature, also influence the switch (e.g. Viney, 1996; Harvey *et al*. 2000; Nolan *et al*. 2004; Minato *et al*. 2008; Stasiuk *et al*. 2012; Sakamoto and Uga, 2013; for more, older, references see Streit, 2008). This inherent temperature dependence allowed identification of the late L1 early L2 stage as the time point when the decision is made by temperature shift experiments (Premvati, 1958*a*; Arizono, 1976; Nwaorgu, 1983; Viney, 1996; Minato *et al*. 2008).

The homogonic – heterogonic switch is believed to be evolutionarily related to the switch between the formation of fast developing L3s and dauer larvae in *C. elegans* (this so-called dauer hypothesis for the evolution of parasitism (Crook, 2014) is discussed in more detail elsewhere in this special issue). Therefore, several studies used candidate approaches based on previous knowledge from *C. elegans* dauer formation and exit. A first approach was characterizing the structure and expression patterns of genes whose *C. elegans* homologues are known to control the dauer switch, and asking if the findings in *Strongyloides* are consistent with a similar role of the gene in the homogonic–heterogonic switch (Crook *et al*. 2005; Massey *et al*. 2005, 2006, 2013; Hu *et al*. 2010; Stoltzfus *et al*. 2012*a*, *b*).

Several attempts were made to further investigate the regulatory machinery controlling the homogonic–heterogonic switch at a more functional level. At the heart of the dauer switch is the nuclear hormone receptor DAF-12 (Antebi *et al*. 2000). DAF-12 when free of ligand promotes dauer formation. When the ligand, dafachronic acid (DA) (Motola *et al*. 2006) is made no dauer larvae are formed and dauer formation can be prevented pharmacologically by the application of exogenous DA. Two groups demonstrated simultaneously and independently of each other that DA also prevents the formation of infective larvae in *S. papillosus* and *S. stercoralis*, respectively (Ogawa *et al*. 2009; Wang *et al*. 2009), indicating that there is a conserved endocrine regulatory module that controls dauer formation in *C. elegans* and L3i formation in *Strongyloides* spp. However, although a clear *daf-12* orthologue is present in *Strogyloides* spp. (Wang *et al*. 2009) the demonstration that the pharmacological effect of DA is through DAF-12 is pending and it is not known yet if the natural ligand of *Strongyloides* DAF-12 is DA. In fact, it is not even known if DA exists in *Strongyloides* spp. (but see below).

Based on these findings and the extensive knowledge about the genetic control of dauer entry and exit in *C. elegans* the Lok lab reported in multiple publications an extensive characterization of the L3i formation and activation in *S. stercoralis*, combining all currently available genetic tools in *Strongyloides* including RNA expression studies, transgenes encoding reporter constructs and wild-type and mutant versions of proteins (for example GFP tagged non-phosphorylatable, phosphomimiking or dominant negative derivatives of the forkhead transcription factor type O (FOXO) FKTF-1b, the orthologue of *C. elegans* DAF-16) and pathway activating and inhibiting chemicals

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[phosphatidylinositol-3 (PI3) kinase inhibitors, 8-bromo-cGMP, cytochrome P450 inhibitors and DA] (Castelletto *et al*. 2009; Stoltzfus *et al*. 2012*a*, *b*, 2014; Massey *et al*. 2013; Albarqi *et al*. 2016). Due to the lack of mutations along with the candidate approach based on *C. elegans* gene function, which will inherently miss *Strongyloides* specific factors, and the much smaller number of man-hours spent on *Strongyloides* research, the picture is not as clear as in *C. elegans*. Nevertheless, the results are most interesting and indicate that dauer/L3i formation and exit are, at least in part, controlled by the same players in *S. stercoralis* and in *C. elegans*. In particular, the most recent of these papers Albarqi *et al*. (2016) demonstrated that inhibition of cytochrome P450 activity by ketoconazole, which in *C. elegans* prevents biosynthesis of DA, has the opposite effect of DA addition in *S. stercoralis* and is suppressible by DA administration. This strongly indicates that in *S. stercoralis* DA or a closely related steroid hormone is involved in the process. Interestingly, however, differences in the expression patterns of several genes as well as varying epistatic relationships between regulatory modules strongly indicated that there are substantial differences in the regulatory logics of the two species.

Conclusions and outlook

Over the years, how genetic information in *Strongyloides* spp. and *P. trichosuri* is passed from one generation to next has been elucidated in quite some detail (see above). Nevertheless, one has to remain open for the possibility that under certain circumstances or in particular species of *Strongyloides* alternatives from what has emerged as general rules for *Strongyloides* spp. are conceivable. Schad (1989), for example, has explicitly warned not to prematurely disregard the reports of parasitic males by Faust and Kreis (Kreis, 1932; Faust, 1933).

The sequencing efforts over the last years have yielded a comprehensive catalogue of genes present in several species of *Strongyloides* and in *P. trichosuri* (Hunt *et al*. 2016). In order to study the functions of these gens, methods to knock them out are highly desirable. No true success with this respect can be reported yet in *Strongyloides* spp. but there is hope. Over the last few years sequence-specific endonucleases such as Zn-finger nucleases, TALENs and the CRISPR/Cas9 system have been established for mutation induction and genome editing in various systems, among them the nematodes *C. elegans*, other species of *Caenorhabditis* and *P. pacificus* (Jinek *et al*. 2012; Wiedenheft *et al*. 2012; Lo *et al*. 2013; Irion *et al*. 2014; Kim and Kim, 2014; Sung *et al*. 2014; Waaijers and Boxem, 2014; Wei *et al*. 2014; Witte *et al*. 2015). In particular, the CRISPR/Cas9-based approach taken by (Cho *et al*. 2013) for *C. elegans* and (Witte *et al*. 2015) for *P. pacificus* looks promising for *Strongyloides*/*Parastrongyloides*. In this approach, the components, namely the endonuclease Cas9 and a bipartite single guide RNA (sgRNA, one part recognizes a 20 bp target site by base-pairing and the other part binds Cas9) are synthesized and assembled into the active complex *in vitro*. The complex is then injected into the gonad of adult hermaphrodites (which in *C. elegans* and *P. pacificus* replace females). Contrary to the approaches taken by the other references mentioned above, which include expression of the RNA and/or Cas9 from transgenes or injected RNAs, this approach does not depend on the availability of promoters or untranslated RNA regions known to work efficiently in the gem line. The modified strategy for *S. ratti* could be as follows (Fig. 2). Inject the Cas9/sgRNA complex designed to recognize a particular gene into the gonads of free-living females. This is expected to introduce double-strand breaks at the recognition site in germ cells, some of which will be imperfectly repaired leading to progenies with small deletions/insertions. These mutations will usually only be present in one of the two copies of the gene such that carriers are phenotypically wild-type (assuming the mutation is recessive). The progeny of the injected mothers are then used to infect host animals and emerging larvae are first tested in batch by PCR and sequencing for the presence of mutations at the desired position. If such a mutation is present among the worms shed by a host individual, gravid adult free-living females are singled out and allowed them to reproduce. Once they have produced a number of progeny they are used for DNA preparation and tested for the presence of the mutation. Single infective larvae derived from heterozygous mutant mothers are then used to infect hosts and establish a culture of heterozygous mutant worms. Single worm infections with *S. ratti* are successful in roughly half of the attempts (Viney *et al*. 1992). For *Parastrongyloides* the passage through the host is not necessary and the free-living progeny of the injected mothers can be tested directly after they produced a number of offspring, which can be used to secure the mutation.

Classical forward genetic approaches with the random introduction of mutations followed by screening for a phenotype of interest would also be most useful. This approach has several advantages. It does not rely on prior assumptions about which genes may be involved in the process of interest and it leads not only to loss of function mutations or alterations with already known consequences (e.g. dominant negatives) but also to hypomorphic (reduced function), hypermorphic (enhanced function) or neomorphic (new function) alleles, which can be highly informative, as is illustrated by one of the best-known mutations in the fruit fly. The phenotype of the mutation *nasobemia* in the

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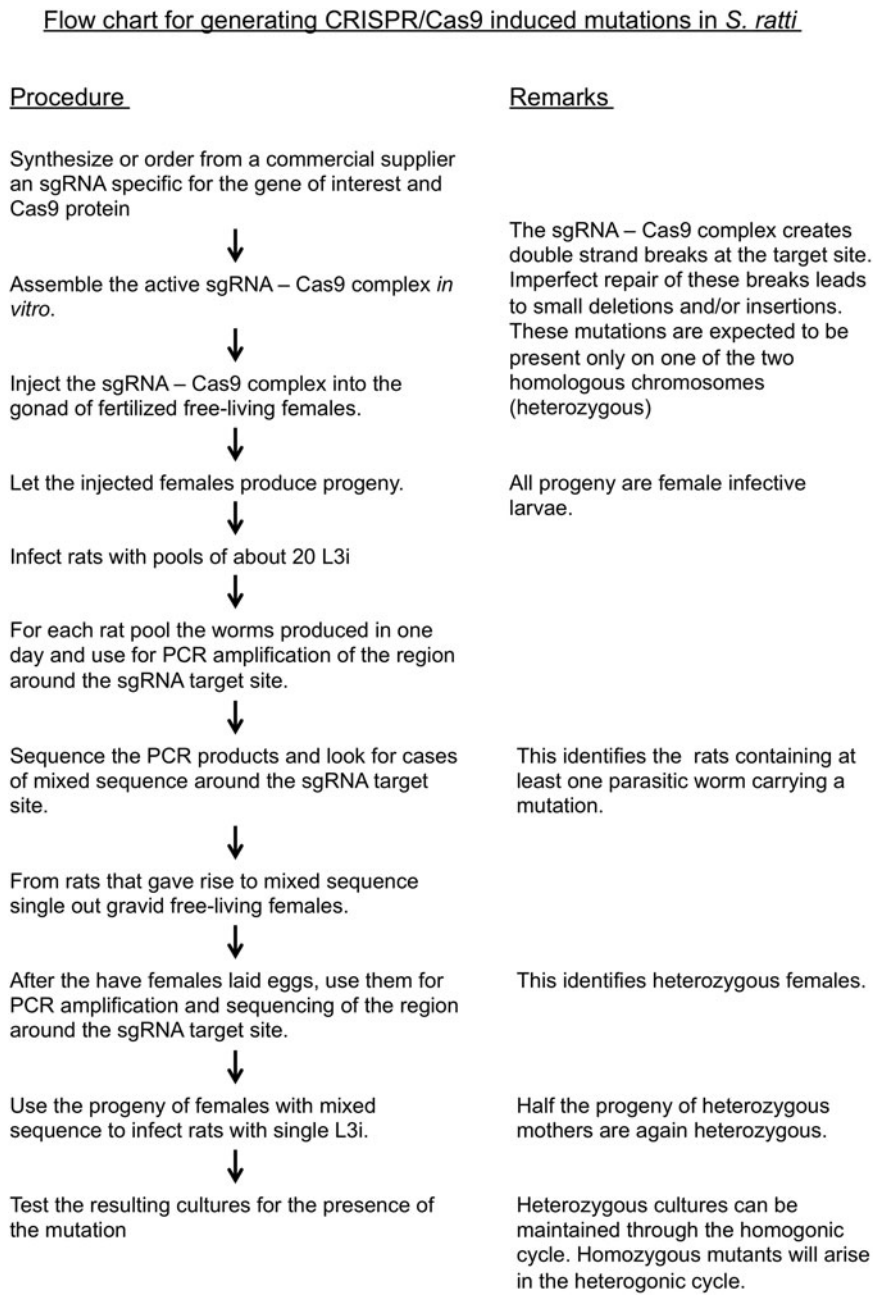

Fig. 2. Proposed strategy for targeted gene knock out in *S. ratti*. This protocol is proposed based the one published for *P. pacificus* (Witte *et al*. 2015) and has not yet been successfully used in *Strongyloides* spp. or *Parastrongyloides* spp. Further modifications, for example the co-injection of a DNA repair template in order to achieve a specific mutation (deletion, insertion or alteration) are possible. For details, see text.

gene *antennapedia*, which eventually led to the discovery of the conserved homeobox (McGinnis *et al*. 1984; Gehring *et al*. 1994) is caused by the mis-expression of the gene (Schneuwly *et al*. 1987). Such a mutation would not have been found in the context of a systematic gene knock out analysis. Protocols for the induction of mutations in *S. ratti* are available and mutant worms were isolated successfully (Viney *et al*. 2002; Guo *et al*. 2015). But, so far forward genetic studies in *Strongyloides* have been hampered by the formidable obstacles to identifying the mutated genes causing the phenotype. However, there is great hope that this will change in the near future. With the recent progress in sequencing technology, requiring less material and becoming more affordable, in the model nematodes *C. elegans* (Sarin *et al*. 2008; Doitsidou *et al*. 2010) and *P. pacificus* (Ragsdale *et al*. 2013) it has become possible to identify mutations by sequencing the genomes of mutant animals and comparing them with the wild-type. In particular, the approach by (Doitsidou *et al*. 2010) looks to be very promising at least for *S. ratti* where an excellent reference genome is now available (Hunt *et al*. 2016). In this strategy (Fig. 3), the mutant line (parental line 1′, which is a mutagenized derivative of a line 1) is

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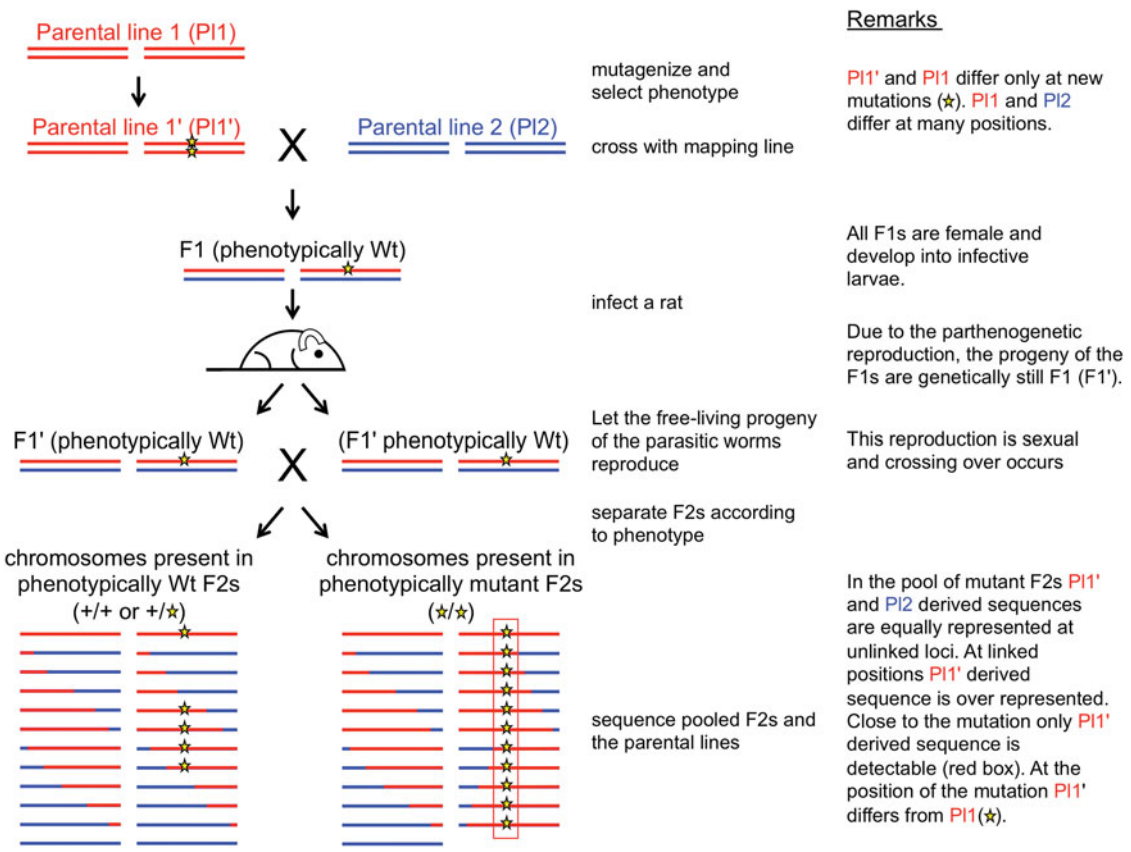

Fig. 3. Proposed strategy for mapping and cloning of mutations in *S. ratti*. This protocol is proposed based on the one published for *C. elegans* (Doitsidou *et al*. 2010) and has not yet been successfully used in *Strongyloides* spp. or *Parastrongyloides* spp. It is assumed that the mutation of interest is recessive and worms with the mutation in the homozygous state are viable. Generating mutations and homozygous mutant lines require at least one host passage but is abbreviated as one step. Red and blue lines represent chromosomes derived from parental line 1 (red) or parental line 2 (blue). Parental line 1 is the one used for mutagenesis. Parental line 2 is a different isolate, whose genome sequence differs from that of parental line 1 at thousands of positions. The mutation to be mapped is indicated by a yellow asterisk. Only one of the two chromosomes not containing the mutation is shown. In the example the chromosome not shown is the X chromosome. The strategy also works for mutations on the X chromosome provided that hemizygous mutant males are fertile. Wt, wild-type.

first crossed with a different strain (parental line 2) with several thousands of known sequence differences compared with line 1. Theoretically parental lines 1 and 1′ differ only at the positions that have been altered by the mutagenesis treatment. The resulting F_1 animals are all heterozygous at all different loci, including the locus of interest and therefore, assuming the mutation is recessive, phenotypically wild-type. The F_1 animals are then crossed among themselves. The resulting F_2 progeny is divided into two pools containing the mutant and all the phenotypically wild-type animals, respectively. From these pools DNA is isolated and quantitatively sequenced. Around the position of the mutation causing the phenotype of interest, all mutant animals carry only alleles derived from parental line 1′. At all positions not genetically linked the allele frequency for both alleles is expected to be 50%. Within the region that in mutants is all parental line 1′ derived, only very few positions will differ between parental line 1′ and line 1. These are the interesting candidates. In order to make this approach workable in *S. ratti* suitable parental lines 1 and 2 need to be established. Since the genomic sequence of the currently most commonly used standard laboratory isolate ED321 has been determined and published (Hunt *et al*. 2016), this strain is a prime candidate for parental line 1. However, ED321 has been maintained in several laboratories for many years and the populations have accumulated rare alleles, which are undetectable by sequencing genomic DNA isolated from large numbers of worms (Guo *et al*. 2015). Selection of individuals with the desired mutant phenotypes represents a very dramatic population bottleneck (the mutant population is derived from the one originally mutant individual and its mates). This will make visible all the rare alleles present in the founding individuals. These variants will appear as differences from the wild-type along with the mutations induced by the mutagen, thereby increasing the number of candidate mutations (Guo

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et al. 2015). Creating two more strongly inbred laboratory strains should therefore be a priority for *S. ratti* geneticists.

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