Parthenogenetic Panagrolaimus species evolve at lower mutation rates, but show increased nucleotide diversity

Laura Villegas[1,*], Luca Ferretti[2], Thomas Wiehe[3], Ann-Marie Waldvogel[1,*], and Philipp H. Schiffer[1,*]

[1]Institute for Zoology, University of Cologne, 50674 Koeln, Germany

[2]BIG DATA INSTITUTE, University of Oxford, Oxford, United Kingdom

[3]Institute for Genetics, University of Cologne, Köln, 50674 Germany

***Corresponding author:** E-mails: lvilleg1@uni-koeln.de; a.waldvogel@uni-koeln.de; p.schiffer@uni-koeln.de

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Abstract

Asexual reproduction is assumed to lead to the accumulation of deleterious mutations (Muller's ratchet), and reduced heterozygosity due to the absence of recombination. Panagrolaimid nematodes display different modes of reproduction. Sexual reproduction through distinct males and females, asexual reproduction through parthenogenesis found in the genus *Panagrolaimus*, and hermaphroditism, found in *Propanagrolaimus*. Here, we compared genomic features of free-living nematode populations with different reproduction modes isolated from geographically distant regions to study genomic diversity and genome-wide differentiation. We firstly estimated genome-wide spontaneous mutation rates per genome for a polyploid parthenogenetic *Panagrolaimus* strain and a diploid hermaphroditic *Propanagrolaimus* species via mutation-accumulation-lines. Secondly, we calculated population genomic parameters including nucleotide diversity and fixation index (F_{ST}) between populations of asexually and sexually reproducing nematodes. Thirdly, we used phylogenetic network methods on sexually and asexually reproducing *Panagrolaimus* strains to understand evolutionary relationships between them. The estimated mutation rate was slightly lower for the asexual strain, as expected for taxa with this reproductive mode. Natural polyploid asexual strains revealed higher nucleotide diversity. Despite their common ancestor, a gene network revealed a high level of genetic differentiation among asexual strains. The elevated heterozygosity found in the triploid parthenogens could be explained by the third genome copy. Given their tendentially lower mutation rates it can be hypothesized that this is part of the mechanism to evade Muller's ratchet. Our findings in parthenogenetic triploid nematode populations seem to challenge common expectations of evolution under asexuality.

Key words: Nematodes, parthenogenesis, mutation rate, diversification, population genomics, asexuality.

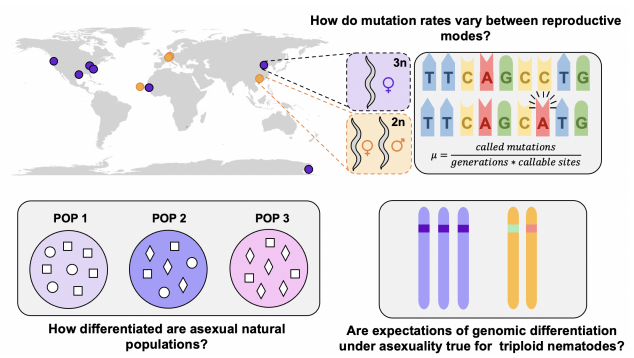

FIG. 1. Graphical abstract. We estimated mutations rates, nucleotide diveristy and population differentiation on sexual and asexual populations of nematodes

Introduction

Dedicated to the memory of Einhard Schierenberg.

Sex is by far the most abundant form of reproduction in Metazoa. The origin of outcrossing and meiosis is closely associated with the evolution of eukaryotes, in which sexual reproduction is dominant. However, under similar ecological and genetic conditions, an individual undergoing asexual reproduction without outcrossing, as found in parthenogenetic taxa, will generate more offspring (each of which is itself capable of generating offspring) than a sexual sibling. The cost of producing males, finding mates, courtship, and of the act itself should give parthenogenetic taxa a huge evolutionary advantage (Otto and Lenormand, 2002). The seeming paradox, between the dominance of sexual reproduction and the apparent advantages of parthenogenesis, has puzzled evolutionary biologists for a century, leading Graham Bell to call it the "Queen of Evolutionary Questions" (Bell, 1982). Consequently, much work has been devoted to the question of the predominance of sex. The main theoretical explanation for the evolutionary benefit of sex rests on the fact that sexual reproduction allows species to disseminate and combine (novel, beneficial) genotypes through meiosis followed by crossover (Smith, 1978). Parthenogenetic taxa are typically found on phylogenetically terminal branches, and this in turn suggests that accumulation of deleterious mutations that cannot be purged by recombination ("Muller's ratchet"; (Muller, 1964)) may lower fitness and ultimately lead to extinction. Consequently, research on obligate parthenogenetic Metazoans has focused on the few old 'evolutionary scandals' (Smith, 1978), the bdelloid rotifers, oribatid mites, and Darwinulid ostracods, as these should have long gone extinct. Among these taxa genomic studies have been conducted in the bdelloids, with a study on the *Adineta vaga* genome suggesting gene conversion in a tetraploid system leading to low allele sequence divergence, and a later study presenting evidence for cryptic sex in the rotifers (Vakhrusheva *et al.*, 2020). While the evolutionary old parthenogens certainly remain of great scientific interest, these cannot really inform on the evolutionary trajectory of incipient parthenogenesis. Contrary to many free-living nematode species, ancient asexuals, such as oribatid mites have long generation times (Pfingstl and Schatz, 2021), making it hard to keep them in culture and perform experiments. Rotifers, on the other hand can be

kept in laboratory cultures. However, they only inhabit aquatic ecosystems (Ejsmont-Karabin, 2019), whereas nematodes can inhabit a variety of ecosystems (terrestrial, freshwater, marine) providing a more comprehensive overview on the evolution of asexuality.

It has been proposed that asexuality impedes adaptation (Engelstädter, 2008), but studies show that asexual taxa can in fact adapt to different niches (Amat *et al.*, 2017; Gibson, 2019; Simón-Porcar *et al.*, 2021), populations of asexually reproducing monkey flowers and parasitoid wasps show adaptation to their environment. Asexual parasitoid wasps have a higher investment on production of eggs on food-depleted environments for adults, whereas the sexual counterpart often found in food-rich environments with patchy distribution of hosts show a higher investment in flight capacity and are more longevous (Amat *et al.*, 2017). Natural populations of asexually reproducing organisms should diverge genetically due to local adaptation, genetic drift, geographic isolation, and generation of distinct mutations on the different populations. The rate in which mutations arise *de novo* on a genome (mutation rate) is expected to be low given that most non-synonymous mutations that occur are deleterious and accordingly under strong purifying selection (Lynch, 2010). For that reason for asexually reproducing organisms it is hypothesized that mutation rates are lower than that of sexually reproducing counterparts. Mutation rates are proposed to be species-specific (Lynch, 2010) but vary according to environmental stress (Waldvogel and Pfenninger, 2021). However, studies on nematodes are limited to species of the *Caenorhabditis* (Denver *et al.*, 2009), (Matsuba *et al.*, 2013) genus or model organisms like *P. pacificus* (Molnar *et al.*, 2011), which are not representative for the phylum. Unlike *Caenorhabditis* nematodes, species from the *Panagrolaimus* genus can be parthenogenetic (asexual reproduction) or gonochoristic (sexual reproduction)(Kiontke *et al.*, 2004). This system allows to compare closely related taxa with different reproduction modes providing information on how their genomic features may differ. Asexual species of hybrid origin are characterized by high heterozygosity (Jaron *et al.*, 2021). For instance, the triploid asexual crayfish *Procambarus virginalis*, has shown to be highly heterozygous even when the expected outcome of its reproductive mechanism would be homozygous (Schwarz, 2017). Parthenogentic *Panagrolaimus* nematodes share a common origin of parthenogenesis through a hybridization event (Schiffer *et al.*, 2019), that might contribute to a higher heterozygosity in parthenogenetic species when compared to sexual sister groups. The variable genomic origins of the homeologous chromosome sets of hybrid asexual strains may be advantageous, as co-occurrence of divergent gene sets could overcome the disadvantages of the loss of ability to recombine with other

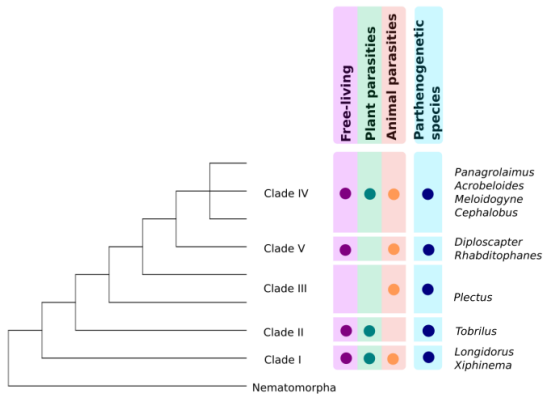

FIG. 2. Cladogram of the 5 nematode clades. Parthenogenetic species are present in many genera across the phylum. In the clade IV genus *Panagrolaimus* parthenogenesis likely has a single origin.

members of the species (Castagnone-Sereno and Danchin, 2014). This might be particularly true for meiotic parthenogens. As the *Meloidogyne* example illustrates there are two general modes of parthenogenesis: meiotic (automictic) and mitotic (apomictic, i.e. direct development of oocytes). While mitotic parthenogens are strictly clonal, they retain their mothers' heterozygosity. However, in many forms of meiotic parthenogenesis (depending on the mode in which diploidy is restored) homozygosity is enforced (Schön *et al.*, 2009). Due to the accumulation of slightly deleterious mutations in many loci under Muller's ratchet this homozygosity should lead to a strong fitness loss over time. Thus, meiotic parthenogens may be on a much steeper trajectory towards extinction than mitotic ones. Parthenogenetic *Panagrolaimus* nematodes are obligate asexual species, unlike other parthenogenetic taxa such as stick insects where facultative parthenogenetic species occur (Larose *et al.*, 2022).

We have shown that meiotic parthenogenetic species in the nematode genus Panagrolaimus are likely to be triploid hybrids, and found evidence for hybridisation in another nematode species, *Diploscapter coronatus* (Kraus *et al.*, 2017). On the other hand, first data from the genera *Plectus* and *Acrobeloides* showed no signal for a hybrid origin in the two species analyzed (Schiffer *et al.*, 2018)(Fig. 2). It is not known how frequently hybridization is involved in the evolution of parthenogenesis in the phylum, but the examples from *Meloidogyne*, *Panagrolaimus*, and *Diploscapter* appear to indicate that it could be frequent.

Results

The spontaneous mutation rate under neutral evolution is marginally lower in parthenogens

We conducted a mutation accumulation line (MAL) experiment with a parthenogenetic strain (triploid PS1159) and a hermaphroditic sexual nematode strain (diploid JU765) over 40 generations (Fig.3). Starting with 100 and 71 lines respectively, 30 hermaphroditic lines and 15 parthenogenetic lines survived. We performed whole genome sequencing with HiSeq2000 and NovaSeq Illumina platforms. A coverage range of 15X was established for each genome copy for the analysis of natural populations, thus asexual triploid lines had a coverage range of 45X, whereas diploid hermaphroditic lines have a coverage range of 30X to allow proper identification of variants per genome copy.

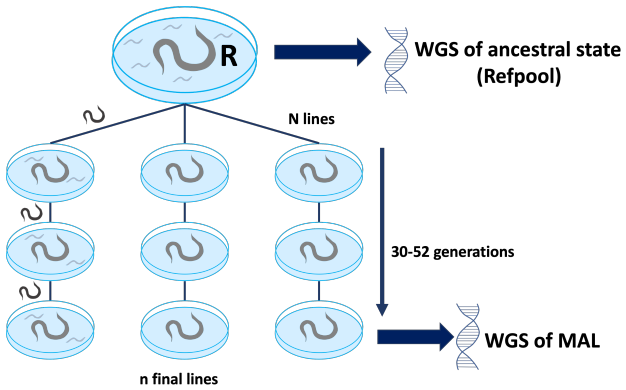

FIG. 3. Experimental setup of mutation accumulation. N refers to the number of starting lines, for PS1159 N=100 and for JU765 N=71; n is the number of final lines, n=15 for PS1159 and n=30 for JU765.

We called mutations using a probabilistic approach (accuMUlate) (Winter *et al.*, 2018) and filtering candidate mutations to only retain high-confidance *de novo* mutations (DNMs). The analysis was performed only on positions commonly covered between all the lines and parental state per reproductive mode, respectively: asexual lines had 31.735 common positions and hermaphroditic lines had 25.812 common positions.

For the asexual lines 72375 candidate mutations were called, whereas 7852 candidates were found for the hermaphroditic lines. After filtering for coverage ranges, read support, unique mutations per line and miss-mapped regions, and manual curation, 11 DNMs with high support remained for the asexual lines and 10 DNMs for the hermaphroditic ones.

Per reproduction mode 6 lines were analyzed, 5 lines for each mode showed at least one DNM. For the asexual lines an average of 7.5×10^{10} callable sites were found and 5.6×10^{10} for the hermaphroditic lines. The mutation rates estimated were 5.828×10^{-10} (credibility intervals $1.2\times10^{-17}, 5.1\times10^{-9}$) and 8.923×10^{-10} (credibility intervals $2.9\times10^{-17}, 6.9\times10^{-9}$) respectively, showing lower mean mutation rate for the asexual line. Mutation rates do not significantly differ between each other, due to wide credibility intervals. The majority of DNMs were transitions. The asexual lines showed 9 transitions and 2 transversions, the sexual lines showed 6 transitions and 4 transitions.

The hermaphroditic sexual lines showed a lower θ_w when compared to the parthenogenetic lines. The lowest nucleotide diversity was found on the parental JU765. Parthenogenetic lines have similar nucleotide diversity values, the lowest π is found on asexual line 4 (al4), which is also the line with the lowest θ_w. Standing genetic diversity is lower on the parental line of sexual refpool than for the parental asexual refpool (Fig. 4).

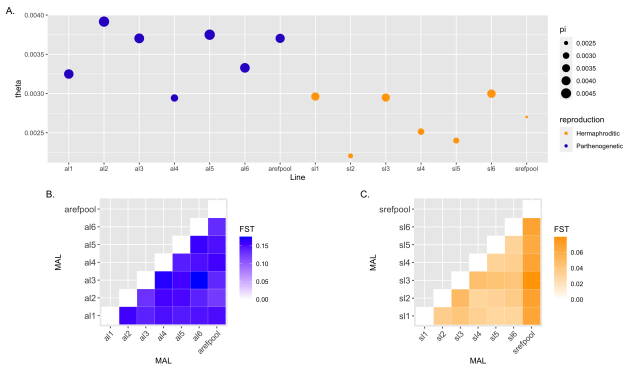

FIG. 4. Nucleotide diversity and population differentiation of (B) asexual and (C) sexual mutation accumulation lines. On average nucleotide diversity is higher on asexual populations. A pattern of differentiation can be seen on sexual populations but not on asexual ones

For the asexual lines, the highest F_{ST} was found between asexual lines 3 (al3) and 6

(al6) (0.176) and the lowest F_{ST} was found between parental PS1159 and asexual line 2 (al2) (0.125), no significant difference was found for the F_{ST} values between parental PS1159 and lines when compared to the F_{ST} between the lines themselves. The hermaphroditic lines showed a significantly higher F_{ST} between the descendant lines compared to the parental state (Welch t-test p-value 6.095×10^{-7}), the lowest population differentiation was found between sexual lines 6 (sl6) and 1 (sl1) (0.03), and the highest one between the parental JU765 and sexual line 3 (sl3).

The population diversity is higher in parthenogens

We compared descendant populations from the MAL experiment from each reproduction mode with regard to nucleotide diversity, heterozygosity and population differentiation. Asexual populations showed a mean nucleotide diversity (π) higher than that found for sexual populations. Asexual population 3 (apop3) was the asexual population with a higher nucleotide diversity (0,206227) and the lowest nucleotide diversity was found for PS1159 (0,0402963). Among the sexual populations, the hermaphroditic JU765 showed the lower nucleotide diversity (0,00128981) and sexual population 3 (spop3) showed the highest one (0,0545159). Nucleotide diversity was on average higher for asexual populations (Fig. 5).

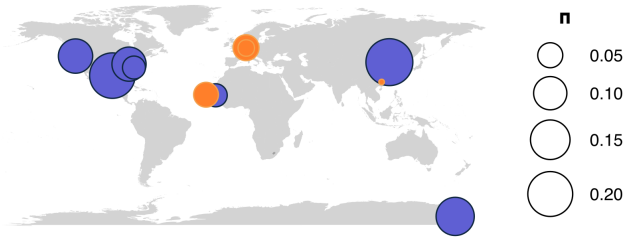

FIG. 5. Nucleotide diversity π on natural populations of nematodes isolated from distant geographical areas. Nucleotide diversity is higher on natural asexual populations whenc ompared to natural sexual populations

Estimator θ_w was estimated genome wide as well as for homologous copies. For diploid sexual populations genome wide estimations did not vary significantly from homologous copies estimations (p-value 0.4). For triploid sexual populations, though not significantly different (p-value 0.0716), the θ_w estimation for homologous copies is much lower than that estimated genome wide (supplementary table 5). On average, asexual populations showed a higher θ_w than sexual populations. Asexual populations 3 (apop3) showed the higher θ_w (0,192175 – 19 polymorphic sites per 100 sites) and PS1159 showed the lowest θ_w values (0,031685).

Higher population differentiation values were found for the sexual populations when compared to the asexual populations. In the sexual populations high values of F_{ST} were obtained, spop1 and spop3 showed highest level of genomic differentiation (F_{ST} =0.851948) whereas spop2 and spop3 are the most similar between each other (F_{ST} =0.743447) (Fig. 6). The asexually reproducing are least differentiated among each other when compared to the level

of differentiation between sexual populations. The asexual populations 1 and 7 (apop1, apop7) were the most distinct between each other (F_{ST} =0.467986) and PS1159 and asexual population 7 (F_{ST} =0.469582), whereas the least differentiated asexual populations between each other were PS1159 and asexual population 1 (F_{ST} =0.0146615).

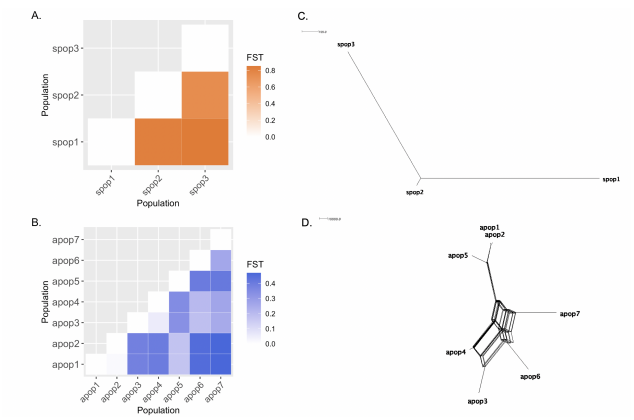

FIG. 6. Population differentiation of (A) natural sexual populations and (B) natural asexual populations. Orthologue gene network of (C) sexual and (D) asexual populations of *Panagrolaimus* nematodes. Sexual populations show higher differentiation than asexual ones. The gene network shows that asexual nematodes analyzed are genetically distinct from each other.

Monophyletic parthenogenetic strains are genetically distinct

Apart from the population level genetic diversity in different parthenogenetic strains, it is also interesting to ask whether these monophyletic strains could be designated as species. As the biological species concept necessarily fails in parthenogenetic organisms we sought to apply the phylogenetic species concept using a split network approach. We first identified for the asexual reference genome (*Panagrolaimus* PS1159), 2173 Universal Single-Copy Orthologues were identified. For each strain reads that corresponded to the coordinates of the orthologues were extracted and then consensus sequences for genes with sufficient read support were created: 375 orthologues were shared on the asexual populations, these genes were aligned and used on the gene network. We conducted a corresponding analysis for the sexual species as proof of principle. For the sexual reference genome 1983 orthologues were found and reads that corresponded to these genes' coordinates were extracted, a total of 213 orthologues were used for the gene network. A Median network for each reproduction mode was constructed using SplitsTree4. We found a total of 23 splits between the parthenogenetic strains, while there were 3 splits between the sexual species.

Discussion

In this work we aimed to analyze divergence patterns and the evolutionary trajectory of parthenogenetic animals using *Panagrolaimus* nematodes as a system, and hermaphroditic *Propanagrolaimus* as a comparator. By running long-term MAL we conducted a classical experiment to measure mutation rates under nearly neutral evolution (Halligan and Keightley, 2009). We also estimated standard population genomic parameters from natural populations, and used phylogenetic networks to understand the complexity of molecular evolution under parthenogenesis. Genome sequencing has seen another drastic advancement in the last years with long-read methods becoming available for single

individuals with sufficient amounts of DNA (e.g. vertebrates), thus potentially allowing for higher resolution in population genomic assays. The data used here is still originating from short sequencing of pools of individuals, as single individual long-read sequencing from tiny organisms remains challenging. Consequently, our study remains reference-based with limited resolution on structural genomic variation (Adewale, 2020). At the same time, short read (Illumina) sequencing still outperforms long-read methods in terms of the cost-coverage-ratio when aiming for deep sequencing studies, which is inevitable for the here conducted MAL experiment to identification of *de novo* mutations.

Lower mutation rates of parthenogens could aid to diminish the effect of Muller's ratchet

Parthenogenesis is assumed to have low evolutionary potential due to the accumulation of deleterious mutations (Muller's Ratchet) (Muller, 1932), lack of recombination and low genetic diversity (Simon et al. 2003). In *Panagrolaimus* nematodes, parthenogenesis has a common origin 1.3–8.5 mya ago involving triploidization (Schiffer *et al.*, 2019). For the nematode *Panagrolaimus* PS1159, here used as a reference for the genomic analysis of natural populations, eggs develop without fertilization and the offspring is exclusively female, asexual meiosis (presence of polar body) occurs without recombination [pers. comment, Caroline Blanc and Marie Delattre, Lyon], as a result offspring are clones of their mother. In sexual *Panagrolaimus* ES5, used as as genomic reference for sexual populations in this study, canonical meiosis occurs [pers. comment, Caroline Blanc and Marie Delattre], recombination occurs between homologs, sister chromatids orient via fused sister-centromeres and segregate to the same pole (Hofstatter *et al.*, 2021). Without the presence of recombination, selection is less effective, linked loci do not allow for selection to act upon target loci independently, leading to the accumulation of mildly deleterious mutations (Bast *et al.*, 2018). Additionally, the lack of recombination is assumed to result in reduced genetic diversity in taxa with this reproductive mode (parthenogenesis) compared to sexually reproducing counterparts (Shreve *et al.*, 2011). To diminish the "lethal" effects of asexuality, asexuals are expected to have lower mutation rates than their sexual counterparts to slow the process of mutation accumulation, since the majority of non-neutral mutations that occur are deleterious (Sloan and Panjeti, 2009). Our findings agree with this expectation, where the parthenogenetic *Panagrolaimus* strain has a lower mean mutation rate when compared to the hermaphroditic *Propanagrolaimus* strain, even though differences between rates did not differ significantly due to broad credibility intervals. Through lower mutation rates, and given the lack of recombination, clonal interference can be reduced when beneficial mutations in a population arise. With higher mutation rates,

if many beneficial mutations are carried on different clones, fixation time of these mutation is increased (De Visser and Rozen, 2005). Mutation rates found for both reproductive modes are one order of magnitude smaller than that reported for *C. elegans* (Denver *et al.*, 2009), showing that measures of model organisms are not true for the whole phylum. Estimation of mutation rates with more MALs measured after shorter accumulation periods will be necessary to reduce variation of the data and allow for statistical validation.

Heterozygosity is maintained in asexual populations

In clonal diploid parasitic populations, differentiation among populations increases when compared to the parent population, clonal populations show a lower genetic differentiation than sexually reproducing counterparts (Balloux *et al.*, 2003; Prugnolle and De Meeûs, 2008). This pattern in not true for the parthenogenetic lines here analyzed of asexual triploid populations, suggesting that the differentiation between these populations is due to random effects. However, on selfing sexual populations, low heterozygosity is expected. Highly inbred laboratory nematode strains previously described such as *C. briggsae* and *C. elegans* show low heterozygosity causing a higher divergence between populations and the parental state than within populations (Barrière *et al.*, 2009; Teotónio *et al.*, 2017). The lower differentiation between hermaphroditic lines compared to the parthenogenetic ones could be explained by (1) lower standing genetic variation in JU675 at the beginning of the experiment and (2) increased heterozygosity for asexuals due to the third genome copy. In asexual lines "inbreeding" does not change the genetic structure of a population in a specific unidirectional way (Bengtsson, 2003). The here found pattern of population differentiation for asexual lines could show the differentiation pattern of natural populations: maintained heterozygosity within offspring due to the lack of allele segregation (Stoeckel and Masson, 2014). It has been proposed that under asexuality heterozygosity can even increase since alleles of a same gene could independently accumulate mutations over generations, this refers to the so-called Meselson effect, not tested here but already proven for obligate asexual oribatid mites (Brandt *et al.*, 2021).

Natural populations of parthenogenetic nematodes show higher genomic diversity than their sexual counterparts

Population estimators obtained for parthenogenetic natural populations showed a mean nucleotide diversity higher than that found for sexual populations, a pattern also found for the amazon molly, an asexual fish of hybrid origin (Jaron *et al.*, 2021), as well as in the asexual bark lice (*Echmepteryx hageni*) (Shreve *et al.*, 2011) when compared to sexual counterparts. θ_w was also found to be higher in the asexual populations with genome-wide

estimations, however, when only homologous genome copies were compared, θ_w estimations were lower, showing how the third genome copy, is a major source for genetic diversity due to the hybrid origin. Asexual reproduction accompanied by high genetic diversity has shown to allow for rapid adaptive responses on parasitic nematodes of *Meloidogyne* species to their hosts, highlighting how studies of asexual populations from different geographical locations, such as the one conducted here, can help understanding the genetic basis of genome structure and evolution of asexual taxa (Castagnone-Sereno, 2006). Estimated nucleotide diversity of natural nematode populations on Hawaiian Islands has been found to be lower than that found here for asexual and sexual (gonochoristic) populations, however, the average genome-wide diversity found for these *C. elegans* populations is very similar to what was obtained for the natural population of *Propanagrolaimus* JU765 (*C. elegans* 0.00109 – JU765 0.00129). Both *C. elegans* and JU765 populations are diploid and selfing hermaphrodites (Crombie *et al.*, 2019). The variable proportion of nucleotide diversity found on different genomic regions, is consistent with the assumed pattern across the genomic landscape. Some regions have very low diversity and could correspond to coding regions, whereas introns are those expected to have a higher nucleotide diversity (Tatarinova *et al.*, 2016). Follow-up studies including the annotation of reference genomes will allow for more precise estimations of the nucleotide diversity across the genomic landscape.

Previous studies on nematode populations showed for the parasitic nematode *Baylisascaris schroederi* that populations isolated from different mountain ranges showed the absence of significant differentiation F_{ST} between three populations of this species, values found in this research ranged from 0.01911 to 0.02875 (Zhou *et al.*, 2013). Natural populations of *C. brenneri* also show low values of differentiation between populations, in this case, from geographically distant regions eastern India and French Guiana (0.092)(Dey *et al.*, 2013). Our results show on average a higher differentiation between populations, for both sexual and sexual populations have high at least two-fold higher F_{ST} values than those reported for *Caenorhabditis* nematodes and *Baylisascaris schroederi*. This higher F_{ST} values could be an initial indication for local adaptation (Hirase *et al.*, 2021) to the distinct regions where the populations were isolated from, and additionally in the asexual populations, due to the Meselson effect.

It was expected that some parthenogenetic taxa appear to be favored in extreme challenging environments (geographical parthenogenesis)(Tilquin and Kokko, 2016) due to their ability to save resources that would otherwise be allocated to finding a mating partner and the potential to found new populations by the dislocation of a single individual. Geographical

parthenogenesis is a pattern observed in plants (Bierzychudek, 1985), flatworms (Lorch *et al.*, 2016) and ostracods (Symonová *et al.*, 2018). However, ambivalent data has been reported on taxa such as salamanders (Greenwald *et al.*, 2016). Our study finding that genomic diversity is high in parthenogenetic populations could support the idea that they are capable to survive in challenging habitats, but more data from strains specifically isolated from such habitats is needed.

Despite a monophyletic origin, parthenogenetic *Panagrolaimus* strains are genetically distinct

Split networks have been successfully applied to compare distantly related taxa (yeast, mammals, *Drosophila* and *C. briggsae*)(Huson and Bryant, 2006), as well as populations of hyperdiverse nematodes (Dey *et al.*, 2013). Our split network analysis based on 375 orthologues in the parthenogenetic strains and 213 orthologues in the sexual species appears to indicate that the former are as genetically distinct as the latter. Notably, the network for the sexual species is tree-like, while it shows more splits for the parthenogens. This is expected in a triploid system, where homeologs are not resolved (or phased in the genome assemblies) and thus recapitulate a pattern usually seen in re-combining populations. The classical biological species concept, defines species as groups of (potentially) interbreeding populations which are reproductively isolated from other similar groups (Mayr, 1999). Naturally the concept is restricted to organisms with sexual reproduction. Asexual organisms can undergo speciation and form significant clusters due to adaptation to different niches or to physical separation (Birky *et al.*, 2010) (William Birky and Barraclough, 2009). It has been suggested that asexual species are characterized by genotypic clusters of long-lasting gaps instead of transient gaps due to random genetic drift and can also form phenotypic clusters that are often cryptic and require genetic analysis (William Birky and Barraclough, 2009). Distinct species have been found for asexual organisms such as bdelloid rotifers, oribatid mites and oligochaete worms by using genomic data (Birky *et al.*, 2010), i.e. applying a phylogenetic or phylogenomic species concept. Similar to these taxa the monophyletic parthenogenetic *Panagrolaimus* nematodes in our study might be seen as species under a phylogenomic concept of the term.

Studying genome evolution in parthenogens requires improved analysis frameworks

In our study it became obvious that studying 'parthenogenomic' complexity is currently limited on two very different levels. Firstly, the availability of high quality genomic resources, i.e. phased reference genomes from tiny invertebrate organisms, with limited amounts of DNA per individual. Secondly, population genetic theory as well as its implementation in analysis tools is not geared towards accounting for variation in

ploidy and mode of reproduction in non-model organisms.

Limitations in sequencing technology still affect inferences of population genomic parameters

The resolution of genome analyses depends on the quality of the underlying genomic data obtained through sequencing. Short-read data, such as the one used here, can provide ample information about a genome, but it is insufficient to distinguish repetitive regions of the genome and cannot be used to properly study structural variants (Adewale, 2020). The maximum resolution that can be derived from short-read data ultimately depends on the quality of the reference genome that is used to map and compare the data to (Waldvogel *et al.*, 2020). Polyploid genomes, as those in the parthenogenetic strains studied here, are technically challenging as they are likely to involve divergent evolutionary trajectories between homeologs, which carry different patterns of variation (Hörandl *et al.*, 2020). To better infer measures as μ and other population divergence measures phased reference genomes should be used and with the current advances in long-read sequencing methods these should be available in the future (The Darwin Tree of Life Project Consortium *et al.*, 2022) even for tiny invertebrate taxa, as nematodes or rotifers.

Population genetic theory and software implementation are not well suited to study complex genomes

With regard to population genetic theory, asexual reproduction adds another level of complexity to our understanding of mutational evolution, selection and adaptation and many expectations of genome evolutionary processes, as e.g. the mode of reproduction violates the basic population assumption of random mating (Fisher, 1923) (Wright, 1931). Even when phased reference genomes become available for tiny invertebrate taxa we currently lack the appropriate software tools to disentangle different genotypes (e.g. autopolyploids or allopolyploids) in a population genomic analysis framework. Tools that account for both ploidy and asexuality in population genomic analysis are non-existing, making current estimations and analysis from data of organisms with these characteristics non precise despite adjustments to reduce bias (Jighly *et al.*, 2019). In this study we aimed to account for potential bias by adjusting coverage ranges to ensure high enough representation of the respective genotypes in the read data, and thus allow for each position on each genome copy to be called equally likely. However, to study such genotypes on a single individual level in polyploid systems it will be necessary to develop more sensitive software tools.

Theoretical expectations about mutation rate evolution need to be adapted to diverse and complex systems

Mutations are the ultimate source of genetic variation, even if most non-synonymous mutations that occur are deleterious. Classically, it has been thought that recombination allows deleterious mutations to be eliminated more efficiently, and while both mutation and recombination rates are

variable along the genome, in obligate asexual taxa recombination can be completely absent, possibly leading to the accumulation of mildly deleterious mutations. We have found indications that natural populations of parthenogenetic *Panagrolaimus* show an elevated level of heterozygosity, potentially due to their third genome copy and the lack of allele segregation. The mutation rate in these organisms appears to be low, thus maybe delaying the effect of Muller's ratchet. The differentiation found within sets of natural populations, as well as the genomic differentiation between strains seems to show the potential for evolution in parthenogenetic animals. Asexual reproduction does not occur in the same way across the tree of life. In some asexual taxa like angiosperms, asexual reproduction occurs with meiosis still present and where gene conversion can occur. In other taxa, such as *Panagrolaimus* nematodes, asexual meiosis happens without recombination, whereas in other nematode species (Castagnone-Sereno and Danchin, 2014) and stick insects (Schwander *et al.*, 2011), mitotic parthenogenesis without meiosis can take place. This diversity necessarily will be reflected in at least equally divers mechanisms of genome evolutionary processes which ultimately cannot be resolved as long as we aim to apply one unifying theory.

Conclusions

In this study we have shown that the mean of the measured mutation rates is lower in parthenogenetic *Panagrolaimus*, than in closely related hermaphrodites, and parthenogenetic populations retain genomic diversity. In combination, both factors could allow them to evade extinction on short evolutionary time-scales and give them adaptive potential in (possibly challenging) environments. Parthenogenetic *Panagrolaimus* strains defy the biological species concept, but might be seen as valid species under a phylogenomic species concept. Our study showed the need for high-quality phased genomes, better analysis software, and the development of improved theoretical models to better understand the complex genomes and population dynamics of tiny invertebrate species.

Materials and Methods

Sampling, sequencing and data pre-processing

DNA was extracted from several plates of standard laboratory cultures (worms are kept at 15°C on low nutrient agar plates inoculated with OP50 *(E. coli)*. Adult nematodes, as well as larvae and eggs were washed off from the plates and cleaned in 3 washing steps. After three rounds of freeze-thaw cycles on lysis buffer, genomic DNA was extracted following a salt-out protocol or using Qiagen's genomic tip. Sequencing of pooled specimens was performed on HiSeq2000 and NovaSeq Illumina platforms. Strains that were used for the estimation of mutation rates were kept in a bottleneck for 30 to 52 generations and sequenced at the starting point of the experiment

(reference) and the end point of the line. DNA extraction was performed as described above.

Paired-end reads were trimmed using fastp (Chen *et al.*, 2018) and mapped against the according reference genome (asexual or sexual) using bwa-mem2 (Vasimuddin *et al.*, 2019). For strains where reads were too short, mapping was done using NextGenMap (Sedlazeck *et al.*, 2013). For strains where the insert size was smaller than double the read length, pear was used before mapping with bwa-mem2. The alignments were filtered to remove duplicates using PICARD tools (MarkDuplicatesWithMateCigar) (Institute, 2018), and low-quality reads (<30) were removed using samtools view (Danecek *et al.*, 2021).

Estimation of mutation rates from a MAL experiment

A reference pool (RefPool) was created by merging several libraries from the parthenogenetic strain *Panagrolaimus* sp. PS1159 and the hermaphrodite *Propanagrolaimus* sp. JU765. To ensure all quality scores were in Sanger encoding seqret (Madeira *et al.*, 2019) and fastqc (Andrews 2010) were used. Alignments from the different mutation accumulation lines (MAL) were merged using samtools merge as input for accuMUlate (Winter *et al.*, 2018), the tool implemented for mutation calling, along with the RefPool.

Putative mutations were filtered by coverage ranges, number of reads supporting said mutation, absence of mutant allele in other samples, absence of mutation in parental state, apparent mutation being caused by mismapped reads (Anderson-Darling test statistic), read pair successfully mapped to the reference genome (Fisher's exact test), the resulting candidates were manually curated using the Integrative Genomics Viewer (IGV) (Thorvaldsdottir *et al.*, 2013). The number of callable sites, sites covered by sequencing depth where *de novo* mutations could be called, for each MAL was estimated as the number of positions within the depth coverage of 10 and 50.

The mutation rates were obtained by dividing the number curated "true *de novo*" mutations by the total of callable sites using

$$\mu = \frac{\text{called mutations}}{\text{generations} \times \text{callable sites}}. \quad (1)$$

Confidence intervals for the estimated mutation rates were obtained using the Bayesian First Aid R package (Bååth, 2014).

Analysis of populations

Population parameters

Pileup files were created using samtools mpileup (Danecek *et al.*, 2021) for all individual strains and for groups of strains (asexual and sexual). For the grouped data, a sync file was obtained using mpileup2sync.jar from Popoolation2 (Kofler *et al.*, 2011b), which served as input for the estimation of the fixation index F_{ST} on non-overlapping 1kb windows using F_{ST}-sliding.pl.

Parameters Waterson's estimator (θ_w) and nucleotide diversity (π) were calculated using previously obtained pileup files as input for Variance-sliding.pl from Popoolation (Kofler

et al., 2011a), using the options –measure θ_w and –measure pi respectively. The effective population size was then obtained as (1) $4Ne=4\mu$ for the sexual populations and (2) $Ne=6$ for the asexual populations, where μ is the mutation rate estimated as described in the following section.

Plots for visualizing θ_w, π, F_{ST} and effective population size results were obtained using the R package ggplot2 (Wickham, 2016).

Phylogenetic gene network

Coordinates of the present orthologous genes were used to generate single gene bam files for each strain using samtools view. To obtain a consensus sequence for each gene, bcftools mpileup and bcftools call were used for variant calling. These served as input for GATK (FastaAlternateReferenceMake) (Auwera and O'Connor, 2020). A gene network was obtained for the consensus gene sequences of orthologues present in of all populations using the median network algorithm from Splitstree4 (Huson, 1998).

Supplementary Material

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Conflict of Interest

The authors declare no conflict of interest.

Data availability

Genome assemblies and sequencing data are deposited under Bioproject PRJNA374706 and are available through Wormbase.

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