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On the evolutionary dynamics of Adineta vaga (Bdelloidea)

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On the evolutionary dynamics of Adineta vaga (Bdelloidea)

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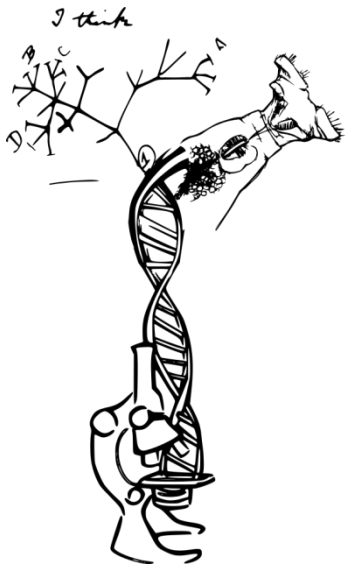

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Summary

Bdelloid rotifers are notorious for their long-term evolution in the absence of meiosis, which led John Maynard Smith to dub them “evolutionary scandals” as it seemed to contravene the general prevalence of sexual reproduction in the animal kingdom. Although bdelloids do not exchange genes via conventional sex, evidence is mounting for their ongoing accumulation of non-metazoan genes through horizontal gene transfers. These transfers have been linked to the capacity of bdelloid rotifers to withstand desiccation at any stage in their life cycle, as desiccation creates multiple DNA double-strand breaks that are repaired upon rehydration. Furthermore, desiccated individuals group together and are easily dispersed by wind over extended geographical ranges. To understand the consequences of asexuality and desiccation on the evolution of bdelloid rotifers, my PhD thesis addressed several questions investigating the evolutionary and ecological dynamics of the widespread bdelloid lineage *Adineta vaga*, for which a draft reference genome was already available.

First, by designing new genetic markers I detected, for the first time, apparent genetic exchanges among *Adineta* individuals sampled in the wild. I observed allele sharing that could be explained by inter-individual recombination among conspecific bdelloid individuals, as delimited using a tree-based approach as well as an allele sharing-based approach (haplowebs and a conspecificity matrix). Moreover, I detected incongruence in species boundaries inferred from distinct genetic markers, suggesting the occurrence of DNA transfers between species. These results were published in Current Biology and are presented in Chapter 1. A research group from Imperial College London challenged those results, and therefore I devoted Chapter 2 to discuss these criticisms and performed additional experiments to validate my observations, as presented in Chapter 3.

Second, I further investigated the possible mechanisms underlying the observed patterns of genetic exchanges by conducting genomic analyses of the regions (<10 kb) surrounding the genetic markers I had previously studied. This revealed intermixed signatures of DNA transfers, gene conversion and asexual evolution, highlighting the highly dynamic nature of the genome of bdelloid rotifers as presented in Chapter 1.

Third, the impact of various ecological parameters (dispersal, reproduction, survival, species interactions and environmental conditions) on the community structure of *Adineta* lineages was studied and presented in Chapter 4. A simple ecological model, combined with quantitative field data, was used to highlight the spatio-temporal dynamics of *Adineta* spp. natural communities. Overall, the model fitted empirical data well, showing that dispersal is the most important factor shaping bdelloid communities. Individuals disperse passively over long ranges, but the frequency of dispersal is correlated with geographical distance, *i.e.* there is more dispersal among close-by communities. In addition, I found that habitat preference varies among *Adineta* species and seems to have an effect on community dynamics. I also found that community dynamics vary with seasons: colonization and population expansion increase from spring to autumn, whereas strong bottlenecks happen in winter. Finally, very local sampling in the town of Namur revealed the dominance of one *Adineta* species in this area that is also present, albeit less abundantly, in other places around Belgium. The distribution of *Adineta* within Belgium is briefly presented in annex Chapter 5.

In this PhD thesis, I developed and applied new methods to study the evolutionary and ecological dynamics of bdelloid rotifer individuals from the genus *Adineta*, from genomes to natural communities. Overall, my results and published data suggest that despite their asexuality, the evolution of bdelloid rotifers reveals apparent genome diversification through chromosomal re-arrangements and DNA transfers; genome homogenization through gene conversion; community diversification through passive dispersal; and habitat specialization. Desiccation may play an important role in all these processes, reinforcing its potential impact on the evolution of bdelloid rotifers. Yet, additional studies quantifying the exact consequences of desiccation at different levels (from genes to communities) will be necessary to resolve the mystery of these "evolutionary scandals".

Résumé

Les rotifères bdelloïdes sont connus pour leur diversification et persistence durant plusieurs millions d'années en l'absence de méiose, ce qui conduit John Maynard Smith à les qualifier de « scandales de l'évolution ». En effet, cela semble aller à l'encontre de la tendance générale qu'ont les espèces animales à se reproduire de manière sexuée. Bien que les bdelloïdes n'échangent pas de matériel génétique via une reproduction sexuée conventionnelle, de nombreuses preuves se sont accumulées quant à leur capacité à intégrer des gènes d'origines non-métazoaires via des transferts horizontaux. Ces transferts ont été corrélés à l'aptitude qu'ont les rotifères bdelloïdes à tolérer la dessiccation, et ce, à n'importe quel stade de leur cycle de vie. Lors de la dessiccation, l'ADN est fragmenté par de multiples cassures double-brins qui sont ensuite réparées lors de la réhydratation. De plus, les individus desséchés ont tendance à se regrouper et semblent facilement dispersés par le vent sur de grandes distances géographiques. Pour comprendre les conséquences de l'asexualité et de la dessiccation sur l'évolution des rotifères bdelloïdes, j'ai lors de cette thèse essayé de répondre à plusieurs questions concernant les dynamiques génétiques et écologiques d'*Adineta vaga*, espèce pour laquelle un génome de référence est disponible.

Premièrement, en développant de nouveaux marqueurs génétiques, j'ai détecté pour la première fois des échanges génétiques entre individus du genre *Adineta* échantillonnés dans la nature. J'ai observé des cas de transfert d'ADN ne pouvant s'expliquer que par des recombinaisons entre individus appartenant à une même espèce. Ces espèces ont été délimitées selon différentes approches basées sur de la phylogénie ou le partage d'allèles (haplowebs et matrices de conspécificité). De plus, j'ai pu détecter des incongruences dans l'assignation de certains individus à une espèce en fonction des différents marqueurs, ceci suggérant des transferts d'ADN entre espèces. Ces résultats furent publiés dans la revue *Current Biology* et sont présentés dans le premier chapitre de cette thèse. Un groupe de recherches de l'Imperial College de Londres ayant remis en doute ces résultats, j'ai discuté en plus amples détails ces critiques dans le Chapitre 2. J'ai également mis au point une nouvelle expérience visant à valider ces observations et dont les résultats sont présentés dans le troisième chapitre.

Deuxièmement, j'ai exploré plus en profondeur les possibles mécanismes expliquant les cas d'échanges génétiques observés en réalisant une analyse génomique des régions (<10kb) bordant les marqueurs génétiques utilisés précédemment. Ceci a permis de révéler différentes signatures de transferts d'ADN, de conversion génique et d'évolution asexuée, démontrant la nature très dynamique du génome des rotifères bdelloïdes comme indiqué dans le Chapitre 1.

Troisièmement, un modèle écologique simple basé sur différents paramètres (dispersion, reproduction, survie, interactions inter-espèces et conditions environnementales), combiné à des données quantitatives collectées sur le terrain, a été utilisé pour comprendre la dynamique spatio-temporelle des communautés d'*Adineta* spp. (Chapitre 4). En général, le modèle collait bien aux données empiriques, démontrant que la dispersion est le facteur le plus important pour expliquer la structure des communautés de rotifères bdelloïdes. Les individus sont dispersés passivement sur de longues distances, mais la fréquence à laquelle ils immigrent dans un patch donné est corrélé à la distance géographique, c'est-à-dire qu'il y a plus de dispersion entre des communautés locaisées

dans des régions proches. De plus, il apparaît que les différentes espèces d'*Adineta* ont des préférences distinctes en matière d'habitat. Les saisons semblent aussi jouer un rôle dans la dynamique des communautés de rotifères : la colonisation de nouveaux habitats et l'expansion des populations étant plus fortes du printemps à l'automne, alors que des « bottlenecks » important ont lieu en hiver. Pour finir, notre échantillonnage concentré sur Namur a permis de révéler la dominance d'une espèce d'*Adineta* dans cette région, alors qu'elle a été retrouvée mais en moins grande abondance dans d'autres endroits de Belgique. La distribution d'*Adineta* à travers la Belgique est présentée dans le Chapitre 5 (annexe).

Durant cette thèse, j'ai développé et appliqué de nouvelles méthodes visant à étudier les dynamiques évolutives et écologiques du genre *Adineta*, de l'échelle du génome à celle des communautés. Au final, mes résultats et données publiées suggèrent qu'en dépit de leur asexualité, l'évolution des rotifères bdelloïdes est caractérisée par une diversification du génome via réarrangements des chromosomiques et transferts d'ADN ; une homogénéisation du génome lors d'événements de conversion génique ; une diversification des communautés par la dispersion passive et la spécialisation à l'habitat. La dessiccation pourrait jouer un rôle important dans ces différents processus, ce qui renforcerait son potentiel impact sur l'évolution des bdelloïdes. Cependant, des études supplémentaires permettant de quantifier les conséquences exactes de la dessiccation aux différents niveaux (des gènes aux communautés) seront nécessaires pour résoudre le mystère de ces « scandales de l'évolution ».

Acknowledgments

First, I would like to thank the jury members, Prof. Y. Michalakis, Dr. D. Fontaneto and Prof. F. De Laender for their detailed reading of the thesis and constructive remarks. Their expertise in the field of evolutionary biology, bdelloid rotifers and ecology contributed to improve the manuscript a lot and enabled interesting and inspiring discussions during the PhD thesis defense. I would especially like to thank Diego and Frederik for their important help in writing Chapters 1 and 5, and Chapter 4, respectively. Without their contribution, those chapters would not have been as accomplished and consistent as I would have expected.

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Preface

"There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved."

Charles Darwin's closing sentence "On the Origin of Species" (1859).

"After sleeping through a hundred million centuries we have finally opened our eyes on a sumptuous planet, sparkling with colour, bountiful with life. Within decades we must close our eyes again. Isn't it a noble, an enlightened way of spending our brief time in the sun, to work at understanding the universe and how we have come to wake up in it? This is how I answer when I am asked -- as I am surprisingly often -- why I bother to get up in the mornings. To put it the other way round, isn't it sad to go to your grave without ever wondering why you were born? Who, with such a thought, would not spring from bed, eager to resume discovering the world and rejoicing to be a part of it?"

Richard Dawkins, "Unweaving the Rainbow" (2000)

"Nothing in biology makes sense except in the light of Evolution"

Theodosius Dobzhansky (1973)

I have always been intrigued by the evolutionary processes that, amongst the infinite number of possibilities, drove Nature to be shaped as we can currently contemplate it in all its magnificence. Yet, of all the evolutionary biology lectures I have attended, of all the evolution congresses I participated in and of all the scientific discussions I took part in, no topic tickled my curiosity more than the evolution of sex and its wide cohort of fascinating and puzzling consequences. Genes, genomic structures, organisms, life cycles, diversity and geographical distribution all play a role in this long and still unsolved mystery. Passionate thoughts started with Charles Darwin, who already questioned the role of sexuality, and vigorous discussions arose for the last two decades with the advent of molecular biology. Why sex? Finding a partner, fertilizing an egg and joining two genomes consist in a complicated and costly path when more straightforward routes are available. To understand the emergence and prevalence of sexual reproduction across higher eukaryotes, most researchers have tried to sort the pros and cons out of the several reproductive mechanisms developed by metazoans. Comparing distinct mechanisms, and by extend distinct species, to point out common key parameters that may answer the question of the prevalence of sex is the basis of scientific reflection. In the same vein, scientists have started to tackle this issue the other way around by asking "If sex is widespread in eukaryotes, it must confer selective advantages. Then do asexual species arise and persist, and how?" The emergence of new tools in the 21st century enables tracking hidden signs of sex in supposedly asexual organisms or to model conditions under which sex can theoretically arise. In this thesis, a wide variety of approaches was used to study a peculiar group of ancient asexual animals, the bdelloid rotifers, hopefully bringing new insights into the question of sex or the question of how asexuals persist in the long term.

General introduction

"Nor do we know why nature should thus strive after the intercrossing of distinct individuals. We do not even in the least know the final cause of sexuality; why new beings should be produced by the union of the two sexual elements, instead of by a process of parthenogenesis… The whole subject is as yet hidden in darkness"

Charles Darwin (1862)

"Sex is the queen of problems in evolutionary biology. Perhaps no other natural phenomenon has aroused so much interest; certainly none has sowed as much confusion."

Graham A. C. Bell (1982)

"… if there is one event in the whole evolutionary sequence at which my own mind lets my awe still overcome my instinct to analyse, and where I might concede that there may be a difficulty in seeing a Darwinian gradualism hold sway throughout almost all, it is this event – the initiation of meiosis."

W. J. Hamilton (1999)

Why did sex appear in eukaryotes whereas prokaryotes successfully evolved asexually over more than 3 billion years? How sex is maintained despite heavy consequences at genetic, cellular, organismal and population levels? To better understand the evolution of sexual reproduction, we have to review the phenomenon of natural selection. The first part of this introduction will aim at giving a broad overview of how natural selection counter-balances the strong costs linked to sex relative to the benefits of being asexual. I will then develop the potential evolutionary mechanisms involved in the transition from sexual organisms to obligate asexuals. Finally, I will describe groups of organisms that evolved and diversified in spite of strict asexual reproduction which will lead us to the main goal of my thesis: how the bdelloid rotifers got the notorious status of ancient asexuals, from genome-level dynamics to community-level dynamics.

The emergence of sexual reproduction as main eukaryotic driver for evolution

Sexual reproduction and the cost of sex

Although the reproductive mode is still obscure in many species, especially in the poorly studied protists, researchers estimate that more than 99.99% of eukaryotes reproduce sexually (Otto, 2008; Schurko et al, 2009; Speijer et al, 2015). The conservation of meiotic mechanisms among the wide range of taxa reproducing sexually incited scientists to accept that sex has probably evolved once at the origin of eukaryotes approximately 2 billion years ago (Cavalier-Smith, 2002; Zimmer, 2009; Goodenough and Heitman, 2014; Lenormand et al, 2016). Sex diversified into several variants involving distinct mechanisms from molecular to organismal levels but the most simple and

generalized definition of sexual reproduction is the ploidy-number reduction (homologous genome separation) through meiosis (Figure 1a) followed by fertilization. This led to two major evolutionary consequences: genders and recombination (Bell, 1982; Bengtsson, 2009). Gender is the consequence of a divergence between the gametes resulting from meiosis, specializing into male (many small and motile cells) and female gametes (few, large and nutrient-rich cells) (Bell, 1982; Lehtonen and Kokko, 2011). This divergence, called anisogamy, often extends back to the whole organism that produces them *e.g.* males and females that are morphologically distinct in many taxa. Recombination is the production of new combinations of genes resulting from segregation and crossing overs. The latter is the consequence of homologous chromosome pairing which is mediated by the induction of numerous DNA double-strand breaks (DSBs) and the chiasmata formation (Figure 1b; Zickler and Kleckner, 2015). As stated by Mendel's second law, pair of genes segregate and reassort independently into gametes during meiosis (as long as the genes considered are sufficiently distant to enable crossing-overs). Fertilization between gametes of sexually reproducing individuals also enables new allelic associations, eventually leading to some advantageous combinations. The diversity generated within the offspring resulting from sexual reproduction is the basis on which natural selection can act and enable evolution. Interestingly, even if meiosis is absent in bacteria and archaea, they can

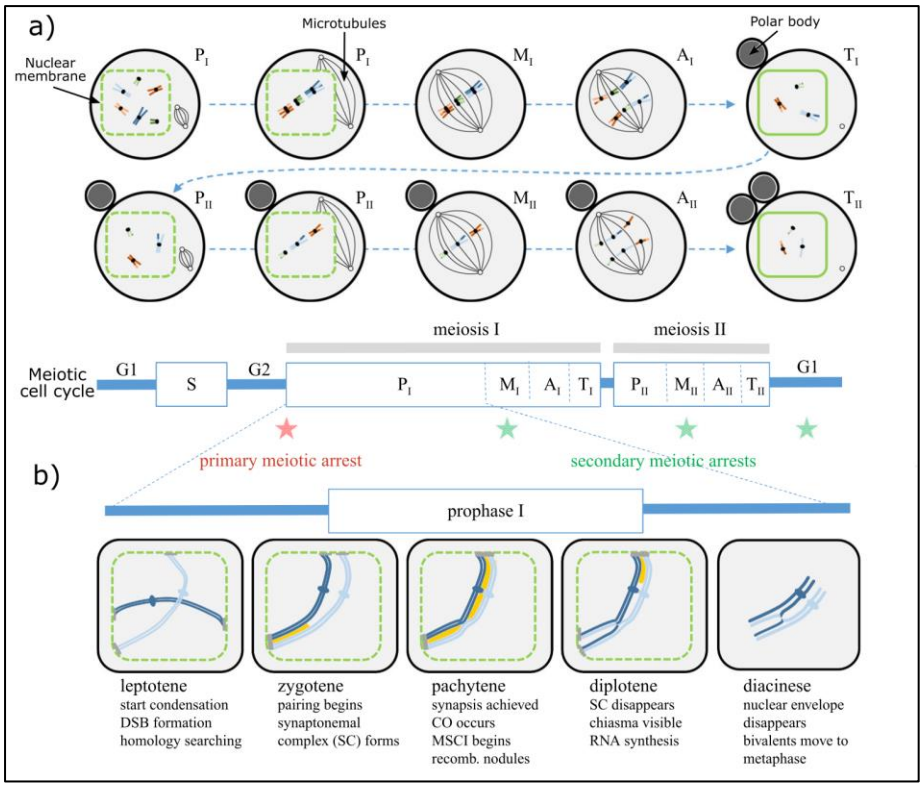

Figure 1 : Schematic representation of the different steps of meiosis (adapted from Lenormand et al, 2016). The top panel illustrates the different phases of a canonical female meiosis for each of the two meiotic divisions: prophase (P, with early and late prophase distinguished), metaphase (M), anaphase (A) and telophase (T). The nuclear membrane is indicated by the green contour (dashed when it starts fragmenting). The small black circles represent microtubule organizing centres and the black lines represent microtubules of the meiotic spindle. First and second polar bodies are shown as grey circles next to the oocyte (chromosomes inside the polar bodies are not shown). Homologous chromosomes are represented with the same colour with slightly different shades (e.g. orange and light orange). Homologous pairs segregate in meiosis I (reductional), then sister chromatids segregate in meiosis II (equational). The middle panel shows the meiotic cell cycle. The timing of the primary meiotic arrest is indicated by a red star, while the timing of the most common secondary arrests in different organisms is indicated by green stars. The lower panel indicates the important steps (DSB formation, crossing overs) occurring during prophase I. The synaptonemal complex is shown in yellow. Chromatin condenses in chromosomes throughout prophase I (only one pair of homologues is illustrated). In most species, telomeres attach to the nuclear envelope. The attachment plate is indicated by a grey bar. MSCI, meiotic sex chromosome inactivation.

occasionally transfer genes (conjugation, transduction and transformation) suggesting that recombination may be essential for their long-term survival.

However, the ubiquity of sex is puzzling when considering the evolutionary costs it implies. First, gender represents a considerable energetic investment in the development of new morphological traits and sex-related behavior (Andersson, 1994). Gender distinction often results in additional evolutionary constraints that do not apply to asexuals, such as sexual selection. Sexual selection was already recognized by Darwin and results in the development of traits giving nature much of its beauty. Peacock males grow a colorful tail for female attraction, fireflies flash through the night to find a mate and plants produce specific perfumes to lure insects that will carry pollen to partners. As a consequence, one gender (most often the male) will invest energy into traits that attract the opposite gender, but which may be detrimental in the context of resource competition for example. Indeed, many of the sexual selected traits turn out to be prejudicial in the strict concept of survival and competition. The peacocks cumbersome tail interferes with its escape from predators, the fireflies flashes signal its presence to insectivores, and plants need to invest energy in the production of complex aromatic molecules. Second, if natural selection is the prevalent force driving evolution, then sex continuously disrupts advantageous gene combinations that have been selected and maintained over generations (Charlesworth and Barton, 1996; Otto, 2009). In other words, it could be risky for an organism well adapted to its environment to mix its genome with another, eventually less adapted individual.

George Williams and John Maynard Smith first pointed out the theoretical disadvantages of sexual reproduction relative to asexual reproduction (Maynard Smith, 1971; Williams, 1971), being qualified as "the twofold cost of sex" (Maynard Smith, 1978). This notorious hypothesis refers to the fact that in sexual reproduction, the unit is the couple (except in a few cases such as hermaphroditism) whereas in asexual organisms the unit is the individual. This means that unless the number of surviving offspring is twice higher in the sexually reproducing couple, the asexual individual will have a better reproductive output per capita (Figure 2). As a result, a sexually reproducing species should rapidly be outcompeted by mutants slowly shifting to asexuality that can randomly appear in the population (Doncaster et al, 2000). This is the case in the fish genus *Poeciliopsis* for which it has been demonstrated that in homogenous stream habitats, clonal lineages deriving from hybridization could eclipse their sexual relatives (Vrijenhoek and Pfeiler, 1997).

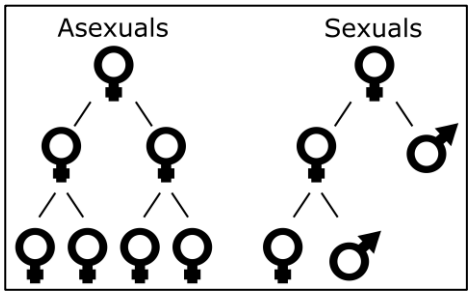

Figure 2 : Representation of the "twofold cost of sex" considering a similar fecundity for both the asexual and the sexual lineages. In the asexual lineage, the female produces two daughters that can then reproduce by cloning. In the sexual lineage, crossing between a male and a female is required leading to a reproductive output twice lower.

In addition to those major costs, sexual reproduction opens new gates for diverse risks linked to frequent outcrossing. Sex facilitated the development and spreading of parasites infecting sexual organs resulting into sexually transmitted diseases. Similarly, since sexual reproduction enables frequent genomic recombination, transposable elements (TEs) propagate among the entire sexual population while it remains clustered within the asexual individuals carrying them and their progeny (Hickey, 1982; Arkhipova and Meselson, 2000). The accumulation of TEs in non-recombining genome portions can be extremely quick as the *Drosophila miranda* neo-Y chromosome accumulated a 19-

fold more TEs in <1 My relative to its homologous neo-X chromosome (Bachtrog et al, 2008). This could be detrimental as those elements may eventually disrupt essential gene functions (Arkhipova and Meselson, 2005; Bast et al, 2015). Moreover, populations reproducing sexually have a reduced capacity to colonize new habitats compared to asexuals since it requires the simultaneous immigration of one male and one female (or a gravid female) within the newly colonized area instead of a single asexual female. Finally, mathematical models have demonstrated that by speeding up the speciation rate, sex could lead to lower biodiversity in some conditions (Meliàn et al, 2012). Indeed, simulations showed that frequent speciation resulted in many species with low abundance, those being more sensitive to species interactions which strongly influenced the extinction dynamics.

The origin of sexual reproduction

Despite all those disadvantages, sexual reproduction is present in almost all eukaryotic clades with extremely rare cases of clades containing distinct long-term obligate parthenogenetic species, *i.e.* the production of a progeny in absence of fertilization ('virgin birth' in Greek). The frequency of sexual reproduction across eukaryotic taxa and the conservation of meiotic mechanisms led to the hypothesis that reductional cell division appeared early in the evolution of eukaryotes (Hamilton, 1999; Cavalier-Smith, 2002; Goodenough and Heitman, 2014, Lenormand et al, 2016). Several hypotheses have been put forward on how this transition happened, but one of the best supported hypotheses argues that a primitive meiosis (often named 'proto-meiosis') took place long before true sex (Wilkins and Holliday, 2009; Zimmer, 2009; Lenormand et al, 2016). Proto-meiosis probably arose as a mechanism to return to haploidy in organisms in which higher ploidy was induced by haploid proto-eukaryote cell-cell fusion (parasexuality) or endoreplication (Lenormand et al, 2016).

Indeed, the presence of an additional set of chromosomes without molecular monitoring by the cell, *i.e.* homologous chromosome pairing, may have promoted the possibility for deadly ectopic recombination (Coop and Przeworski, 2007; Carvalho and Lupski, 2016) between DNA fragment presenting micro-homologies. In this context, meiotic chromosome synapsis offers a non-negligible novelty to pair homologs tightly and favor accurate recombination (Wilkins and Holliday, 2009).

With the evolution of meiosis and the underlying benefits, eukaryotes slowly shifted from a mostly haploid existence to a diploid life with episodic chromosome number reduction (haploid) necessary for sexual reproduction (Zimmer, 2009; Goodenough and Heitman, 2014). Yet, the two-fold cost of sex had to be overcome to explain its evolutionary maintenance.

One hypothesis is that during periods of stress, rare sexually reproducing individuals emerged within asexual populations as it is observed in certain species. For example, sexuals can persist in a population of cyclical parthenogenetic monogononts when only a few individuals switch to sexual reproduction during stress conditions (Stelzer, 2011). Thus, genes responsible for sexual reproduction may have been transmitted from one generation to the other during periods of stress, especially in facultative asexuals (*i.e.* the alternating cycles of sexual and asexual reproduction) with low fitness, in order to segregate out of detrimental genomic backgrounds (Hadany and Otto, 2007; Hadany and Otto, 2009). Although it is now well admitted that sex arose in cyclical parthenogens, its maintenance and the evolution of obligate sexuality require additional explanations. Hadany and Beker (2007) demonstrated through a mathematical model that when some individuals are attractive enough to reproduce with a high amount of mates, they could have more offspring than simply by cloning themselves. Mutations favoring the sexiness of a few individuals may, thus, have promoted

the spreading of selfish sex genes in the population until asexual individuals eventually disappeared (Hadany and Beker, 2007; Kleiman and Hadany, 2015).

The benefits of sex

Why sex persists as the main reproductive mode among eukaryotes for approximately 2.0 billion years (Miyamoto and Fitch, 1996) is another question. Mutants able to reproduce clonally often emerge within obligate sexual populations (Schön et al, 2009; Schwander et al, 2010) and those clones, freed from the twofold cost of sex, ought to take over their sexual relatives. However, given their rarity within the metazoans, something must restrain the spreading and persistence in the long-term of asexuals. Extensive theoretical work has investigated the advantages of sexual reproduction despite its costs, some being outlined here, but it is not clear whether those advantages offset the twofold cost of sex.

First, sex is known to speed up adaptation by bringing favorable mutations together faster than in asexuals and thus to enable quicker responses to frequently changing environments (related to seasonality, resource depletion, crowding …) (Fisher, 1930; Müller, 1932). New beneficial mutations entering an asexual population have good chances to arise in distinct competing lineages, only one of which will fix in the population (Figure 3). In a sexual population beneficial mutation appearing in distinct lineages can be brought together through recombination, increasing the speed of adaptation (yellow dot on Figure 3). This has been tested using the baker's yeast *Saccharomyces cerevisiae,* with micro-evolution experiments demonstrating that sexual strains could fix and combine beneficial mutations more efficiently than asexual strains, resulting in an overall higher fitness (Goddard et al, 2005; Gray and Goddard, 2012; McDonald et al, 2016). In contrast, those studies could not find any difference in fitness between asexual and sexual strains under permissive conditions. Under these considerations, one might think that sex would be favoured in unpredictable environments because it maintains heterogeneity in the populations. Yet, it is only the case under certain conditions where the association between loci frequently changes (fluctuating epistasis). In example, if two loci determine the adaptation to two environmental factors (temperature and humidity) that do not correlate (cold always associated with wet conditions and hot with dry conditions), there is no epistasis and recombination does not make any difference. In contrast, if associations between those environmental conditions vary over time resulting in a fluctuating epistasis, recombination can be favored because loci association is more frequently disrupted (see Barton et al, 2007). Similarly, sexual lineages of the fish genus *Poeciliopsis* were shown to colonize a broader range of ecological niches than there asexual relatives (Vrijenhoek and Pfeiler, 1997). The more genetically diversified sexual *Poeciliopsis* lineage presented a higher average fitness overall than the asexual lineages but a lower fitness for a given niche (Figure 4). In this example, sex is advantageous because recombination tends to maintain populations at linkage equilibrium.

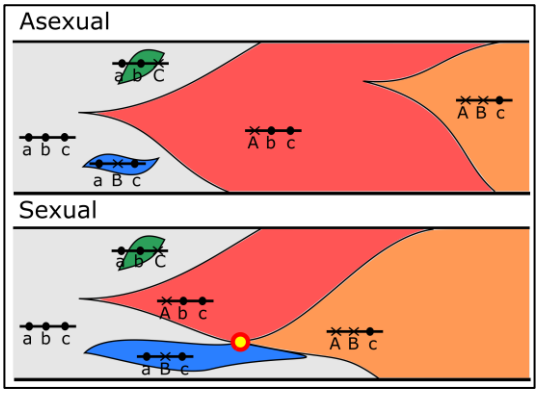

Figure 3 : Favourable mutations arise independently in distinct asexual lineages. For example, A being advantageous over a, B over b and C over c. In the asexual lineage they have to accumulate one after the other in the same genome. In the Sexual population, favourable mutations found at different loci (a, b and c) can be combined rapidly through recombination (yellow dot).

Second, sexual reproduction is a powerful mechanism to purge the genome from harmful mutations. Natural selection eliminates less efficiently mutations if they are slightly deleterious (Lynch and Gabriel, 1990). If not lethal, deleterious mutations can fix in the population when they are linked to selected advantageous ones, a process known as genetic hitchhiking (Maynard Smith and Haigh, 1974; Rice, 1987). Inversely, beneficial mutations can be eliminated from the population when they are associated to unfavorable ones *i.e.* background selection (Charlesworth et al, 1993). Those interactions among linked loci, named Hill-Robertson effect, are more pronounced in asexuals whereas meiotic recombination frequently disrupts associations between loci (Hill and Robertson, 1966; McVean and Charlesworth, 2000). As a result, mutations can fix and accumulate over generations in asexuals, a process known as "Muller's Ratchet", until permanently reducing the fitness and population expansion (Muller, 1932; Barton and Charlesworth, 1998). In contrast, homologous chromosomal recombination taking place during meiosis in sexual organisms can replace a defective version of a gene with the wild-type, recovering the ancestral beneficial genotype. In other words, sexual reproduction makes natural selection more efficient at sorting beneficial mutations from detrimental ones (McDonald et al, 2016). For example, both beneficial and detrimental mutations fixed in populations of asexually reproducing *S. cerevisiae* whereas only beneficial mutations could fix in out-crossing populations highlighting the role of recombination in decoupling advantageous mutation from their genetic background (Figure 5; McDonald et al, 2016). In a recent population genomic study, Lovell et al (2017) observed a higher mutation load (H_0 = 0.23) in asexuals than in sexual individuals (H_0 = 0.15) from sympatric populations, those deleterious mutations being more often present in the asexual genomes.

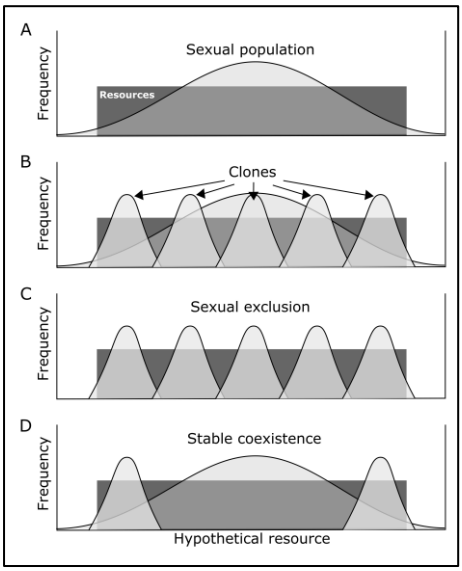

Figure 4 : Frozen Niche variation and asymmetric competition between new clones and their sexual progenitors. (A) A sexual population exhibits genetic variation for the utilization efficiency of a natural resource that is evenly distributed. (B) A range of genotypes is frozen among clones produced by the sexual ancestor. (C) Too high a rate of clonal formation will eclipse resource use by the sexual ancestor and lead to its extinction. (D) Natural selection rapidly eliminates clones that substantially overlap one-another and the centrally distributed sexual phenotypes, leading to stable coexistence if rate of clonal origins is not too high.

Third, sex turns out to be an efficient defense against the infection by parasites. Mathematical models indicated that host-parasite interactions resulted in their co-evolution, also known as the Red Queen hypothesis (Hamilton, 1980). In this context, asexuality is particularly disadvantageous as parasites able to infect the most frequent host strain will be favored (Koskella and Lively, 2009; Wolinska and Spaak, 2009). Meanwhile, sexual organisms can permanently produce new resistant strains that can

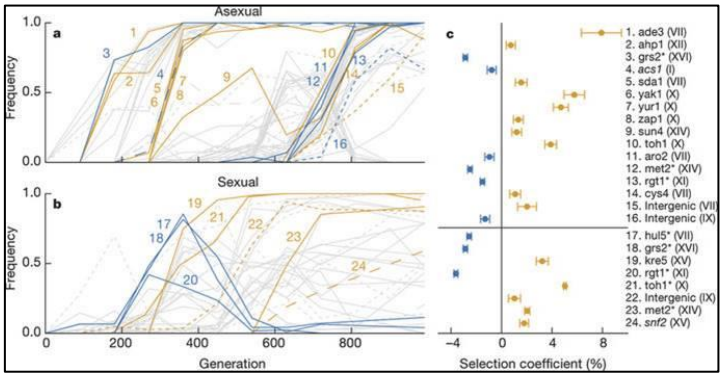

Figure 5 : Fitness effects of individual mutations (from McDonald et al, 2016). a) and b) Mutation trajectories in an asexual (a) and sexual (b) line. Orange mutations are significantly beneficial; blue are deleterious; gray are unmeasured or consistent with neutrality. c) Identities and fitness effects of significantly beneficial or deleterious mutations (chromosome number in parenthesis).

thus expand rapidly in the population until a parasite finally gets adapted to this strain. Those population boom-and-bust cycles were experimentally highlighted in the nematode *Caenorhabditis elegans* and its parasite *Serratia marcescens* interaction (Morran et al, 2011). In control conditions, reproduction by selfing was constantly more frequent than outcrossing in the *C. elegans* population. Inversely, outcrossing was preferred when *S. marcescens* was present and coevolution took place (Figure 6). Identical results were observed in natural conditions in the freshwater snail *Potamopyrgus antipodarum* in which sexual populations were generally less infected by the parasitic trematodes *Microphallus sp.* than the coexisting asexual populations (Vergara et al, 2014).

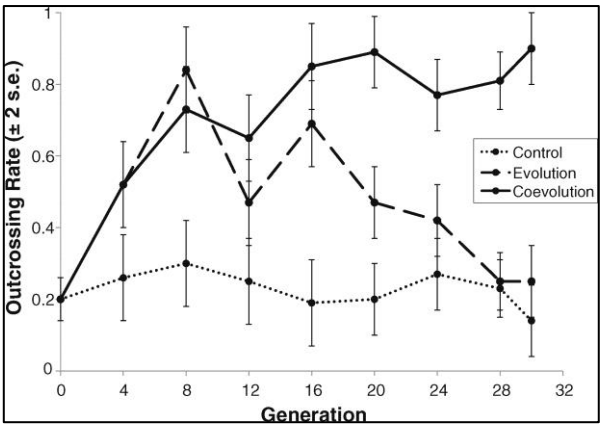

Figure 6 : Wild-type outcrossing rates over time (from Morran et al, 2011). Outcrossing rates in wild-type populations were not manipulated and free to evolve during the experiment. The wild-type populations were exposed to three different treatments: control (no *S. marcescens*; dotted line), evolution (fixed strain of *S. marcescens*; dashed line), and coevolution (coevolving *S. marcescens*; solid line) for 30 generations. Error bars, 2 SEM.

Finally, the two-fold cost endured by a sexual population can be counter-balanced by the access to a wider range of resources than its asexual competitor (Pound et al, 2002, Crummett et al, 2013). For example, asexual lineages of the fish genus *Poeciliopsis* were shown to co-exist with their sexual relatives under heterogeneous environmental conditions (Vrijenhoek and Pfeiler, 1997). In that example, clonal lineages derives from generalist sexual species but as a given genotype is inherited and possibilities for recombination are eliminated, the resulting parthenogenetic lineages became specialists (*i.e.* each clonal lineage has a subset of all the niches that were suitable for the sexual ancestor) (Vrijenhoek and Parker Jr, 2009). This evolutionary concept was described as the Frozen Niche-Variation (Figure 4; Vrijenhoek, 1979). Because intra-specific competition is stronger than competition between species (Chesson, 2000), the accumulation of asexual clones in a sexual population will substantially be slowed (Doncaster et al, 2000; Pound et al, 2002).

The emergence of asexual taxa in metazoan

New asexual lineages derive from sexual ancestors

The evolutionary advantage conferred by sexual reproduction is obvious when looking at the eukaryotic tree of life since most taxa contain species reproducing sexually. Asexual species are restricted to a few tips of the eukaryotic tree. However, many groups have not been investigated in detail yet (Figure 7; adapted from Speijer et al, 2015). The restricted distribution of asexual taxa despite the frequent apparition of asexual lineages among sexual populations suggests that asexuality is a short-term strategy relative to sex (Paland et al, 2005; Schurko et al, 2009; Neiman et al, 2009), that speciation is less frequent in asexuals and/or that they rapidly revert to sex (Schwander and Crespi, 2009). Indeed, it has been estimated that on average only 1 out of 1000 species is asexual (Vrijenhoek, 1998) and half of them are less than 500,000 years old and, thus, relatively young in comparison with the average age of a species, e.g. primate species are ≈4 My old (Neiman et al, 2009). However, parthenogenesis can be much more frequent in particular taxa as it

has been demonstrated in the hexapode *Aphytis* for which 38% of the described species are asexual (van der Kooi et al, 2017). Moreover, it is commonly admitted that all the described asexual taxa are deriving from sexual ancestors (Bell, 1982; Neiman et al, 2014). There are indeed several mechanisms involved in the transition to obligate parthenogenesis, such as infection by micro-organisms, interspecific hybridization and mutations in meiotic genes.

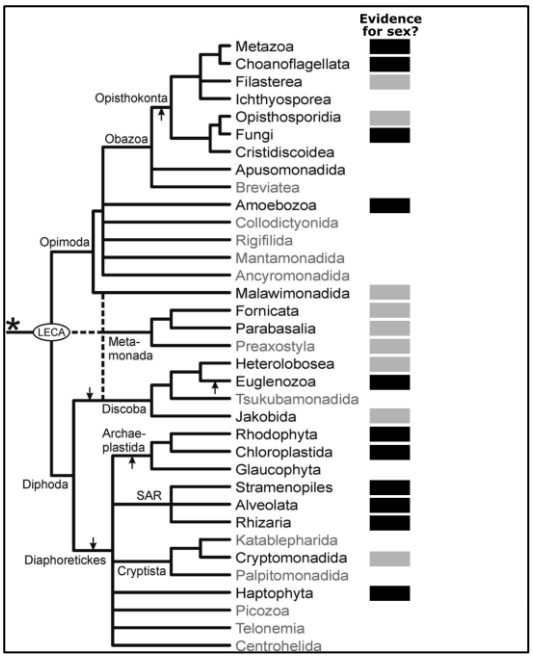

Figure 7 : Distribution of (meiotic) sex and selected sex-related features in eukaryotes (adapted from Speijer et al, 2015). The schematic phylogeny is a consensus of recent literature. *Root position (uptake of an α-proteobacterium at the origin of the eukaryotes); root positions suggested by other studies are indicated by small arrows. For each lineage, previously reported or assumed presence of sex is indicated: black boxes, well-documented sex; gray boxes, limited evidence for sex (rare direct observations, indirect inference from genomic data); no boxes, no published evidence. Absence of boxes does not directly imply absence in a lineage, especially for those given in gray, with limited (or absent) genome-scale sequence data.

Infection by endosymbiotic bacteria

Many cases of transitions from sex to parthenogenesis have been linked with the presence of intra-cellular endosymbiotic bacteria, especially in insects (reviewed in van der Kooi et al, 2017). The most notorious example is the interaction between *Wolbachia* and its parasitoid wasp host but surveys suggested that more than 30% (Duron et al, 2008) of sampled arthropods are infected by common endosymbiotic bacteria, *i.e. Wolbachia, Cardinium* and *Rickettsia* (Weeks and Breeuwer, 2001; Werren et al, 2008, Cordaux et al, 2011), that feminize the populations. The intra-cellular endosymbiotic bacteria are located within the host cells cytoplasm and, thus, can only be transmitted by the female to the next generation as only the nuclear content of male sperm is transmitted to the progeny. Thus, it is beneficial for the endosymbiont to favor the production of infected daughters in spite of sons in order to ensure its own transmission. In *Wolbachia*, this is achieved by various mechanisms such as the feminization of genetic males, the killing of male progeny from infected females, the induction of egg-sperm cytoplasmic incompatibilities and the development of unfertilized eggs i.e. parthenogenesis (Werren et al, 2008; van der Kooi and Schwander, 2014a). Interestingly, parthenogenetic females were demonstrated to be able to revert to sex once treated with antibiotics that removed their parthenogenesis-inducing endosymbiont (Stouthamer et al, 1990). Parthenogenesis induction by endosymbionts is tightly linked to haplodiploid species because, in this system, females can

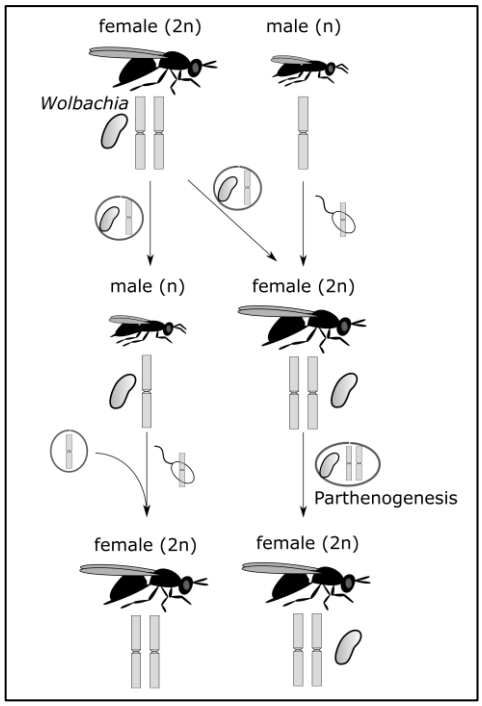

Figure 8 : Mechanisms responsible for the propagation of *Wolbachia* in haplodiploid species. As Wolbachia is dwelling in its host cytoplasm, it is transmitted to the next generation through female oocytes but not by male sperms (as only the nuclei content are transmitted). As a result, *Wolbachia* induces parthenogenesis in its host females in order to avoid the dead-end represented by males.

produce progeny without males (Figure 8; Cordaux et al, 2011, van der Kooi et al, 2017).

Hybridization of distinct species

Parthenogenesis may be retrieved when interspecific hybridization occurs as it has been observed in vertebrates (Neaves and Baumann, 2011). Around 80 taxa of asexual vertebrates have been described so far, specifically among fishes, amphibians and squamate reptiles (Kearney et al, 2009; Neaves and Baumann, 2011). In most known instances, asexuality has arisen through interspecific hybridization, resulting in a high heterozygosity level that may play a significant role in their persistence over thousands of generations (Brown et al, 1995; Ament-Velásquez et al, 2016). In the peculiar reproductive mode hybridogenesis, the reduced oocytes produced by the female contain only the genetic material that came from the mother lineage. When fertilized by a sperm of a male from another species (Figure 9a), the descendant carries both parental haploid genomes in the somatic cells, but only the maternal haploid non-recombinant set of chromosomes is transmitted to the next generation as the paternal genome is selectively excluded during oogenesis. This mode of reproduction is only viable when the unisexual (all-females) lineage is tightly ecologically associated with its sexual parent species (Lamatsch and Stöck, 2009). Because distinct species often contain rearranged chromosomes and disrupted synteny, hybrids often have an inefficient meiosis resulting in a low viability. Interspecific hybridization may therefore result in the development of alternative mechanisms to rescue viable egg production, such as obligate parthenogenesis (Schwander and Crespi, 2008). Gynogenesis, another mode of unisexual reproduction occurring in some fishes and amphibians, could have also led to obligate parthenogenesis. In this case, the embryogenesis of the unreduced oocyte produced by the female will only start its development when stimulated by the sperm of a related sexually-reproducing species (Figure 9b; Neaves and Baumann, 2011). In this process too, it is tempting to imagine that divergence leading to cytological incompatibilities between the oocyte and the sperm may impede the stimulus necessary for the embryo development and leave the female with no other option than go through obligate parthenogenesis, although this has, to our knowledge, not been documented yet. Finally, the emergence of asexual females from a sexual population can happen when erroneous meiotic divisions produce unreduced eggs that hatch spontaneously, a mechanism called tychoparthenogenesis (Schwander et al, 2010). In populations where males are rare, some females may fail to find a mate and produce daughters through a low rate of tychoparthenogenesis (Figure 9c). As a consequence, the biased sex-ratio increases and it has been hypothesized that this may lead to asexuality through a positive feedback loop as observed in some *Timema* stick insects (Schwander et al, 2010).

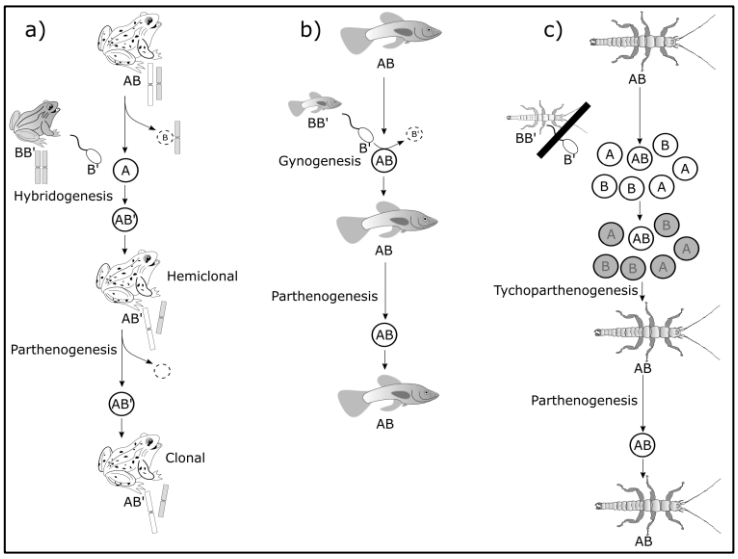

Figure 9 : Distinct reproductive mechanisms that may favour the induction of parthenogenetic lineages. a) Hybridogenesis, b) gynogenesis and c) tychoparthenogenesis.

Alteration of sex/asex cycles

Transition to obligate asexuality can be the consequence of spontaneous mutations in meiotic genes, particularly in facultative or cyclical asexual species that regularly switch from sexual to asexual reproduction (Serra and Snell, 2009; Stelzer et al, 2010). If the meiotic machinery is disrupted, those species can only reproduce through parthenogenesis. Facultative asexuality, the production of seeds both through sexual and asexual mechanisms, is widespread in plants. Cyclical parthenogenesis has essentially been observed in animals among which cladocerans, trematodes, cynipid insects and monogonont rotifers and it is the reproductive mode in which asexual generations alternate with sexual ones (Neiman et al, 2014). In both facultative and cyclical asexuality, switching from the asexual reproductive mode to sexuality has been associated with stress, sexual reproduction and the adaptive power of recombination predominating under unfavourable conditions. In those species, no novel adaptations are required as all the genetic and developmental machinery for parthenogenesis is constitutively present facilitating the transition to obligate asexuality (van der Kooi and Schwander, 2014b). Simple genetic changes could alter the pathways inducing sexual reproduction, for example, by shifting the threshold of the environmental signal triggering parthenogenesis to a level beyond natural range. Furthermore, in cyclical parthenogens recessive alleles of the genes responsible for obligate asexuality may evolve and remain undetected for several generations in heterozygous individuals until their frequency is high enough for homozygotes to appear in the population resulting in a transition to obligate asexuality (Stelzer et al, 2010). This has been observed in the monogonont rotifer *Brachionus calyciflorus* for which self-fertilization of heterozygous individuals produced obligate parthenogens at a ratio 1:3, the frequency of homozygous offspring. Stelzer et al (2010) hypothesized that this switch was probably due to a single locus *op* (obligate parthenogenesis) although they could not totally exclude the possibility for epistatic interactions among several unlinked loci.

The different types of asexuality and their consequences

Asexual taxa offer unique opportunities to understand how they survive without sex and to study the evolutionary consequences of the absence of sexual reproduction. In 2011, Neiman and Schwander listed distinct modes of asexual reproduction and how studying those cases could give

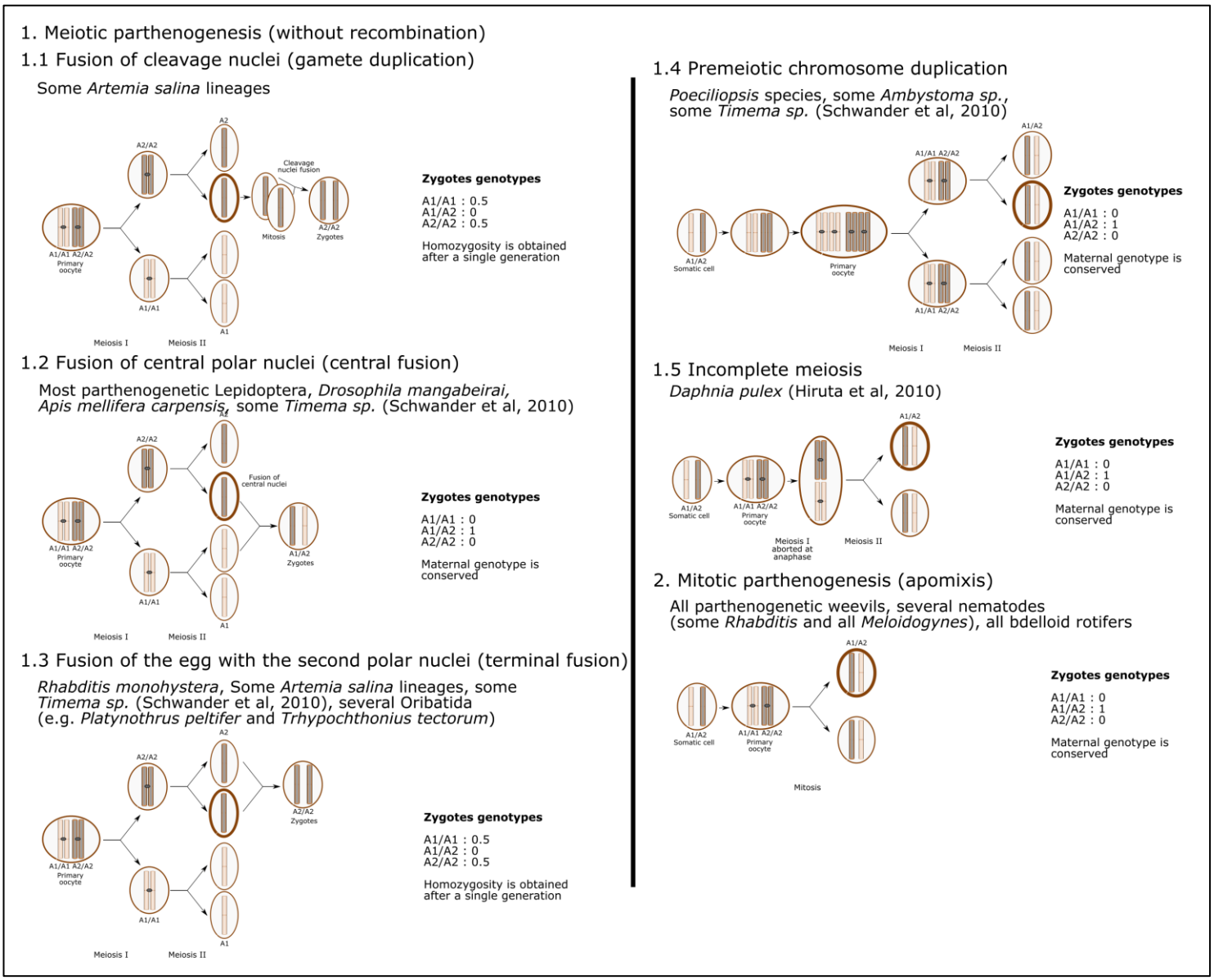

Figure 10 : The major parthenogenesis modes in animals and their consequences for the maintenance of heterozygosity and genetic variation among offspring (adapted from Neiman and Schwander, 2011).

insights in the prevalence of sex (Figure 10 adapted from Neiman and Schwander, 2011). First, comparing meiotic and mitotic parthenogens can provide information about the theoretical role of meiosis in limiting deleterious mutation accumulation. Indeed, because meiotic parthenogens maintain recombination and segregation, they should accumulate deleterious mutations slower than purely mitotic lineages (Haccou and Schneider, 2004). Second, if diversity is one of the major elements explaining the success of sex, assemblages comprising independently-derived asexual lineages should be more persistent than single-origin clonal lineages (Maynard Smith, 1978). Third, meiotic parthenogenesis can either be characterized by a rapid loss of heterozygosity (gamete duplication or terminal fusion) or heterozygosity excess (central fusion, premeiotic chromosome

duplication or incomplete meiosis) whereas mitotic parthenogens (apomixis) are expected to maintain heterozygosity (Figure 10; Neiman and Schwander, 2011). As a result, comparing different types of parthenogenesis could bring new insights into the evolutionary importance of heterozygosity. Similarly, asexual lineages originating from hybridization should present some advantages over non-hybrid parthenogens. Finally, evaluating the ploidy levels of old sexual lineages may be relevant as polyploidy confers a temporary protection against recessive deleterious alleles (Otto and Whitton, 2000; Selmecki et al, 2015).

Even though most of the asexual eukaryotic species currently described are relatively young (Neiman et al, 2009), a few groups of organisms have persisted and diversified in absence of sex for several tens of million years, namely the bdelloid rotifers (Mark Welch and Meselson, 2000; Flot et al, 2013; Tang et al, 2014), the darwinulid ostracods (Schön et al, 2009) and the oribatid mites (Domes et al, 2007; Laumann et al, 2007). Studying the different parthenogenetic lineages, their origin, the number of times they arose independently and their age may finally give a chance to put an end to the numerous discussions concerning the "implications of sex" that have appeared throughout the history of evolutionary biology (Table 1, adapted from Neiman et al, 2009).

Table 1 : Current estimates of asexual lineage age across a diverse set of eukaryotic taxa (adapted from Neiman et al, 2009).

Taxon(a)	Type	Age (thousands of years)	Origin(b)	No. of origins
Vertebrates				
Ambystoma	Salamander	<25	H	Single
Cnemidophorus	Lizard	<20	H	Multiple
Cobitis	Fish	<100-342	H	Multiple
Darevskia	Lizard	?	H	Multiple
Heteronotia	Lizard	<300	H	Multiple
Lacerta	Lizard	<5-200	H	Multiple
Menidia	Atherinid fish	?	H	Multiple
Poecilia	Poeciliid fish	<100	H	Multiple
Poeciliopsis	Poeciliid fish	<100-150	H	Multiple
Arthropods				
Aramigus	Weevil	?	H	Multiple
Artemia	Brine shrimp	3000	H?	?
Aspidiotus	Scale insect	1000	I?	Single
Bryobia	Phytophagous mite	?	I	Multiple
Calligrapha	Beetle	300-3100	H	Multiple
Daphnia	Water flea	<20-200	C	Multiple
Darwinulidae	Ostracod	<200000	?	Single?
Eucypris	Ostracod	<250-4000	S	Multiple
Heterocypris	Ostracod	<500-13000	S	Multiple
Oribatida	Mite	<200000	?	Multiple
Otiorhynchus	Weevil	?	H	Multiple
Rhopalosiphum	Aphid	"Recent"	C	Multiple
Timema	Stick insect	250-1500	H, S	Multiple
Warramaba	Grasshopper	?	H	Multiple
Molluscs				
Campeloma	Prosobranch snail	100-500	H, S	Multiple
Lasaea	Clam	5500-7600	H	Multiple
Potamopyrgus	Prosobranch snail	<40-1000	S	Multiple
Nematodes				
Meloidogyne	Nematode	40000	H, S	Multiple
Platyhelminthes				
Schmidtea	Flatworm	<500-1500	H, S?	Multiple
Rotifers				
Bdelloidea	Bdelloid rotifer	<100000	?	?

(a) All taxa are genera, except Bdelloidea (class), Oribatida (order) and Darwinulidae (Family)

(b) C = contagion; H = hybrid; I = infection, usually Wolbachia; S= spontaneous, usually autopolyploidization

Something of an evolutionary scandal: the bdelloid rotifers

"I formed the strong impression that the study of parthenogenesis is, at least, beginning to tell us something about the evolutionary significance of sex. Much remains to be done. The Bdelloids remain something of an evolutionary scandal. We need to know the age of at least some natural clones; the extent of genetic diversity that can arise within them; and whether deleterious mutations accumulate in them by the ratchet mechanism […]. The suggestion that sex is needed in the evolutionary race against parasites should be investigated."

J. Maynard Smith (1986)

In the present thesis, we studied the bdelloid rotifers (etymology: *bdella,* leech; *rota + ferre*, wheel-bearer), the most notorious "ancient asexuals" currently known within metazoans (Mark Welch and Meselson, 2000; Schön et al, 2009; Flot et al, 2013; Fontaneto and Barraclough, 2015). Bdelloid rotifers have apparently persisted without meiosis or males for 40-100 My according to fossil evidence and estimations from molecular data (Poinar and Ricci, 1992; Tang et al, 2014). The paradox of their long-term survival without meiosis or sex has long been recognized as "something of an evolutionary scandal" (Maynard Smith (1986). Nonetheless, those microscopic animals (100 µm to 1.5 mm) have diversified into hundreds of morphologically described species (Segers, 2007) colonizing different types of limno-terrestrial habitats in which temperature, humidity or salt content vary unpredictably (Ricci and Balsamo, 2000). More recently, studies using genetically-based species delimitation tools have highlighted that the bdelloid morpho-species *Rotaria rotatoria* and *Adineta vaga* were actually composed of several independently evolving entities refuting the hypothesis that sex is

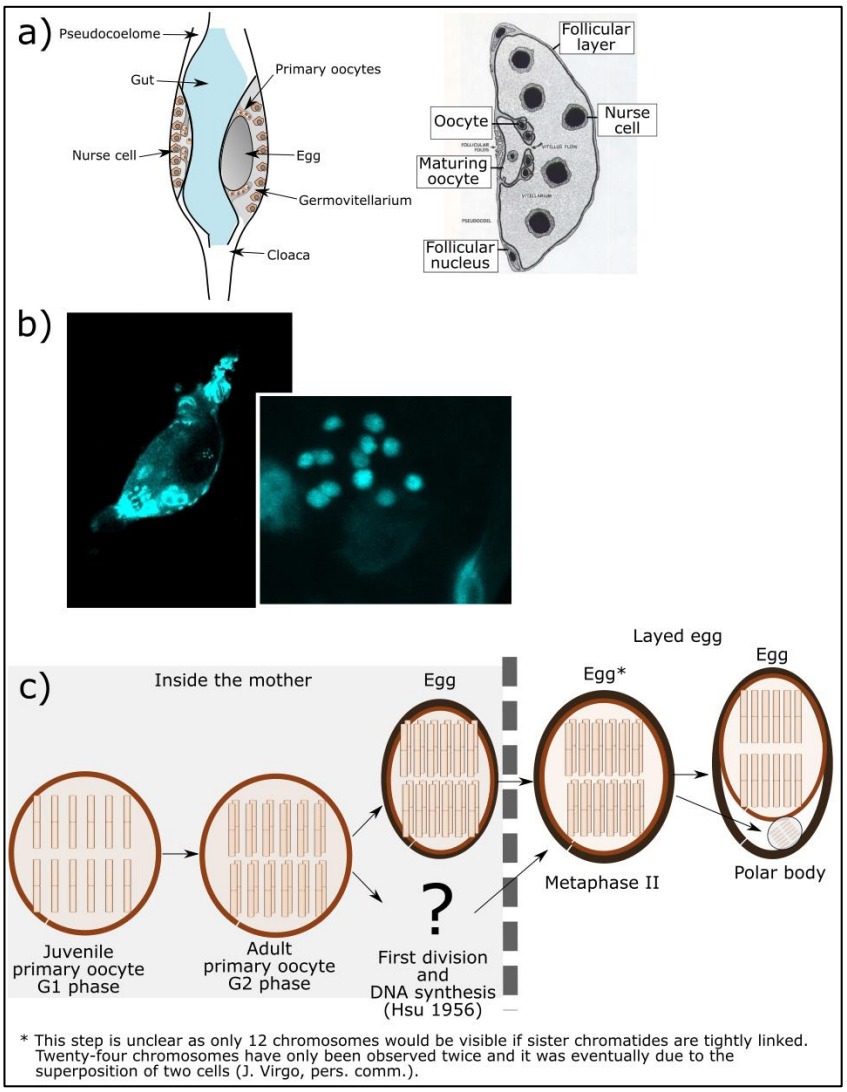

Figure 11 : Reproductive organs and oogenesis in bdelloid rotifers. a) Schematic and b) photograph of *A. vaga*'s germovitellarium showing its organization around the gut. c) Egg formation through the formation of two polar bodies as expected if apomixis derived from meiosis with the two reduction divisions conserved or d) with only one reduction division.

necessary for diversification into evolutionary species (Fontaneto et al, 2007; Fontaneto et al, 2008; Fontaneto and Barraclough, 2015).

Here, I will detail the distinct pieces of evidence for the asexuality of bdelloid rotifers. I will then describe their ecology and report studies proving the existence of species in bdelloids. Finally, I will develop some possible scenario for the origin of bdelloid from their sister clade monogononta.

Evidence for long-term asexuality

The most straightforward evidence of bdelloid asexuality is that males or at least male organs have never been reported since bdelloid were first observed almost 350 years ago (van Leeuwenhoek, 1677 and 1702), whereas females ovaries (two) are clearly distinguishable along the digestive system in all species (Figure 11a and b). Given the number of studies on bdelloid rotifers and the number of individuals observed since the invention of the microscope, Birky Jr (2010) estimated that, if bdelloid males exist, their frequency would approximately be 8.1×10^{-6}. This estimation takes into account the uncertain report of Wesenberg-Lund (1930) who wrote "*With great hesitation I venture to remark, that twice I saw among the thousands of Philodinidae a little creature, unquestionably a rotifer male […] both times I failed to get it isolated*", even though its description most likely corresponds to a male monogonont that happened to be in the same sample (Mark Welch et al, 2009). Bdelloid males existing or not, they are extremely rare and the frequency of sexual reproduction appears too low to exert an influence on the evolutionary processes of bdelloids.

In addition to the wide range of morphological data available, the asexual status of bdelloid rotifers was confirmed by two cytological studies that investigated the ploidy level during oogenesis (Hsu, 1956a and 1956b). In both *Philodina roseola* and *Habrotrocha tridens*, there are two phases of chromosome doubling and maturation divisions, after which one polar body (two in total) is extruded to restore the initial ploidy, but chromosome pairing was never observed (Hsu, 1956a and 1956b). Yet, those two steps of chromosome doubling have not been reported since then and do not seem to fit the observations of *Adineta vaga* oogenesis (M. Terwagne, pers. comm.). Another study on *Macrotrachela* sp. demonstrated that the total number of oocytes in the germarium was fixed in young individuals, with no new oocytes being produced during its life-cycle (eutely, *i.e.* fixed number of cells) (Figure 11a; Pagani et al, 1993). In mature individuals (2 days old after hatching) the chromosomes number in all the primary oocytes doubles and subsequently, mature oocytes form an egg, one at a time (Pagani et al, 1993). Flow cytometry studies on *A. vaga* demonstrated that oocytes presented twice the amount of DNA observed in somatic cells, indicating that oocytes were blocked in G2 phase until mitotic division in the egg (M. Terwagne, pers. comm.). Indeed, only 12 condensed chromosomes are visible in eggs blocked at metaphase within the mother (Figure 11b; J. Virgo, pers. comm.).

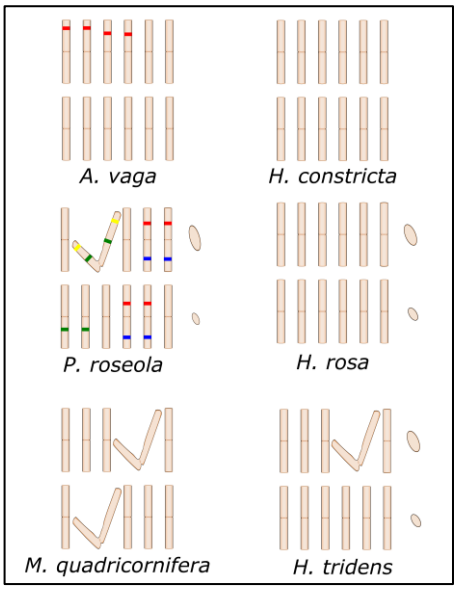

Figure 12 : Representation of the genomic organization of six bdelloid species adapted from Mark Welch et al, 2009. Most chromosomes have approximatively the same size and shape. In *P. roseola*, *M. quadricornifera* and *H. tridens*, one big chromosome is V-shaped. In some species, there are also additional "dot" chromosomes. A limited number of regions have been mapped to chromosomes by FISH: red = region around *hsp82* gene, blue = region around *Hox5*-like gene, green = region around *Hox6*-like gene, yellow = unsequenced YAC clone.

Intriguingly, one polar body is produced after this first mitotic division and is clearly visible in the egg. It is currently hard to tell if something occurs in the oocyte between the pause in G2 phase and the first mitotic division in the egg (Figure 11c). However, the occurrence of a first reduction division as depitcted by Hsu (1956a and b) seems impossible as the resulting progeny would be highly homozygous, which was never observed. Thus, our current view of *A. vaga* oogenesis is inconstistent with canonical meiosis, ruling out the possibility for sex. Interestingly, *P. roseola* and *H. tridens* harbor 13 chromosomes: ten indistinguishable ones, one twice bigger than others and two small "dot" chromosomes of unequal sizes (Mark Welch et al, 2004a). This odd number of chromosomes is incompatible with predictions that previous karyotypes corresponded to anaphases. It also suggested that aneuploidy or genomic rearrangement occurred in bdelloids which is incompatible with conventional meiosis too (Figure 12; Mark Welch et al, 2009).

The most direct evidence supporting the long-term absence of meiosis in bdelloid rotifers come from the peculiar structure of their genome. Chromosome staining by FISH (Fluorescent *In Situ* Hydridization) with allele-specific probes on *P. roseola* embryos indicated that genes are organized in quartets of four homologous regions that can be classified into two pairs of alleles (A1/A2 and B1/B2) (Mark Welch et al, 2004b). Within allelic pairs, the two gene copies are on average 3.8% different at the nucleotide level while the distance between ohnologous pairs (As vs Bs) is on average 26.4%, a structure qualified as 'degenerate tetraploidy' (Figure 13a and b, Mark Welch and Meselson, 2000; Mark Welch et al, 2004b; Mark Welch et al, 2008; Hur et al. 2009; Flot et al, 2013). The availability of the first bdelloid rotifer genome confirmed this degenerate tetraploidy, with ≈40% of the 49.300 genes being present in four copies within *Adineta vaga*, the remaining genes presenting lower ploidy levels (Flot et al, 2013). This degenerate tetraploid structure is likely explained by whole-genome duplication followed by the loss of several gene copies over generations and the divergence of ohnologous pairs in the absence of sex (Mark Welch et al, 2008; Flot et al, 2013).

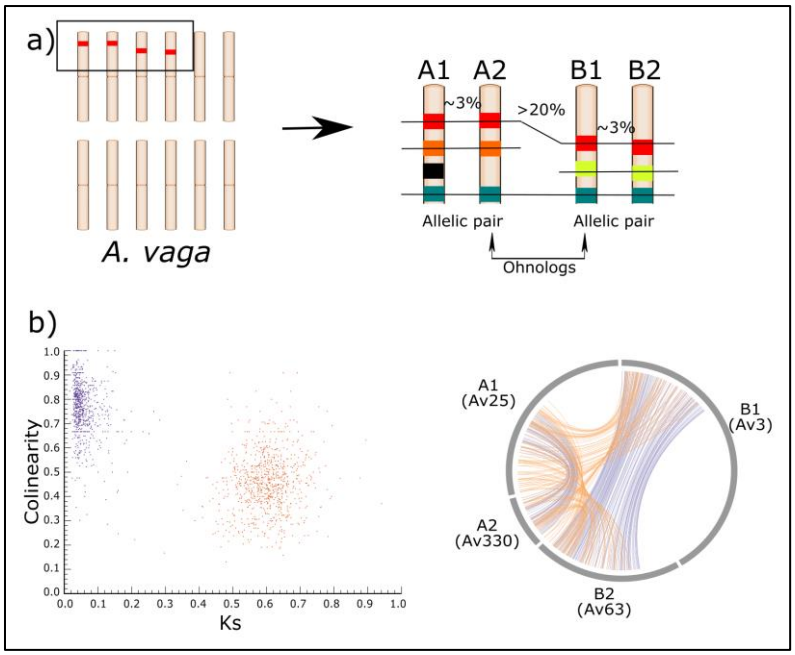

Figure 13 : Degenerate tetraploidy as observed in *A. vaga*. a) The genome is constituted of 12 chromosomes of similar size. b) Chromosomes are organized in quartet composed of two allelic pairs A1/A2 and B1/B2. The genetic distances and the collinearity is conserved within pairs (3% of nucleotide divergence and identical gene contents), but only partially between pairs (>20% of nucleotide divergence and gene re-arranged). c) In pairwise comparisons, allelic pairs are represented by high collinearity and low Ks (in purple) whereas ohnologs are characterized by a drop in collinearity and higher Ks (orange) (from Flot et al, 2013). d) Circos plot of four aligned scaffolds constituting a quartet (from Flot et al, 2013).

In addition, chromosome rearrangements were detected in the genome of *A. vaga*, while the karyotypes of *P. roseola* or *H. tridens* contain one big odd chromosome probably representing a chromosome fusion (Mark Welch and Meselson, 1998). Flot et al (2013) also presented evidence for

17 palindromic regions, *i.e.* regions where blocks of colinear genes (allelic copies) are present in reverse direction on the same chromosome and nowhere else in the genome, and 3 regions harboring direct allelic repeats (Figure 14a). Furthermore, homologous allelic blocks could be defined locally but do not appear to extend to the chromosome level, *i.e.* breakpoints in the collinearity are observed, the gene order being altered among chromosomes (Figure 14b; Flot et al, 2013). With such structure, it is impossible to have a conventional meiosis which requires the pairing of entire, homologous chromosomes and separate the genome into distinct haploid sets (Figure 14c). However, even if peculiar genomic structures have been observed in *A. vaga*, one has to confirm this in other bdelloid families to confirm the asexual status of all bdelloids.

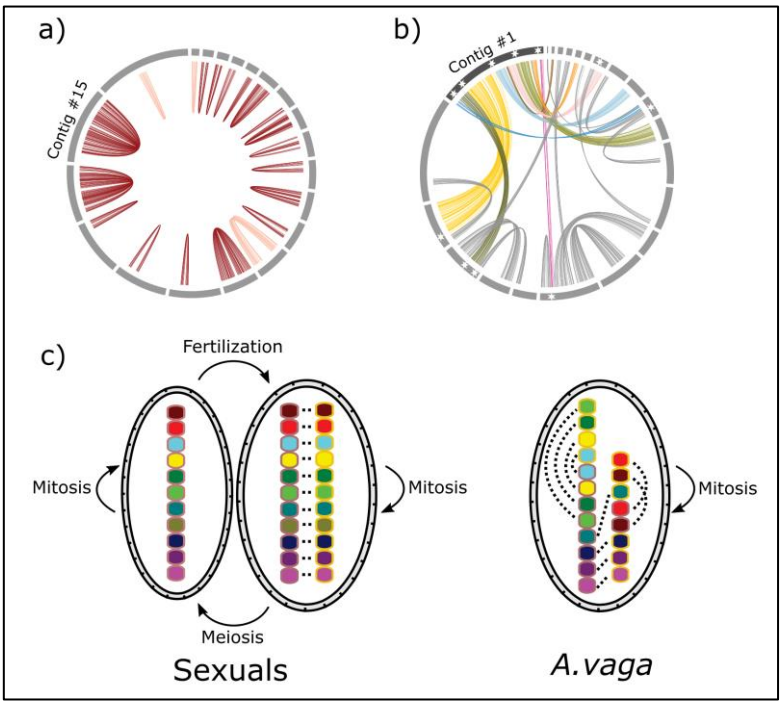

Figure 14 : *Adineta vaga* genome organization (Flot et al, 2013). a) Palindromic regions are found on 17 scaffolds (dark red) and direct repeats on 3 scaffolds (light red). b) Collinearity breakpoints (stars) indicate regions where homologies with a given scaffold stop highlighting the absence of homologies between chromosomes along their full-length. c) Those signatures (a and b) are incompatible with conventional meiosis as chromosomes organization does not allow for pairing, but does not impede mitosis.

Sex-related genes has also been investigated in *A. vaga* and in the transcriptome of *A. ricciae* with the hypothesis that missing key genes for sperm-production or meiosis would rule out the possibility of sex (Schurko and Logsdon, 2008; Flot et al, 2013).

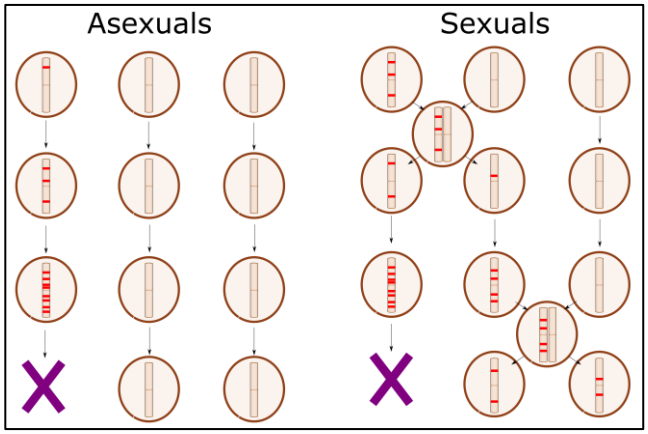

Figure 15 : Transposable elements (in red) can increase within an asexual lineage, but if they have deleterious effects on their host genome, lineages that carry them will eventually go extinct. In a sexual population, transposable elements can spread horizontally through the whole population.

Contrasting results have been obtained, some genes involved in male gametogenesis or meiosis are present in bdelloid rotifers (*Spo11*, *MutS*, *Msh4*, *Msh5 …)* but others are missing (*Msh3*, *Rad52* …) (Hanson et al, 2013; Flot et al, 2013). It is difficult to draw any conclusions based on the presence/absence of those genes since genes related to sex can be non-functional or have alternative functions, *e.g.* the meiosis-specific gene *Spo11* is present in *A. vaga* but its function of DSB creation could eventually be used in bdelloid rotifers to induce breaks when not desiccated, promoting DNA DSB repair and gene conversion (homogenizing the genome) (Flot et al, 2013). Desiccation is the ability to tolerate long periods of drought, often by entering a paused metabolic state named “tun” for

bdelloid rotifers (Ricci et al, 2007; Hespeels et al, 2014). It has been demonstrated that under desiccation residual water constituted only 6.5% of *A. vaga* weight and that DNA double-strands breaks accumulated over time (Hespeels et al, 2014). Inversely, bdelloid rotifers are able to efficiently repair their fragmented genome upon rehydration (Gladyshev and Meselson, 2008; Hespeels et al, 2014). Accurate pairing of homologous regions could be mediated by proteins originally involved in meiosis when DNA strand breaks are repaired using homologs as template (Mark Welch et al, 2008; Flot et al, 2013; Hespeels et al, 2014). Finally, the absence or the low frequency of several classes of transposable elements found in bdelloid rotifers compared with sexual species fits the prediction that asexual lineages should be purged of most TEs (Figure 15) or at least that bdelloids have a machinery inactivating them (Arkhipova and Meselson, 2000; Arkhipova and Meselson, 2005; Flot et al, 2013).

To conclude this part, the apparent absence of males and the peculiar genomic structure impeding traditional meiosis seem to support the long-term asexuality of bdelloid rotifers (Danchin et al, 2011; Fontaneto and Barraclough, 2015). Even though the presence of extremely rare males and an alternative meiotic mechanism could still be plausible (M. Meselson comment on Flot et al, 2013, available on *Nature* website), additional research needs to be conducted.

Asexuals, yet diversified

Throughout their asexual evolution, bdelloid rotifers have diverged into more than 460 morphospecies (Segers, 2007; Figure 16) which are all characterized by a small, elongated and transparent body (approximatively 1000 nuclei forming different syncitia, confirmed by FACS J. Virgo and M. Terwagne, pers. comm.) divided into three regions: the head, the trunk and the foot (Ricci and Balsamo, 2000). Most bdelloid species use the characteristic ciliated region on their head, namely the corona, to propel in the water column and feed by creating a flow that goes through their mastax, composed of two jaws, where organic debris, bacteria and algae are filtered and crushed. The digestive, excretory and reproductive organs are contained in the trunk, wrapped in the pseudocoelome. The foot, often ending in a few toes, is used for adhesion to the substratum by the secretion of a sticky compound. All those anatomical features can vary a lot in size and shape across species and have therefore been used by taxonomist for classification (Donner, 1965; Fontaneto et al, 2007a; Birky Jr et al, 2011). For example, in the Adinetidae family, the corona is located ventrally and used to scratch the substratum while sliding on the surface (Figure 16). Those

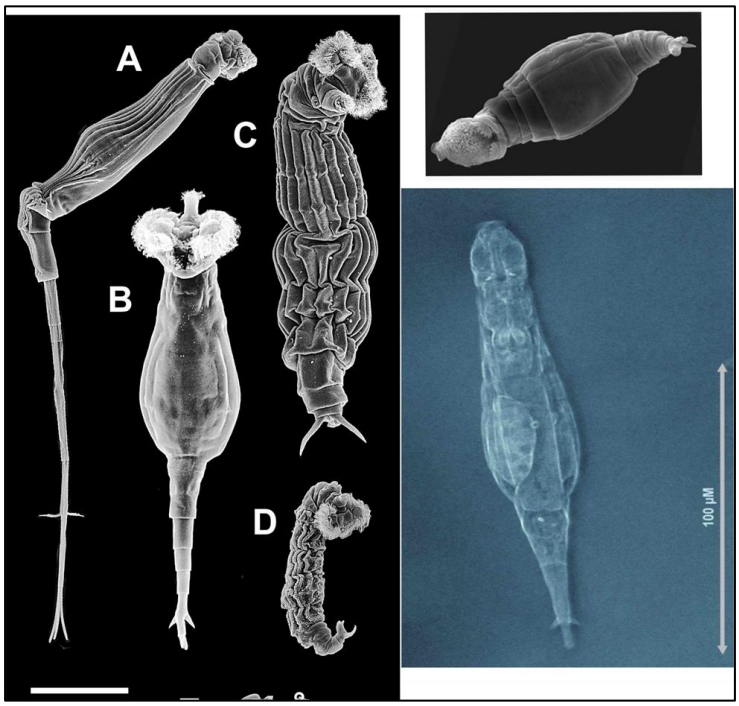

Figure 16 : SEM Pictures of Some Species of bdelloid rotifers (Fontaneto et al, 2007). (A) *Rotaria neptunia*, lateral view; (B) *Rotaria macrura*, ventral view; (C) *Rotaria tardigrada*, dorsal view; (D) *Rotaria sordida*, lateral view. Scale bars: 100 µm. (E) *Adineta vaga* (picture of G. Melone) and (F) *Adineta vaga* (picture of B. Hespeels).

different morphospecies present contrasting life-cycles (Ricci, 1983; Ricci and Caprioli, 2005) and ecological requirements (Fontaneto and Ambrosini, 2010; Fontaneto et al, 2011), reinforcing the designation of species within this clade, despite the sexual criterion underlying the species definition. More recently, DNA-based taxonomy have provided evidence for independently evolving entities even within morphological species (Fontaneto et al, 2007b; Birky Jr et al, 2011; Fontaneto, 2014, Tang et al, 2014). Those studies mainly used the mtDNA and ribosomal sequences to delimit genetic clusters in the genus *Rotaria* and *Adineta* revealing the existence of more species than described from morphology, *i.e.* cryptic species (Fontaneto et al, 2007b; Birky Jr et al, 2011). For example, more than 70 cryptic species were identified genetically within the species complex *Rotaria rotatoria* (Fontaneto, 2014).

In addition to the wide diversity of morphologies, bdelloid rotifers also vary in their ecology. Most species colonize freshwater environments over a wide range of temperatures and pH, *e.g.* Antarctic species withstand temperatures below -40°C and heating above 100°C (Radzikowski, 2013). Those freshwater species are particularly frequent in temporarily humid habitats (mosses, lichens, pools, birdbaths …) where populations can reach high densities, *e.g.* Fontaneto and colleagues (2011) counted 347 specimens in a 2.5cm² lichen patch. Their success in semi-terrestrial environments is due to their ability to survive alternating periods of desiccation-rehydration by entering a paused metabolic state at any stage of their life-cycle (Ricci et al, 2007; Hespeels et al, 2014). Desiccation, also known as anhydrobiosis, is widespread in nature with a variety of bacteria, plants and protists and able to tolerate it, suggesting its ancient acquisition probably to colonize land mass (reviewed in Rebecchi et al, 2007). However, in metazoans desiccation is rarer with only a few clades capable to whitstand it and only three phyla in which desiccation can occur at any stage of the life cycle: tardigrades, nematodes and bdelloid rotifers (Rebecchi et al, 2007). When desiccated, the resistance of bdelloid rotifers to extreme conditions is surprising with records of individuals tolerating exposure to liquid helium (-269°C), heating above 150°C, hyper-gravity (20G) or high doses of radiations (Ricci et al, 2005; Gladyshev and Meselson, 2008; Radzikowski, 2013; Hespeels et al, 2014). However, a few bdelloid species are exclusively aquatic living in streams or even in marine habitats. One of the best examples of ecological specialization described yet is the niche partition of two related *Rotaria* species, both living as epibiont on the crustacean *Asellus aquaticus,* but on distinct part of their host (Fontaneto and Ambrosini, 2010).

The evidence obtained by genetic and morphological methods to delimitate species within bdelloid rotifers questioned the role of sex in speciation (Fontaneto and Barraclough, 2015). It has been suggested that ecological and geographical isolation act as the main vectors for speciation in asexuals whereas reproductive isolation represents an additional parameter in sexual species.

How bdelloids avoided the fate of other asexual species

How did bdelloid rotifers avoid the fate of countless other species that go extinct when abandoning sex is not fully understood, but several bdelloid features may play an important role in their survival and diversification as asexuals? A first element is the absence of the expected Meselson effect in bdelloid rotifers (Mark Welch et al, 2008; Flot et al, 2013). Without meiotic recombination, homologous alleles should evolve independently, resulting in an increased level of heterozygosity between alleles (Mark Welch and Meselson, 2000). However, in bdelloid rotifers, the expected high divergence between alleles was not observed. It has been suggested that the observed signatures of gene conversion between alleles within the genome of *A. vaga* may play a role in preventing the

accumulation of mutations since colinear regions are used as template to repair the multiple DNA double-strand breaks (DSBs) apparent when desiccated for a prolonged period (Mark Welch et al, 2008; Gladyshev et al, 2008; Flot et al, 2013; Hespeels et al, 2014) (Figure 17). Frequent gene conversion should indeed slow down the accumulation of deleterious mutations that makes asexuality an evolutionary dead-end. Interestingly, meiosis is initiated by genetically programmed DSBs which can reach a surprisingly high number (de Massy, 2013), it would therefore be interesting to evaluate if the number of DSBs resulting from prolonged desiccation and meiosis are comparable in order to estimate if those mechanisms can result in a similar amount of gene conversion. Similarly, studying the exact role of genes present in the bdelloid genomes and coding for proteins involved in the ZMM pathway (*Msh4* and *Msh5*), a pro-crossover machinery, could eventually reveal mechanisms for homologous chromosome recombination without meiosis. Another interesting point to consider is the existence of bdelloid species living in permanently humid habitats and if those species present higher heterozygosity than frequently desiccated species. Alternatively, studying the role of *Spo11* in those non-desiccating species could reveal why this meiotic gene is conserved in bdelloid rotifers and its eventual role in generating DNA breaks to initiate gene conversion without desiccation. On the one hand, Birky Jr reports on his website (http://www.eebweb.arizona.edu/faculty/birky/BirkyLab.html) that isolated *A. vaga* individuals could only survive for 22 generations when kept hydrated but in the other hand going through cycles of desiccation-rehydration drastically reverse this fitness decline (Ricci et al, 2007) indicating that bdelloids not only tolerate such stresses but depend on it. Additionally, Brandt and co-authors (2017) recently showed that, in oribatid mites, large population sizes (≈ 350 000 individuals/m²) could efficiently reduce the impact of mutation accumulation that vow asexuals to extinction. This observation was mathematically demonstrated by the fact that huge effective population can relax the effect of genetic drift, natural selection remaining as the only prevalent force driving evolution in that case (Lynch et al, 1993). Although further studies on this aspect have to be done on bdelloids, it would not be surprising to

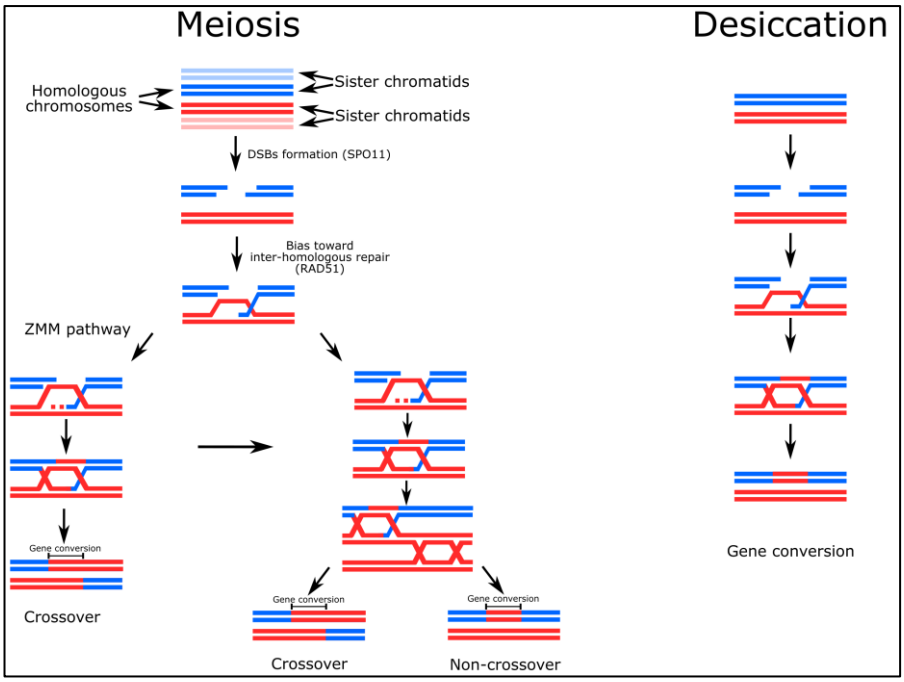

Figure 17 : Comparison between molecularly and desiccation induced DNA breaks. During meiosis, *spo11* induces DNA double-strand breaks in order to trigger the chiasma formation that will result in the pairing of homologous chromosomes. DNA DSBs repair through strand invasion and Holliday junction resolution can either result in gene conversion or in crossovers. Similarly, DNA DSBs resulting from desiccation can be repaired through strand invasion which will result in gene conversion as observed in Flot et al, 2013. It is currently unknown if crossovers can be produced by this mechanisms in bdelloid rotifers.

find similar results given the observed densities of bdelloid (347 bdelloids in a 2.5cm² moss patch represents >500 000 individuals/m²).

Second, desiccation plays an important role in the evolution of bdelloid rotifers by reducing the impact of co-evolving parasites predicted by the Red Queen theory. It has been demonstrated that bdelloid populations were rapidly infected and killed by endoparasitic fungi of the genus *Rotiferophthora* which spores develop into hyphae when ingested. However, long desiccation periods could reduce the amount of bdelloid killed by their parasites as the former withstand anhydrobiosis much better (Wilson and Sherman, 2010). The survival rate and establishment of a new clonal population of bdelloid was even more frequent when long desiccation periods were combined with subsequent wind dispersal as it is often the case in nature (Wilson and Sherman, 2010; Wilson and Sherman, 2013). Therefore, decoupling from parasites and pathogens by playing a never-ending hide-and-seek game could substantially reduce the co-evolutionary burden imposed to asexual lineages.

Third, desiccation has been hypothesized to promote diversification in bdelloid rotifers by facilitating DNA acquisition from non-related organisms, *i.e.* a process known as horizontal gene transfer (HGT). Such transfers are common among bacteria, but the integration of foreign elements in nucleus-wrapped genomes makes it more complex in eukaryotes, especially in multicellular ones where the transfers have to occur specifically in the germline in order to be heritable (Andersson, 2005). In many cases, the acquired gene may be incompatible with the receiver genome and experience strong negative selection. However, integration in the recipient genome through homologous recombination requires only 25 bp at one or both ends of the donor segment and can be effective even between highly divergent regions (Majewski and Cohan, 1999; Popa et al, 2011). Several genes from non-metazoan origins have been detected in bdelloid rotifers genome representing 8-10% of their genes set (Gladyshev et al, 2008; Flot et al, 2013; Eyres et al, 2015), some of which having been shown to be expressed (Hespeels et al, 2015; Eyres et al, 2015). This high amount of HGTs have been attributed to the ability of bdelloid rotifers to undergo cycles of desiccation that result in DNA breakage and repair upon which foreign genes could be integrated (Gladyshev et al, 2008; Flot et al, 2013; Hespeels et al, 2014). Indeed, Eyres et al (2015) highlighted a significant correlation between the frequency of desiccation and the acquisition of novel DNA. Yet, bdelloid species living in permanently aquatic habitats harbor unique foreign genes indicating that other mechanisms may favor HGTs (Eyres et al, 2015). Feeding activities (Yue et al, 2013; Grant et al, 2014), or alternatively weakened eggs exposed to extreme environments (Hotopp et al, 2007; Yue et al, 2012, Soucy et al, 2015), have often been linked with gene transfers. Even though diversification and acquisition of novel functions through horizontal gene transfers may play an important role in bdelloid evolution, the tetraploid structure of their genome could already be a key parameter in itself. Indeed, in addition to masking deleterious recessive alleles, polyploidy can provide the bases for neo- or sub-functionalization of genes could result in novel genetic variation and adaptation (Soltis et al, 2014; Blanc-Mathieu et al, 2017).

Finally, whole-genome sequencing of *A. vaga* revealed that only 3% of the genome was composed of transposons, much less than other metazoans (Flot et al, 2013, Arkhipova and Rodriguez, 2013, Blanc-Mathieu et al, 2017). The diversity of TEs reported in that study was surprisingly high (255 different TE families found) but most of them were present in one or two full copies and often restricted to telomeric regions. It seems that bdelloid rotifers harbor efficient RNA-

mediated silencing processes to suppress TEs mobility and maintain genome integrity (Rodriguez and Arkhipova, 2016). Interestingly, it appears that TEs are co-located with foreign genes and that RNA-based silencing processes help to integrate and domesticate them.

Asexuality, degenerate tetraploidy and desiccation tolerance, causes or consequences?

A last point I want to address before going through the main objectives of the present thesis is the origin of bdelloid rotifers. Several hints of the possible scenarios leading to the emergence of bdelloids have been put forward in between other observations but no studies have really focused on this topic (Donner, 1965; Ricci, 1998; Mark Welch and Meselson, 2000; Hur et al, 2008; Mark Welch et al, 2008). From those published studies, we can admit that Bdelloidea is a monophyletic Class composed of species that are all asexually reproducing (Donner, 1965; Mark Welch and Meselson, 2000; Mark Welch et al, 2008), desiccation-tolerant although some aquatic species have lost this ability secondarily (Ricci et al, 1998; Eyres et al, 2015) and tetraploids (Hur et al, 2008). For this last point it is difficult to determine if bdelloids common ancestor was a real tetraploid or if it had already partially lost some gene copies. If those traits have seemingly been inherited from a common ancestor to all bdelloids, the question remains to understand which scenario could have led to the origin of this ancestor.

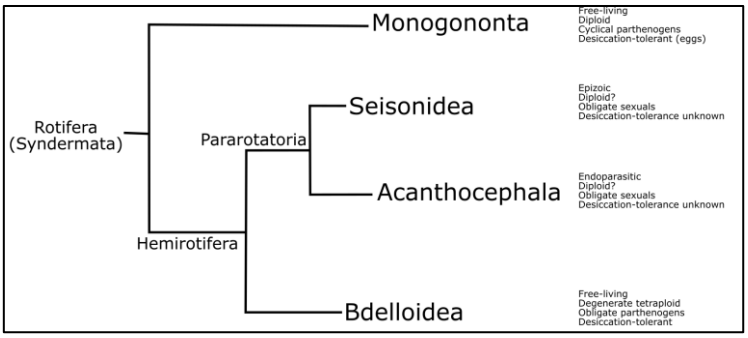

Figure 18 : Phylogenetic relation among the four classes of animals constituting the Phylum Rotifera. Information on their ecology, genome organization, reproductive modes and desiccation tolerance are also provided.

The relationship of Class Bdelloidea with other Rotifera (Monogononta and Seisonidea) is commonly accepted but the phylogeny inside this clade has been widely debated, especially due to the potential relationship with one additional clade (Acanthocephala) (Mark Welch, 2000; Fontaneto and Jondelius, 2011; Tang et al, 2014; Sielaff et al, 2016). The most recent phylogeny based on whole mitochondrial genome sequences, in agreement with previous assumption based on morphological and ecological data, groups Seisonidea and Acanthocephala (Pararotatoria) with Bdelloidea (Hemirotifera), Monogononta being considered as the sister clade of Hemirotifera (Figure 18; Sielaff et al, 2016). Given the topology of this phylogeny, it is tempting to hypothesize that Monogononta and Hemirotifera have inherited diploidy, cyclical parthenogenesis and desiccation-tolerance from a common ancestor, the latest as a prerequisite to colonize terrestrial habitats (Radzikowski, 2013). On the one hand, parthenogenesis was possibly lost in Pararotatoria which specialized into parasitism, sex offering its non-negligible advantages in the context of host/parasite co-evolution. On the other hand, Bdelloidea may have emerged as a result of an autopolyploidization i.e. whole-genome duplication, or an allopolyploidization event i.e. hybridization of two closely related species (Mark Welch and Meselson, 2000). Even though both scenarios are theoretically possible to explain the peculiar genomic structure observed in bdelloid rotifers, allopolyploidy seems more likely. Indeed, autopolyploidy implies that a diploid genome A||B would have duplicated into two pairs A||A’ and B||B’ within which genetic divergence would be null or close and the synteny between A and B (i.e. among all four copies in the tetraploid descendant) would be conserved in the ancestor in order to perform correct meiosis. It is more likely that hybridization between two related species with

genotypes A||A' and B||B' that have diverged over time, would result in offsprings presenting syntenic discordances. This additional set of chromosomes impeding the successful functioning of meiosis and leading to the rare production of apomictic eggs (Figure 19). The possibility for hybridization is appealing if we consider that the common ancestor of Bdelloidea could have been cyclically parthenogenetic and was able to produce males and reduced eggs. Furthermore, hybridization between individuals with remarkably different genome sizes has been observed in the monogonont from the *Brachionus* species demonstrating the possibility of the present hypothesis (Riss et al, 2017). In the successive generations of hybrid lineages, the relaxed pressure for tight chromosomes pairing contributed to the loss of synteny and gene copies loss (i.e. degenerate tetraploidy) through a positive feedback loop until the frequency of meiosis totally disappeared.

Although the hybridization between two species of monogonont-like ancestors could nicely explain the tetraploidy and the loss of sex in bdelloid rotifers, it is necessary to include the acquisition of "all-stages desiccation-tolerance" in the scenario to explain how asexuality was maintained over evolutionary scales. Monogonont are able to produce resting eggs that tolerate desiccation when reduced eggs are fertilized by haploid males. It is possible that desiccation, essential for propagules dispersion in the ancestor, could have evolved in Bdelloidea to keep genomic integrity through gene conversion. Because sex was loss, desiccation became a major mechanism impeding mutation load and enabling survival to parasites. Selection for individuals able to enter desiccation at any stage of their life cycle led bdelloid rotifers may thus have counter-balanced the lack of sex. The early apparition of "all-stages desiccation-tolerance" in Bdelloidea is supported by the fact that an important fraction of the HGTs observed in their genome are common between distinct bdelloid Families and even with the ones found in non-desiccating species (Eyres et al, 2015). It is thus likely that the bdelloid ancestor acquired those HGTs found in all bdelloid species, eventually upon DNA repair that took place after desiccation events. A few bdelloid species secondarily lost this ability when they returned to a fully aquatic life (Ricci, 1998). All-stages

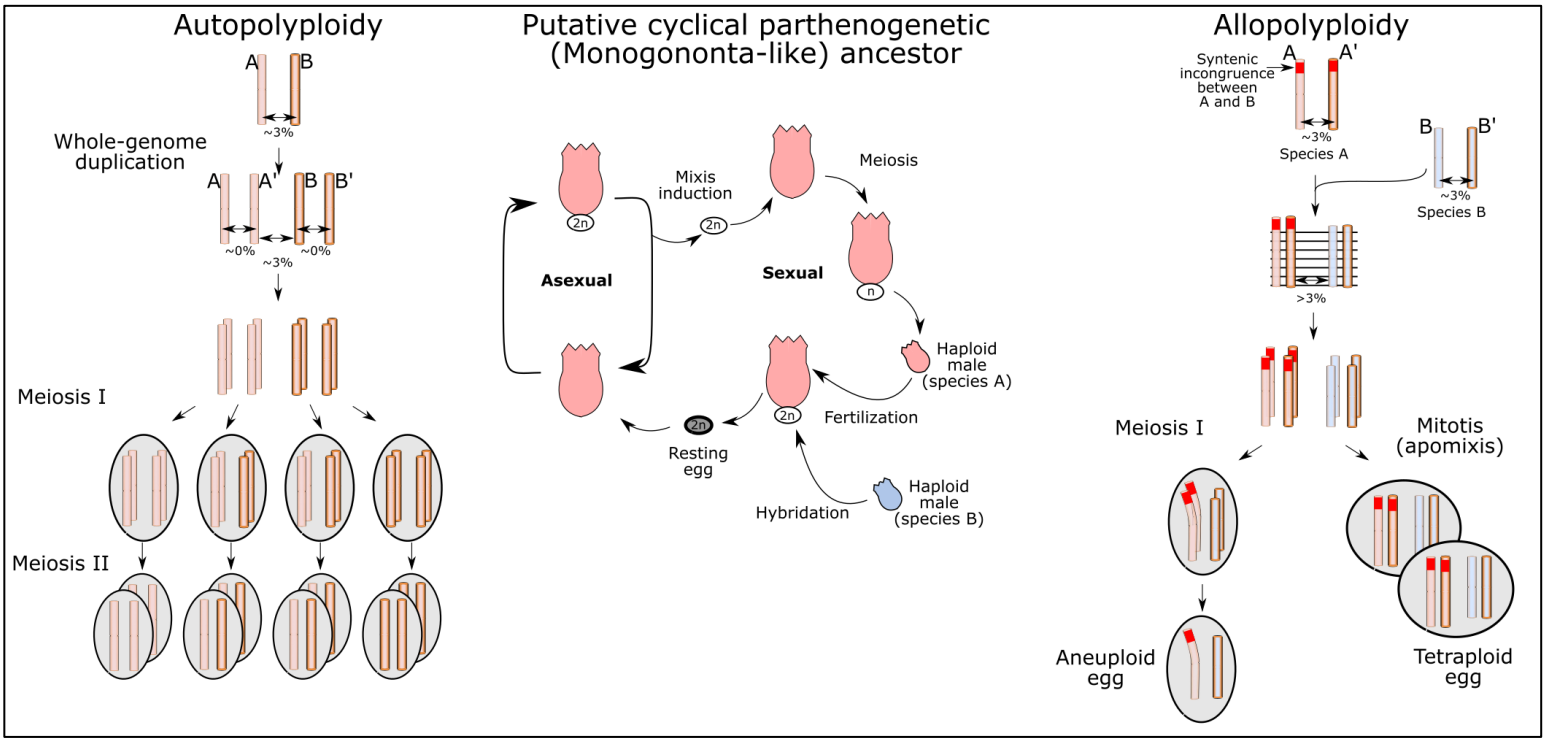

Figure 19 : Hypothetical origin of class Bdelloidea from a cyclical parthenogenetic ancestor. The two main accepted mechanisms that could have resulted in the degenerate tetraploidy are presented (allopolyploidy and autopolyploidy).

desiccation-tolerance probably required several intermediate adaptations such as modifications of the molecular pathways inducing desiccation in the egg, induction of efficient DNA repair mechanisms and organs wrapping. Nonetheless, the selective advantages conferred by hybridization and polyploidization (Ament-Velásquez et al, 2016; Alix et al, 2017; Blanc-Mathieu et al, 2017) could have given sufficient times for those adaptations to fix.

Goals of the present thesis

In this introductory chapter, I have presented the "paradox of sex" and theoretical advantages conferred by sexual reproduction. I have then summarized a few mechanisms that are known to result in the origin of parthenogenetic lineages. Finally, I have developed the case of the bdelloid rotifers which have evolved and diversified over millions of years apparently without sexual reproduction. Even though many studies have contributed to a deeper understanding regarding the existence of such successful asexual taxa, there is much left to do.

First, despite a genomic structure theoretically incompatible with conventional meiosis, there is still a possibility for bdelloids to engage in rare cryptic sex (parasexuality, *Oenothera*-like meiosis, or other undescribed mechanisms). In Chapter I, I will present the first part of my thesis during which we set up an unprecedented study combining population genetics and genomics to detect plausible recombination events.

Questions addressed:

1) Are individuals collected from a very local-scale study genetically diversified or purely clonal?
2) If bdelloid rotifers accumulate gene of non-metazoan origins, are interbdelloid transfers possible?
3) If so, what are the underlying mechanisms enabling those transfers?

Second, I will develop and clarify several issues imposed by the experimental settings used in the first chapter and pointed out by colleagues and other scientists in the field (Chapter II).

Questions addressed:

1) How can cross-contamination signals be distinguished from sequencing background noise?
2) Are whole-genome amplification reactions biasing the results?
3) Are the patterns of intra- and interspecific transfers resulting from cross-contaminations?

Third, an additional study to detect cryptic sex or alternative recombination mechanisms, taking into account all the interesting and relevant remarks and thoughts, will be described in chapter III. This third chapter was done in collaboration with Matthieu Terwagne (Post-doc, LEGE, UNamur) and with the helpful technical support of Ludovic Herter (Technician, LEGE, UNamur).

Questions addressed:

1) Can we retrieve signals of intra- and interspecific DNA transfers in bdelloid without whole-genome amplification step?
2) Is genetic distance among donor and receiver a barrier to those transfers?

3) What is the size of the transferred DNA fragments?

The second part of this thesis will be sensitively different, with experiments targeting the impact of asexuality at community levels. The peculiarities of bdelloid rotifers in term of reproduction, life cycles, stress tolerance, dispersion have diverse theoretical eco-evolutionary consequences that have been widely studied, especially in connections with spatial distribution. However, there is currently a lack of studies focusing on the communities spatio-temporal dynamics. In Chapter IV, I will present the results of a spatio-temporal dynamics experiment that was combined with an ecological model to understand which parameters shaped bdelloid communities (stochasticity, seasons, habitat …).

Questions addressed:

1) Which ecological parameters could explain the bdelloid community structure observed in chapter one?
2) Can several cryptic Adineta species co-occur in a same patch or do species exclude each others?
3) Are species assemblage changing through seasons or are communities stable?

In the fifth chapter, I will briefly present the results on bdelloids communities obtained within the framework of the SPEEDY project (Spatial and environmental determinants of Eco-Evolutionary Dynamics) which aims at understanding community changes throughout a gradient of anthropogenic disturbances.

Questions addressed:

1) Are bdelloid rotifers communities affected by urbanization gradients or not?
2) Are species assemblages observed around Namur similar to the ones found throughout Flanders?

Finally, I will briefly present the different species I started to isolate from nature and describe the different methods tested to keep them in lab conditions. I decided to include this sixth chapter as a side project because, overall, culture maintenance and optimization represented several hours of work weekly. In addition, this collection represents a unique clone bank that is widely used as a basis for several projects conducted by the LEGE laboratory (RADseq, comparative genomics, karyotypes…).

Those six chapters will be concluded by a wide and, I hope, comprehensive discussion about the interest of this thesis and the few new thoughts it provides for the understanding in the evolution of bdelloid rotifers, asexuality and the “paradox of sex”.

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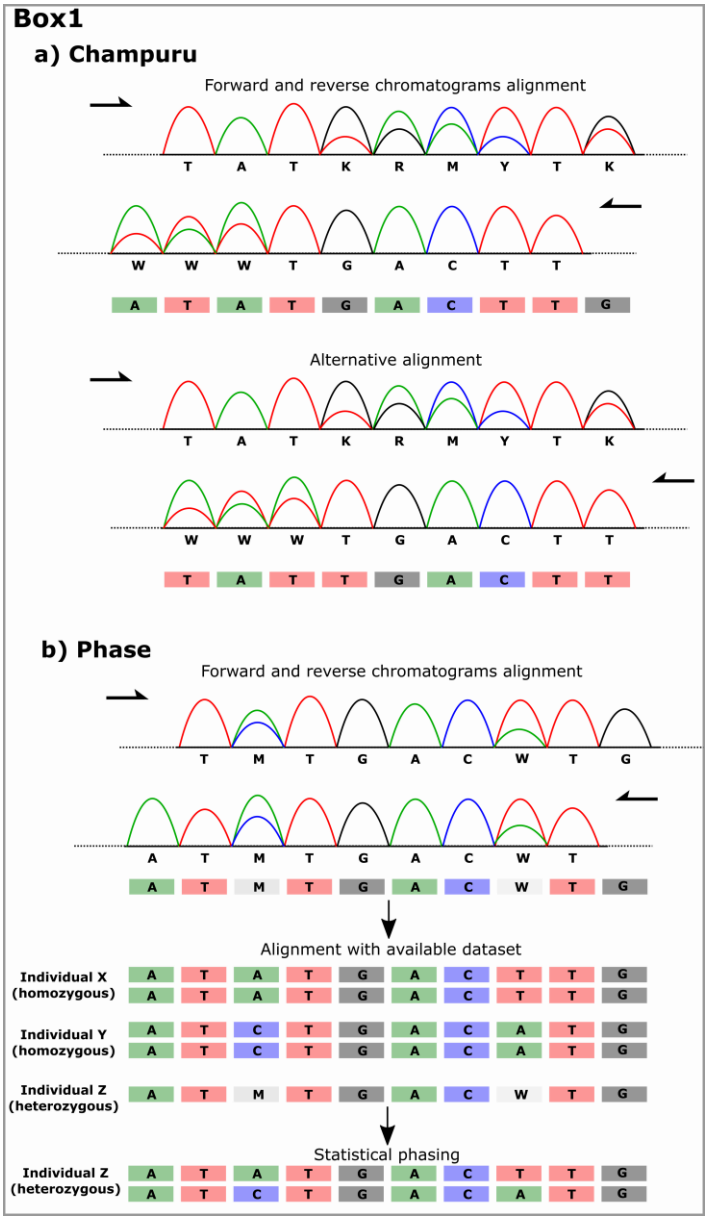
Details on the methods used throughout the thesis

A few methods specific to species delimitation, *in silico* sequence phasing and genetic diversity representation were repetively used throughout the present thesis; it may thus be worth to provide some details on those methods for none specialists.

Champuru and Phase

In box 1, two methods developed to reconstruct sequences from the patterns of double-peaks observed on the chromatograms of heterozygous individuals are presented. The most widely used option to genotype heterozygous individuals is to PCR-amplify both alleles using a same primer pair and then insert the resulting amplicons into plasmids that will be inserted in bacteria for replication. Each of the resulting bacterial colonies contains only one of the two original alleles and is used for plasmid extraction and Sanger-sequencing. Screening several colonies increases the chance to retrieve each allele. Even though this method is efficient and allows obtaining "clean" sequences with low background noise, it is time consuming and expensive, especially in large studies when hundreds of individuals are genotyped. Alternatively, directly Sanger-sequence in both directions (forward and reverse) the PCR amplicons containing the two alleles of heterozygous individuals can enable efficient genotyping too. The chromatograms obtained will represent of mix of two distinct sequences (clear double-peaks) and background noise (underrepresented contaminants, PCR-induced errors). Two methods enable to reconstruct the two original alleles in silico based on the patterns of double-peaks observed when the forward and reverse chromatograms of an individual are aligned. a) Champuru relies on the pattern of double-peaks produced by the presence of two similar sequences distinguish by indels. Double-peaks will start to be frequent after the site at which the indel occurred. Aligning the forward and reverse chromatograms in the two possible alternatives can efficiently enable to reconstruct both alleles without cloning into plasmids. b) In cases where the two alleles are not distinguished by an indel, the patterns of double-peaks are not sufficient to resolve the problem. In this example, the only two mutations lead to four possible combinations (ATATGACTTG,



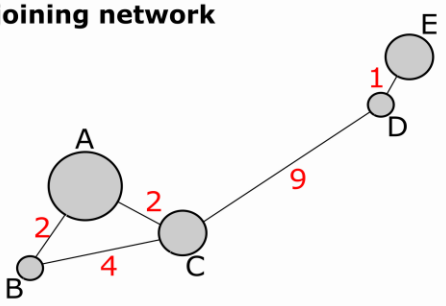
ATATGACATG, ATCTGACTTG and ATCTGACATG), each having 25% chances to be correct. Using a larger dataset containing other individuals from the population will enable phasing with higher probabilities if for example the allele ATATGACTTG and ATCTGACATG were observed in the population but never the two others.

Haplotype networks and haplowebs

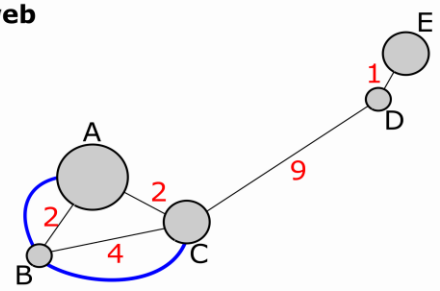
In box 2, methods to visualize the genetic diversity and the sequences distribution across a sampled population are presented. a) A haplotype network is a network in which each haplotype (a given haploid sequence from the population, often a mitochondrial marker) is represented by a circle. The size of each circle is proportional to the frequency of the corresponding haplotype in the population. In the example, haplotype A is the most frequent whereas B and D are rare. The haplotypes are linked and the number of mutations separating each haplotype is indicated (in red). Two mutations distinguish haplotype A from B and two other mutations distinguish A from C. As a result, four mutations separate B from C. The polymorphic sites can also be indicated instead of the number of mutations, but for clarity we rarely show this information. b) Haplowebs are networks on which sequences co-occuring in one heterozygous individual for a given locus (*i.e.* alleles) are linked (blue curves). In the example, heterozygous individuals present genotype A||B for the marker sequenced but most individuals are homozygous A||A as represented by the higher frequency of A. It is also possible to make the width of the blue curve vary with the frequency of the corresponding heterozygote in the population. c) Alleles that are linked directly or indirectly form fields for recombination (FFRs) that can be regarded as species (*i.e.* species are individuals that are susceptible to exchange alleles through sex). In the example, individuals harbouring genotype A||B and individuals harbouring genotype B||C are present in the sampled population. As a result, alleles A, B and C form one FFR because individuals harbouring genotype A||C are virtually possible. In contrast, even if a single mutation distinguishes D from E, they never co-occurred in one heterozygous individual in the present dataset and are thus considered as distinct FFRs (*i.e.* belonging to different species). This method of species delineation is obviously influenced by the sampling size, the frequency of each genotype and the ability to phase the alleles correctly as larger samples, homogeneous distribution of genotype frequencies and correct phasing will impact the number of links (blue curves) on the haplowebs.

Box2

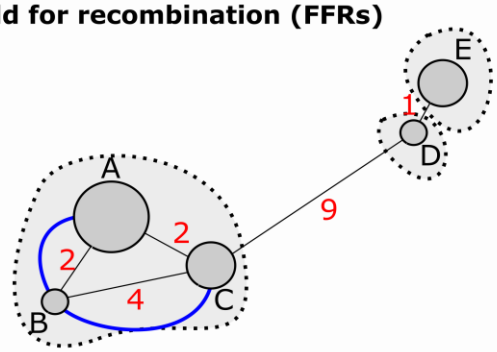
a) Median-joining network



b) Haploweb



c) Field for recombination (FFRs)

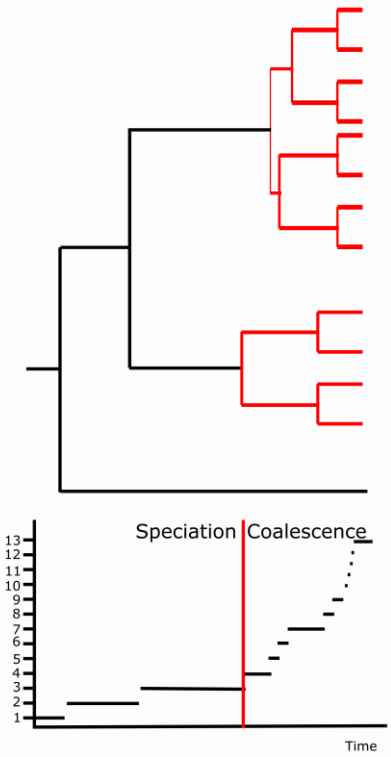


Generalized mixed Yule-coalescent method (GMYC)

In box 3, a tree-based method for species delineation, named generalized mixed Yule-coalescent method (GMYC), is presented. In this method a statistical test is applied to an ultrametric phylogenetic tree to distinguish if the branching pattern follows a distribution attributable to speciation or coalescence. On longer timescales genetic drift and selection have time to influence the evolution of the locus under scope, *i.e.* speciation. In contrast, on shorter timescales, those parameters have lower (or nul) impacts on the sequences distribution; the resulting tree topology is thus due to coalescent processes. In the GMYC method a threshold distinguishes speciation from coalescence is statistically determined based on the branching rate. Branching occurring after the threshold corresponds to coalescence and the resulting clusters of sequences correspond to species. In this example, two clusters (in red) are delimited, a third branch correspond to the outgroup used to root the tree.

Box3

Generalized mixed Yule-coalescent method (GMYC)



Chapter 1: Allele sharing among bdelloid rotifers of the genus *Adineta*, evidence for sexuality or for horizontal gene transfer?

In this first chapter we have conducted a population genetic study coupled with genomics data to study the genetic diversity present among individuals of the species *Adineta vaga* collected at a local scale within a park in Belgium. As demonstrated in a few published studies, morphospecies within the bdelloid rotifers appear to contain genetically diverse entities, akin to cryptic species (Fontaneto et al, 2007; Fontaneto et al, 2009; Kaya et al, 2009). Therefore, using genetic markers enabling species delimitation within the morphospecies *A. vaga* was required here to define at which taxonomic scale the observed diversity occured. A few genetic markers had already been used in previous studies, some of which based on fosmid libraries (Hur et al, 2009) containing the histones genes or the four copies of the *hsp82* genes (Mark Welch and Meselson, 2000; Hur et al, 2009). However, those markers are too conserved and this lack of variability makes them unsuitable for population genetics studies. Other nuclear markers were designed around the widely used 18S and 28S rDNA subunits but did not present satisfying variability compared to the mtCOI marker (Fontaneto et al, 2007; Tang et al, 2012). As our aim was to study the genetic diversity among individuals of the focal species *A. vaga*, we first tried to design genome-scale markers by developing microsatellites and AFLP markers in a preliminary study, but without success (Dr. Xiang Li thesis and N. Debortoli master thesis). Alternatively, we took advantage of the first *A. vaga* genome that had just been published (Flot et al, 2013) to design new variable exon-primed intron-crossing (EPIC) nuclear markers that could work on all bdelloid species, or at least the entire Adinetidae family. We designed our markers in order to amplify the two copies of one allelic pair simultaneously and directly Sanger-sequenced the resulting amplicons without cloning to limit time and money waste (Stephens, 2001; Flot et al, 2006; Flot, 2007; Flot, 2010).

At the time the experimental design of my population genetic study was established, the first evidence of horizontal gene transfer (HGT) from non-metazoan origin within bdelloid rotifer genomes were published (Gladyshev et al, 2008; Boschetti et al, 2012), with additional studies confirming this high level of HGT within this animal clade (Flot et al, 2013; Eyres et al, 2015) . Indeed, around 8-10% of the genes in the *A. vaga* genome were predicted to be horizontally acquired and coming from bacteria, plants and fungi. It was hypothesized that this high amount of HGTs was desiccation-mediated (Boschetti et al, 2012; Eyres et al, 2015). During desiccation bdelloid rotifers were shown to accumulate DNA DSBs (Hespeels et al, 2014) which may help the incorporation of DNA fragments from their environment during DNA repair upon rehydration. Even though the exact mechanism responsible for those numerous HGTs is still unknown, the fact that the bdelloid genome is inclined to the integration of foreign DNA resulted in the hypothesis that genetic transfers among bdelloid individuals may also occur.

Starting in 2012 a population genetic study targeting the genetic diversity found within a single bdelloid morphospecies sampled in the wild by using new genetic markers expected to be highly variable (exon-primed intron-crossing markers), we hypothesized that our experimental setup could also identify whether transfer of genetic material between bdelloid rotifers may occur. Here, it

is important to emphasize that we sampled more than 500 *Adineta vaga* individuals inhabiting habitats that dry out frequently (i.e. lichen patches on trees and soil samples) and within a small geographic region, being the park Louise-Marie, in order to detect such exchanges. Moreover, bdelloid rotifers were shown to harbor allelic copies with on average nucleotide divergences around 3% (Mark Welch et al, 2008; Flot et al, 2013) despite their asexual evolution. The limited accumulation of mutations among alleles is probably the result of frequent gene conversion events between alleles, probably during DNA DSB repair (Flot et al, 2013). If DNA transfers among bdelloid individuals are rare, focusing on recent exchanges may increase the chances to observe such cases, gene conversion eventually masking them over longer timescales. In addition, eventual transfers among genetically identical clones, *e.g.* sisters, would be undetectable emphasizing the importance to work on a high number of distinct individuals (high intra- and interspecific diversity). The more SNPs separating the received and the original DNA sequence, the better to exclude the possibility for the accumulation of identical SNPs independently.

The results of this chapter were published in Current Biology:

Debortoli, N., Li, X., Eyres, I., Fontaneto, D., Hespeels, B., Tang, C. Q., ... & Van Doninck, K. (2016). Genetic exchange among bdelloid rotifers is more likely due to horizontal gene transfer than to meiotic sex. *Current Biology*, *26*(6), 723-732.

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Published paper: Genetic Exchange among Bdelloid Rotifers Is More Likely Due to Horizontal Gene Transfer Than to Meiotic Sex.

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HIGHLIGHTS

- Bdelloid individuals of the genus *Adineta* exchange DNA within and between species
- Genomic signatures are found of asexual evolution, gene conversion and recombination
- Horizontal genetic exchange appears more likely than sex in bdelloid rotifers

Summary

Although strict asexuality is supposed to be an evolutionary dead end, morphological, cytogenetic and genomic data suggest that bdelloid rotifers, a clade of microscopic animals, have persisted and diversified for more than 60 Myr in an ameiotic fashion. Moreover, the genome of bdelloids of the genus *Adineta* comprises 8-10% of genes of putative non-metazoan origin, indicating that horizontal gene transfers are frequent within this group and suggesting that this mechanism may also promote genetic exchanges among bdelloids as well. To test this hypothesis, we used five independent sequence markers to study the genetic diversity of 576 *Adineta vaga* individuals from a park in Belgium. Haplowebs and GMYC analyses revealed the existence of six species among our sampled *A. vaga* individuals, with strong evidence of both intra- and interspecific recombination. Comparison of genomic regions of three allele-sharing individuals further revealed signatures of asexual evolution and/or gene conversion adjacent to patterns of inter-individual recombination. Our findings suggest that bdelloids evolve asexually but exchange DNA horizontally both within and between species.

Keywords

asexuals, haplowebs, HGT, gene conversion, cryptic species, recombination

Introduction

Bdelloid rotifers are microscopic, aquatic animals often considered an evolutionary scandal because they have apparently evolved asexually for more than 60 Myr [1]. Evidence for their long-term evolution in the absence of conventional sex (here defined as the alternation of meiosis and fertilization events) has accumulated since their first observation by van Leeuwenhoek [2] and has recently been summarized by Fontaneto and Barraclough [3]. Earlier cytological studies on two bdelloid species described egg production by two maturation divisions from primary oocytes without chromosome pairing or reduction in chromosome number [4][5]. This absence of meiosis was corroborated by the recent publication of the draft genome of *Adineta vaga,* which appears devoid of homologous chromosomes, hence ruling out the possibility of conventional meiosis [6]. However, these results could not dismiss the presence of alternative mechanisms of genetic exchange among bdelloid rotifers.

The idea that bdelloids acquire genes horizontally was first suggested by the observation that 8-10% of the genes found within *Adineta*'s genome [6][7] and transcriptome [8] are of putative non-metazoan origin, indicating that bdelloids are receptive to horizontal gene transfer (HGT). Although the exact molecular mechanisms behind these HGTs remain unidentified, they were hypothesized to result from the periods of desiccation experienced by bdelloids in their ephemeral habitats (such as lichens and mosses) [6][7]. Indeed, Hespeels et al. [9] demonstrated that desiccated *A. vaga* individuals accumulate multiple DNA double-strand breaks (DSBs) that get repaired upon rehydration, opening an avenue for the horizontal integration of foreign DNA. In addition desiccation may also compromise the integrity of cell membranes, thereby facilitating the entry of foreign genes into rotifer cells [8]. Consistent with this desiccation hypothesis, Eyres et al. [10] demonstrated recently that the level of horizontal gene transfer is higher in bdelloid species of the genus *Rotaria* that experience regular desiccation events in their semi-terrestrial habitats than in other species inhabiting permanent water bodies and unable to resist desiccation.

The observation of high numbers of horizontally transferred genes in bdelloids led us to hypothesize that they could also exchange genes among themselves [6]. This hypothesis was recently supported by a study reporting signatures of allele sharing between three individuals within one mitochondrial clade of the bdelloid genus *Macrotrachela* [11]. However, the authors of this study interpreted their results as evidence for sexual reproduction, possibly occurring via an atypical *Oenothera*-like mode of meiosis requiring neither chromosome pairing nor segregation [11]. In *Oenothera,* heterozygous chromosomal translocations prevent homologous pairing and therefore only telomeres pair and recombine, resulting in the formation of meiotic rings in which alternating parental chromosomes co-segregate into two linkage groups (alpha and beta). Since only alpha and beta gametes can cross, heterozygosity is maintained within the population (hence the term "permanent translocation heterozygosity" (PTH) to designate this system). The consequence of this atypical meiosis is that chromosomes are non-recombining along 90% of their length and the 10% that do recombine contain very few genes, if any; hence haplotypes are maintained from one generation to the next and the whole genome behaves as a single linkage group [12]. Reproduction in PTH *Oenothera* species is predominantly by selfing, although hybridisation between PTH forms may occur (resulting in new PTH forms akin to species) [12] [13] [14]. In their study of bdelloid rotifers of the genus *Macrotrachela*, Signorovitch et al. [11] observed matching allele-sharing patterns among 3 bdelloid isolates at 4 genomic loci (ranging in size from 2.8 to 9.7 kb) and hypothesized that such PTH type of crossing was responsible for these patterns. This suggestion opened the intriguing possibility that bdelloids may be engaging in meiosis and sexual reproduction despite their lack of homologous chromosomes.

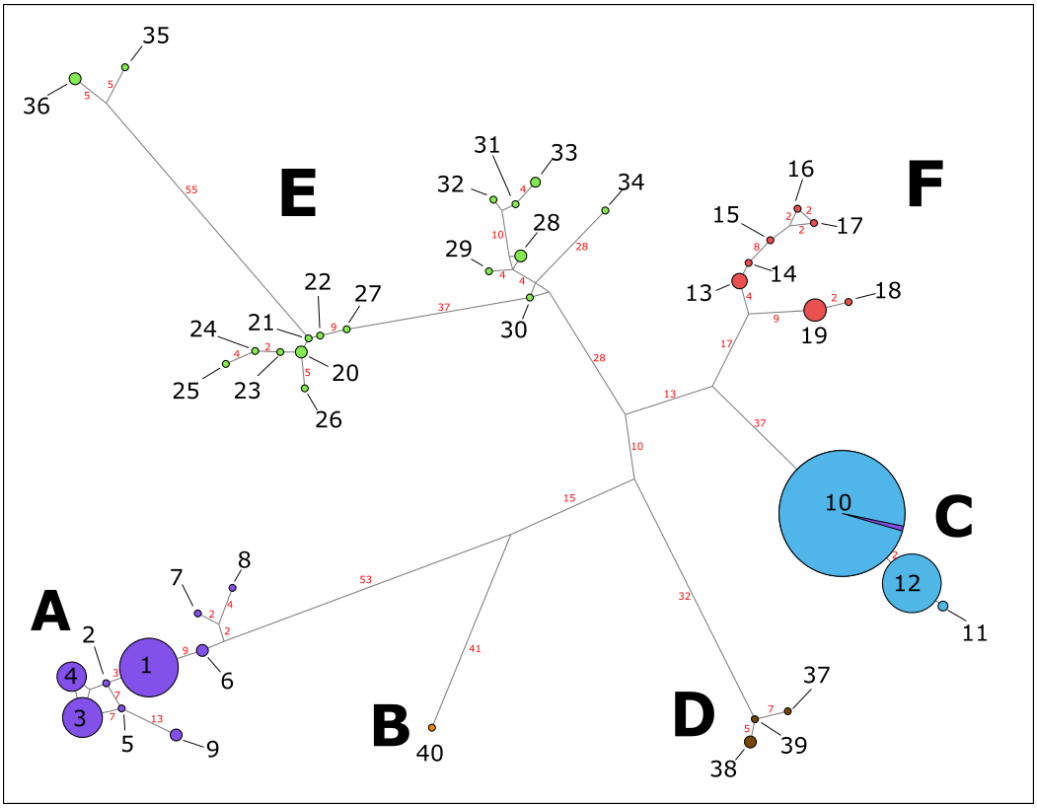

Figure 1 : Median-joining haplotype network of the COI sequences obtained from the 576 *A. vaga* individuals collected. The frequencies of the forty COI haplotypes identified are proportional to the circle size. Each individual is coloured according to its assignment to one of the six species (A-F) determined using the conspecificity matrix (Figure 2). The number of mutations (SNPs) separating two haplotypes is indicated in red when higher than one. One individual assigned to species A according to the conspecificity matrix (Fig. 2) harboured a COI sequence (haplotype 10) attributable to species C.

To examine whether the observation of Signorovitch et al. [12] held for all bdelloids, we performed a population genetic study of the bdelloid rotifer species complex *A. vaga* by taking advantage of its available genome sequence. More than 500 individuals were sampled at a local scale (one public park in Belgium) and species were delineated using tree-based and allele sharing-based approaches [15] on five independent markers. We acknowledge that the term 'species' may not be

appropriate for organisms that do not have meiosis and such term may encounter some difficulties in some readers. Yet, the definition of 'species' may be multifaceted, and we here make explicit use of this term to identify independently evolving entities that represent arenas for evolution analysing the patterns in genetic diversity, as they would be expected in other more traditional sexual species in animals. Such DNA taxonomy approach is commonly applied in comparisons between sexual and asexual rotifers [1] [3]. Our study revealed the presence of several cryptic species in our dataset as well as signatures of intra- and interspecific genetic exchanges. We further sequenced the genome of three conspecific allele-sharing individuals and assembled contigs around each of our four nuclear markers in order to look at recombination patterns alongside their genome.

Results

Evidence for cryptic species within A. vaga

A total of 576 bdelloid rotifers morphologically identified as *Adineta vaga* were isolated from 36 lichen and soil patches distributed in one park in Belgium. A portion (631 bp) of the mitochondrial cytochrome *c* oxidase 1 gene (COI) was successfully sequenced in all these 576 individuals, yielding a total of 40 distinct haplotypes (Fig. 1). Eighty-two individuals representative of the different COI haplotypes and distinct patches were selected for further sequencing of four independent nuclear markers. Three of these markers (28S, EPIC25 and EPIC63) were successfully amplified and sequenced for all these individuals, whereas amplification of the Nu1054 marker did not work for 18 individuals. Haploweb analyses of each nuclear marker yielded six (28S), fourteen (Nu1054), fifteen (EPIC63) and eighteen (EPIC25) fields for recombination (FFRs), *i.e.* putative species (see Fig. S1) [16] [17], among the 82 individuals sequenced. The 40 haplotypes in our COI dataset were considered as FFRs in the downstream analysis, which integrated the information from all markers into a single conspecificity matrix. Upon clustering, the matrix revealed six distinct blocks,

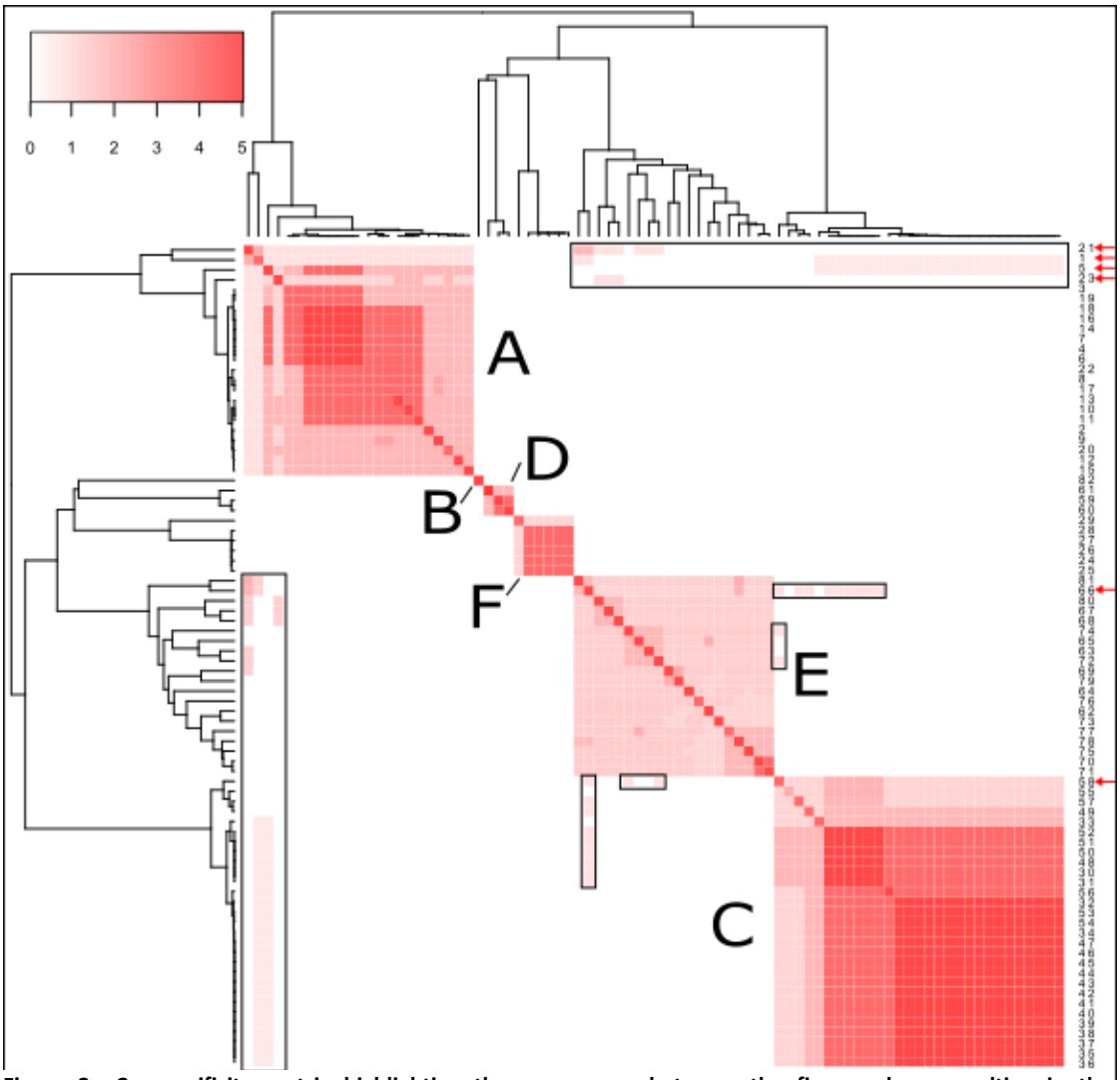

Figure 2 : Conspecificity matrix highlighting the congruence between the five markers, resulting in the delineation of six species (A-F) among the 82 *A. vaga* individuals sequenced. This matrix was obtained computing, for each pair of individuals, a conspecificity score equal to the number of independent markers supporting the hypothesis of their conspecificity, , then reordering the rows and columns to maximize the scores along the diagonal. The highest scores (5 out of 5) are shown in red, whereas the lowest scores are shown in white (and the intermediate scores in various shades of pink). The black frames indicate incongruent species assignment for six individuals (red arrows). The twelve individuals (Ind38-47, 54 and 55) isolated from the same patch and harbouring the same COI haplotype 12 form one dense red block on the bottom right corner of the matrix.

i.e. six consensus clusters akin to species (labelled A-F on Fig. 2). Overall, species delimitation using the conspecificity matrix on all markers and the Generalized Mixed Yule-Coalescent approach on the COI database (GMYC, Fig. S2) [18][19] [20] gave identical results: six genetic clusters were found within the sampled *Adineta vaga* dataset, referred to as six distinct species (A to F) in the rest of the

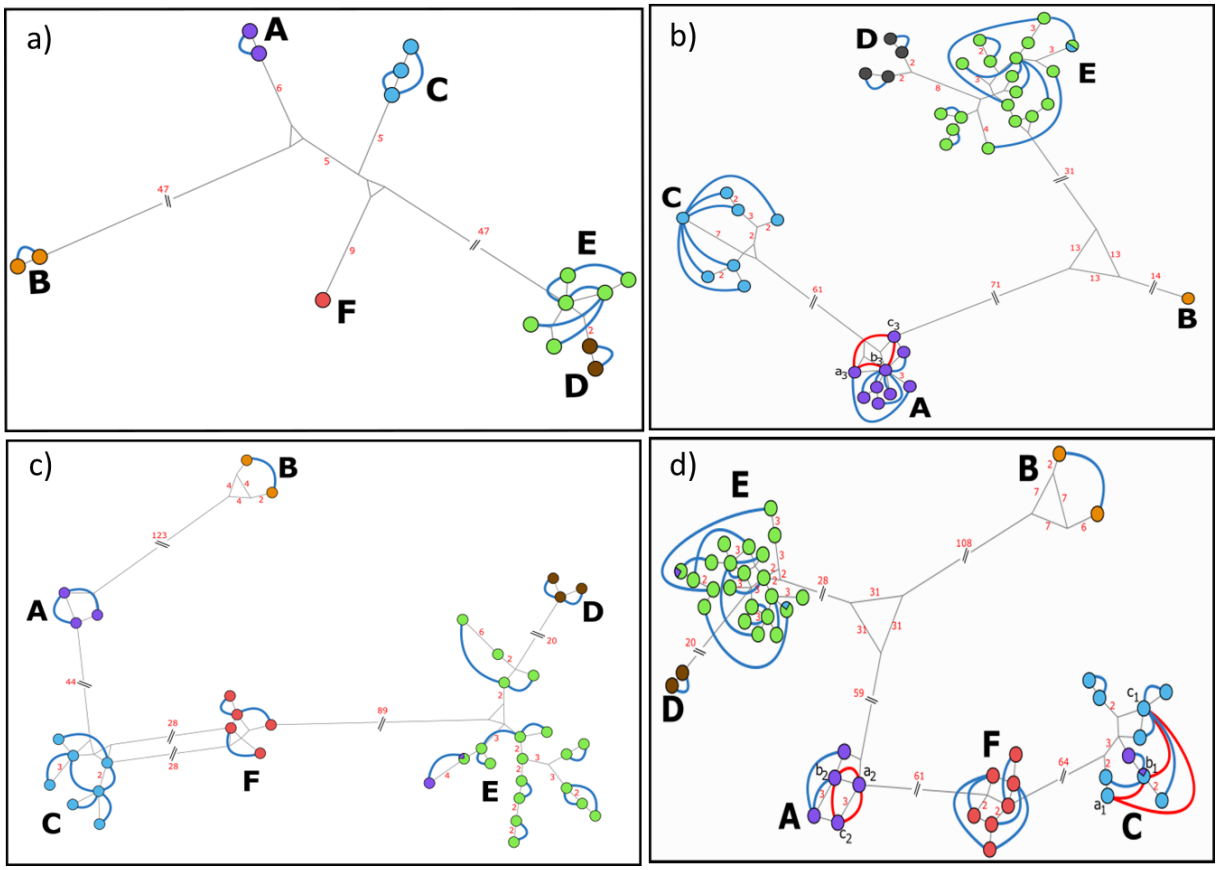

Figure 3 : Haplowebs of the nuclear markers a) 28S rDNA, b) Nu1054, c) EPIC63 and d) EPIC25 amplified in 82 *A. vaga* individuals. Haplowebs consist in median-joining haplotype networks on top of which links (here shown in blue and red) are added between haplotypes (alleles) that co-occur within heterozygous individuals. Each individual is coloured according to its assignment to one of the six species (A-F) determined by the conspecificity matrix. One individual from species C had the same sequence than individuals from species E for the Nu1054 marker (Fig. 3b). For the EPIC63 marker, one individual from species A had one allele identical to individuals from species E and its other allele was closely related to species E (Fig. 3c). Two individuals assigned to the A and C species according to the conspecificity matrix harboured an EPIC25 sequence identical to individuals from species E and one heterozygous individual from species A presented two alleles similar to individuals from species C (Fig. 3d). The red links highlight individuals belonging to haplotype trios and represent signatures of allele sharing. The number of mutations (SNPs) separating two haplotypes is indicated in red when higher than one. The frequency of each haplotype is not represented on this figure as all circles were drawn with equal sizes.

manuscript. The COI network and the haplowebs of each nuclear marker were coloured according to the delimitation obtained from the conspecificity matrix, revealing either perfect (28S) or high congruence (COI, EPIC25, EPIC63, Nu1054) with the six molecularly defined species (Fig. 1 and 3).

All the nuclear genotypes of the 12 individuals harbouring the COI haplotype 12 (Fig. 1) were identical (aside from one individual harbouring a single SNP on the 28S marker), consistent with a clonal mode of reproduction for species C. Indeed, the clonal diversity indexes for this species ranged from 0.005 to 0.571, suggesting that the "true" clonal diversity for this species was quite low. For species A and B these calculations were inconclusive: for species A it was because the range was very large (0.070 for COI haplotypes vs. 0.909 for multilocus genotypes), whereas for species B it was because only one specimen of this species was available. However, for species D, E and F the clonal richness index [21] calculated using only COI haplotypes ranged from 0.316 to 0.696 whereas the

clonal richness index calculated from the multilocus genotypes were all equal to 1.000 (Suppl. Data), suggesting frequent recombination.

Inter-specific DNA Transfers

We observed six individuals (Ind1, 5, 21, 23, 58 and 66) that were assigned to distinct species according to different markers, as indicated by the framed rectangles on the conspecificity matrix that did not fall within the diagonal (Figure 2). The species assignment of each individual

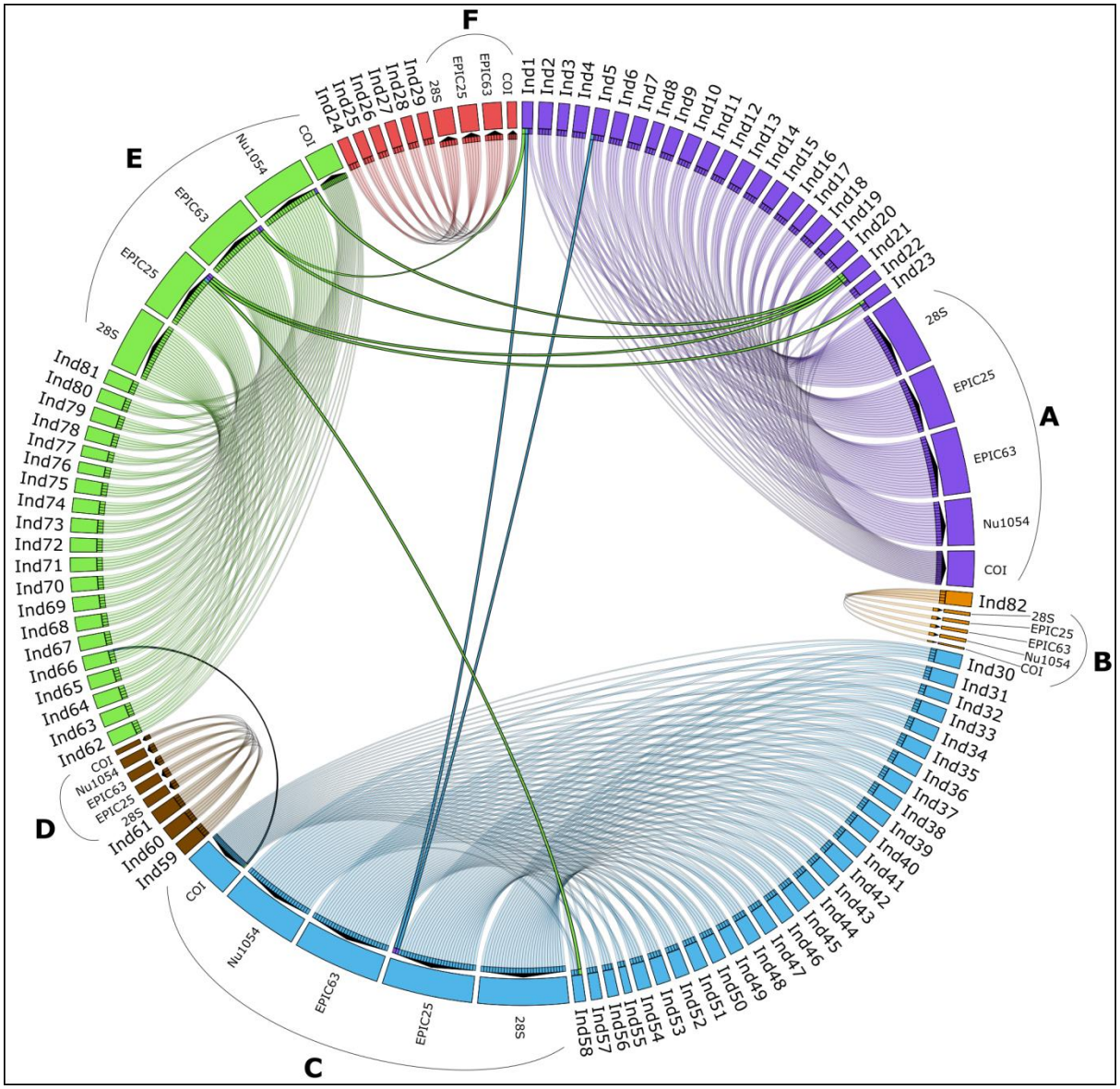

Figure 4 : Circular plot summarizing the species affiliation of each of the 82 *A. vaga* individuals according to each of our five markers (except markers/species combinations for which amplification and/or sequencing did not work). Each individual (Ind) is connected by links to each of the five markers sequenced. The colours correspond to the six species (A-F) defined by conspecificity matrix. Six individuals have sequences being assigned to different species as indicated by the arrows.

according to each genetic marker is summarized on Figure 4: the 82 individuals analysed are connected to the genetic sequence retrieved for each of the five markers, and the six species are colour-coded as in the haplowebs and labelled A to F. In total, 76 individuals were assigned to a single species (blurred links) congruently by all markers. In contrast, five individuals (Ind5, Ind21, Ind23, Ind58 and Ind66) had genetic markers (bright colours) attributed to two distinct species, *e.g.* Ind5 had COI, 28S, EPIC63 and Nu1054 sequences from species A but harboured EPIC25 sequences

from species C. These five individuals were homozygous at the loci that were incongruent. In addition, one individual (Ind1) assigned to species A, harboured genetic markers belonging to two other distinct species (C for EPIC25 and E for EPIC63, Fig. 4); this individual was heterozygote for these two latter markers (Fig. 3c and 3d).

Intra-specific Haplotype Sharing

We observed two cases of EPIC25 haplotypes being shared by heterozygous individuals in a cyclic fashion (Fig. 3d), and one such case with the Nu1054 marker (Fig. 3b). Each of these cycles comprised three haplotypes and we therefore refer to them as "haplotype trios" (displayed as red links on the figures 3b and 3d). Each haplotype trio occurred within a species, *i.e.,* no individual of two different species shared haplotypes in a cyclic fashion. Although the observation of three distinct haplotypes a, b and c in heterozygous individuals with genotypes (a||b) and (b||c) may be explained by mutations and gene conversions alone, their co-occurrence in three genotypes (a||b), (b||c) and (c||a) can only be explained by recombination between individuals. Indeed, the haplotypes (or alleles) a_1, b_1 and c_1 in the first trio (in species C on Fig. 3d) were separated by four to eight point mutations, making convergence unlikely. In the second trio (species A of marker EPIC25, Fig. 3d), a single nucleotide polymorphism (SNP) distinguished the a_2 and b_2 haplotypes whereas the b_2 and c_2 haplotypes differed only in their number of short tandem repeats (TTC_4 and TTC_5 respectively). As a tandem repeat difference was not enough to exclude the hypothesis of convergent evolution or sequencing error, the flanking regions were sequenced till a length of 761 bp, yielding five additional SNPs between haplotypes b_2 and c_2 (results not shown). The third cycle was observed for the Nu1054 marker: a single SNP distinguished alleles a_3 and b_3, whereas two SNPs separated b_3 and c_3. These patterns of genetic recombination among conspecific individuals were confirmed by re-sequencing each marker directly using the original gDNA of each individual instead of the WGA products, yielding identical results.

Genome Dynamics

The genomic data of the three individuals forming the trio of species C for EPIC25 marker (with genotypes $a_1||b_1$, $a_1||c_1$ and $b_1||c_1$) were heavily contaminated by gut and environmental bacteria (mostly *Pseudomonas*), and the sequencing coverage was highly heterogeneous due to the whole-genome amplification step conducted prior to library preparation. Nevertheless, we managed to assemble contigs of 5 to 10.5 kb around each of our nuclear markers (Fig. 5). Although the posterior probabilities of our reconstructed haplotypes using PHASE were rather low (see Suppl. Data), the sequences of our markers were identical for both Sanger and Illumina sequencing, confirming that our haplotype reconstruction method from the patterns of double peaks was highly accurate. We did not detect any additional copy of the EPIC25, EPIC63 and Nu1054 markers, nor any additional 28S rDNA sequence type, in any of the three genomes assembled.

Analysis of the alignments using RDP3 detected two recombining regions of respectively 1865 bp and 1031 bp on the contigs harbouring the EPIC25 marker and one recombining region of 4547 bp on the contigs harbouring the EPIC63 marker (Fig. 5b, grey areas). In contrast, no recombination was detected in the trio of the Nu1054 region.

The maximum-likelihood trees built from each region showed three contrasting patterns (Fig. 5a). First, the phylogeny obtained for the Nu1054 region was consistent with expectations for asexually evolving nuclear alleles: both alleles within each individual are diverging independently in the absence of recombination and therefore clustered in separate clades [22]. Second, other regions presented signatures of inter-individual recombination (2831-4695 bp and 8110-9141 bp on EPIC25 and 2574-7121 bp on EPIC63) as confirmed by phylogenetic analyses with each of the three individuals sharing one allele with the two others (segregation and recombination). Last, the regions adjacent to those signatures of inter-individual recombination presented phylogenetic signatures of gene conversion events, *i.e.* the replacement of one allele by a copy of its homologue in the same individual.

Interestingly, the longest contig assembled around the EPIC25 marker (10 539 bp) showed a patchwork of signatures of asexual evolution, gene conversion and recombination, with the

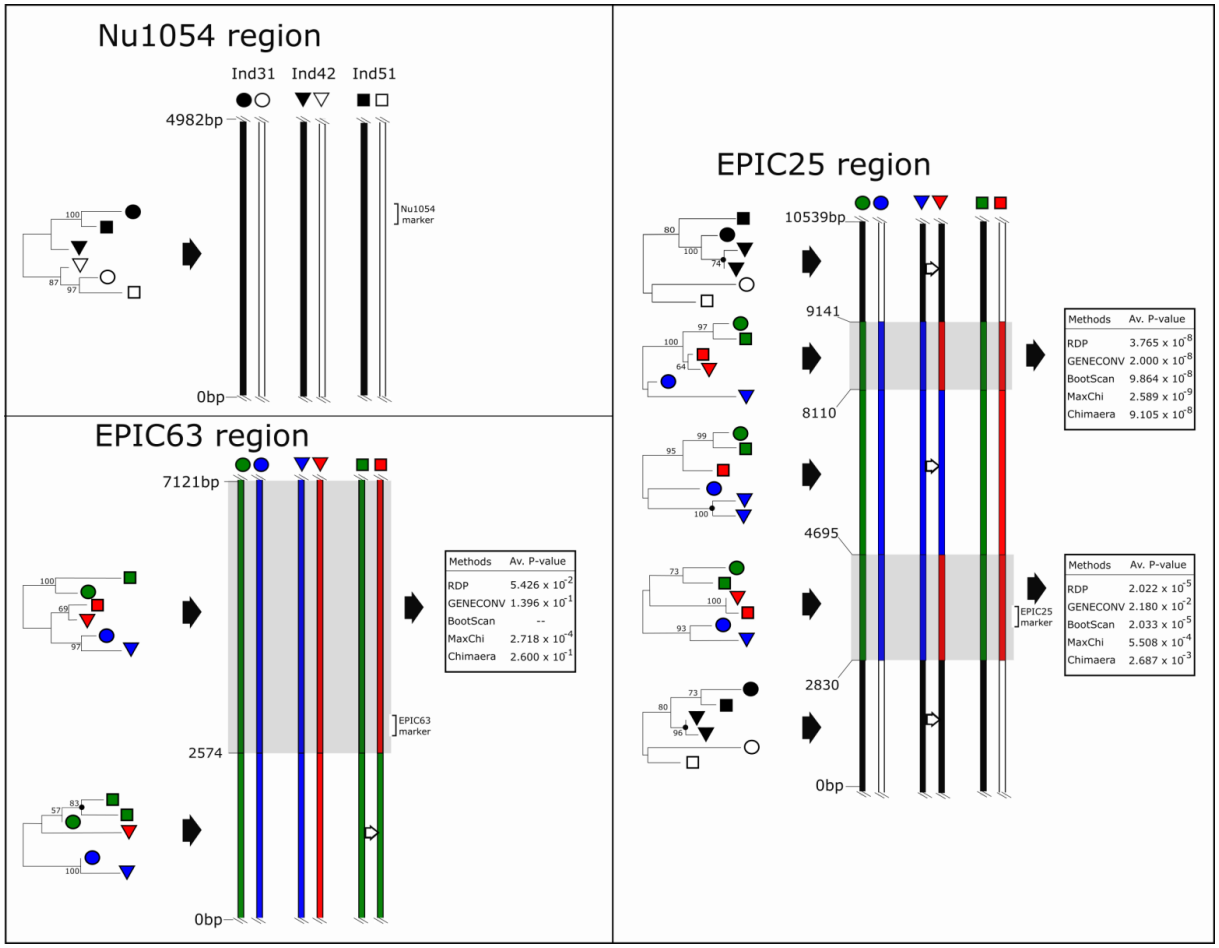

Figure 5 : Representation of the contigs assembled around the Nu1054, EPIC25 and EPIC63 markers for the three individuals (Ind31, Ind42 and Ind51) whose genome was sequenced. The size of each aligned region is indicated. The maximum-likelihood (GTR+G+I) trees indicate the evolutionary pattern for each portion of the contigs. Coloured tree labels correspond to the recombination patterns highlighted (grey areas) in the alignments. The tree built from the Nu1054 regions is characteristic of asexual evolution; the trees built from the other regions indicate allele sharing (*i.e.* recombination) among individuals and gene conversion events (shown with black dots on the trees and with white arrows on the phylogenies). The trees were rooted using the *Adineta vaga* homologous sequences from [6]. The tables show the results of the five recombination detection methods implemented in RDP3.

conversion events having occurred within individual 42 on both sides of the recombined region (the two identical alleles are shown in red and blue on Fig. 5a).

For the three individuals (Ind 31, 42, 51) of species C whose genomes were sequenced, we also assembled contigs around the heat-shock protein (*hsp82*) and histone *his3* genes (for which

recombination was reported within the genus *Macrotrachela* [11]). The *hsp82* assembly (1399 bp) produced a tree indicating allele sharing (*i.e.* recombination) between our three genotypes $a_1||b_1$, $a_1||c_1$ and $b_1||c_1$, whereas the four copies of *his3* (1642 bp when aligned) presented a pattern consistent with asexual evolution combined with gene conversion (Fig. S3).

Discussion

Our molecular approaches delimited six distinct species among wild-sampled bdelloid rotifers of the morphospecies *A. vaga*. This corroborates previous studies that detected cryptic species within the bdelloid morphospecies *A. vaga* [23] as well as in other bdelloid and monogonont rotifer species (see [20] [24] [25]). Although most of the genetic exchanges we detected occurred within these six cryptic species, some individuals combined marker sequences attributed to different species (Fig. 2 and 4). This provides strong evidence that inter- and intraspecific DNA exchanges occur within the bdelloid rotifer genus *Adineta*.

Sequencing the genomes of three species C individuals showing allele sharing (Ind 31, 42 and 51) revealed a striking patchwork pattern of regions exhibiting signatures of asexual evolution, inter-individual recombination and/or gene conversion (Figure 5). This pattern is unlikely to arise in the case of PTH (*Oenothera*-like) meiosis since haplotypes are transferred as entire blocks and only the relatively small and gene-poor telomeric regions experience intra-individual recombination (Fig. 6) [26]. Although such pattern may be produced by conventional meiotic recombination besides HGT (Fig. 6), our previous analysis of the genome structure of *A. vaga* showed that it lacks homologous chromosomes, making meiotic pairing and allelic segregation impossible and therefore conventional meiosis unlikely [6].

As only a total of five independent nuclear regions ranging in size from 5 to 10 kb were assessed in the present study and in the one of Signorovitch et al. [11], the question whether horizontal exchanges occur along the entire genome remains open. However, an indication might be found in the distribution of interkingdom HGTs (*i.e.* HGTs originating from bacteria, fungi and plants): 195 such genes of putative non-metazoan origin (AI>45) were distributed all across the ten largest scaffolds (1.08-0.93Mbp, ~4.1% of the total genome) of the *A. vaga* draft genome (Fig. S4). This result suggests that HGTs in the bdelloid *A. vaga* occur across their entire genome, and since the interkingdom HGTs are up to 8067bp long (see Suppl. Data), it seems plausible that the intra- and interspecific recombination patterns observed here and in Signorovitch et al. [11] also result from horizontal exchanges.

The recombination events observed in the bdelloids *A. vaga*, *Macrotrachela* [11] (integrating both non-metazoan and bdelloid DNA) and *Rotaria* ([10] for non-metazoan DNA) may be mediated by various mechanisms of DNA repair taking place after the DSB accumulation experienced during prolonged desiccation events [8]. For example, distantly related genetic material, e.g. of non-metazoan origin [6], could be integrated during DSB repair by non-homologous end-joining mechanisms requiring no homology between the repaired fragments. In contrast, DNA exchange between closely related individuals may be mediated by DSB repair through homologous recombination (HR), since the frequency of homologous recombination is strongly correlated with the degree of identity between the recombining DNA fragments and dramatically declines as the sequences diverge [27]. This hypothesis is reinforced by the observation that no additional copy of the EPIC25 locus was found in our *A. vaga* individuals, as would have been expected if new copies had been integrated into the genome in addition to the original copies. Instead, the transferred sequences replaced the original ones in the recipient individuals. As a consequence, closely related species should be more prone to genetic exchanges, which would explain why our study, focusing on intrageneric variation within the morphospecies *A. vaga,* detected multiple cases of genetic transfers.

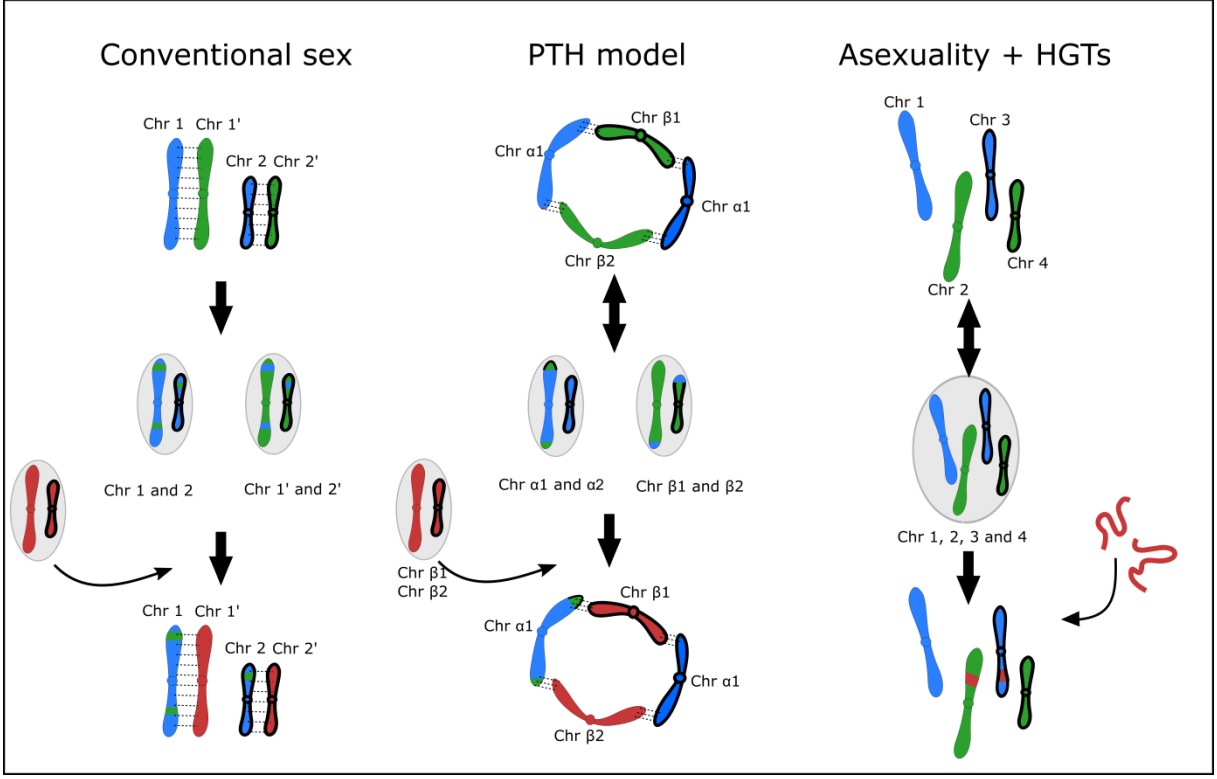

Figure 6 : Representation of the signatures expected under three potential mechanisms leading to allele sharing in regard to the mosaic genome structure observed for the individuals (Ind31, Ind42 and Ind51) whose genome was sequenced. Conventional sex refers to classical meiosis during which paternal and maternal (blue and green) chromosomes pair, segregates and form gametes that fuse with the gametes of another individual (red) leading to genome-wide exchanges. Colinearity is maintained because chromosome pairing is required at each generation. In the PTH model (*Oenothera*-like meiosis), colinearity is not mandatory as pairing is restricted to the chromosomes extremities. Chromosomes form two linkage group (blue and green) that segregate into two distinct gamete types ; fertilization only occurs between opposing gamete types. As for *Oenothera*-like meiosis, asexuality does not require chromosome pairing nor segregation. Frequent horizontal gene transfers (HGTs) between asexually diverging individuals lead to a patchwork pattern of signatures of allele-sharing mixed up with other regions exhibiting asexual evolution and/or gene conversion.

Another intriguing observation is that we did not detect any *A. vaga* individual harbouring two alleles of two distinct species at any locus studied. Instead, five of the six interspecific recombining individuals (Ind5, Ind21, Ind23, Ind58 and Ind66) were homozygous at the loci transferred, whereas the sixth individual (Ind 1) was heterozygous at the two transferred loci (EPIC25 and EPIC63) but presented in both cases two alleles from the same donor species. We speculate that after the integration of DNA, gene conversion promptly copied the integrated DNA on its homologous region (or vice versa). This is consistent with previous calculations showing that gene conversion occurs frequently in *A. vaga* (namely, 25 times more often than point mutation) [6].

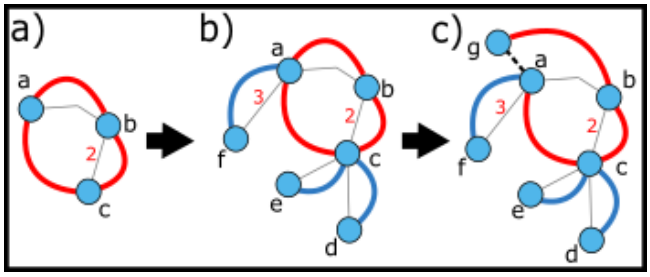

Figure 7 : Hypothetical evolutionary scenario explaining the transition from a pattern of shared alleles between three genotypes (left, as in species C for locus EPIC25) into a pattern of open cycles indicating past intraspecific recombination (as in species C for locus EPIC63 locus). a) Following recombination between individuals with genotypes a||b and c||b, a third genotype is formed (a||c), yielding a haplotype trio for that specific locus (in red). b) Subsequent point mutations accumulate, producing new genotypes in the population (c||d, c||e, a||f). c) After a few generations, the original haplotype trio may not be detectable anymore (either because of insufficient sampling or of genotype extinction) but open cycles (blue dashes) are still present as vestiges of past recombination events.

Our phylogenetic analysis of assembled genomic regions of the three individuals belonging to species C also presented a pattern indicating past allele sharing for the EPIC63 marker (Fig. 5), although no haplotype trio was observed in the haploweb for the EPIC63 marker (Fig. 3c). This is because only closed cycles were considered for detecting haplotypes trios, whereas open cycles (*i.e.,* groups of haplotypes that would be joined in a cyclic

fashion if one would neglect a few mutations) may indicate more ancient genetic exchanges followed by SNP accumulation (Fig. 7). If we include such open cycles when detecting genetic exchanges, all the individuals of species A for the EPIC25 marker show evidence of past recombination, as well as most individuals from species C (Fig. 3d). An open cycle is also found among species A at the Nu1054 marker, although here the individuals involved seem to have accumulated more mutations since the recombination events. These results suggest that the inter-individual recombination in bdelloids may be even higher than suggested by the number of haplotypes trios detected in our analyses.

The two species A and C for which we observed intraspecific recombination were also the most frequent in the community (see Fig. 1). This may suggest that intraspecific recombination is rare and only detectable under large sampling. Alternatively, intraspecific transfers could increase the fitness of those individuals, as suggested by Signorovitch et al. [11]. One possible explanation for the persistence of species in bdelloids could be niche differentiation [3]. To test this hypothesis, we analysed whether the assemblages of *A. vaga* species and haplotypes within each patch sampled, differed significantly from the null hypothesis of random assortment. Both Fisher's exact test and an analysis of phylogenetic structure of communities (see Suppl. Data) revealed highly significant departures from randomness and significant phylogenetic clustering. Because dispersal does not seem to be limiting in bdelloid rotifers [20] ecological specialisation may be the major parameter influencing spatial clustering which would support the hypothesis that differences in ecology maintain bdelloid species boundaries despite interspecific genetic exchanges. Moreover Hespeels et al. [9] reported that *A. vaga* individuals tend to group before entering desiccation, a phenomenon that may favour genetic transfers between related individuals sharing the same niche.

To conclude, our observations do not support the hypothesis of an *Oenothera*-like meiosis in bdelloids but are consistent with intra- and interspecific horizontal genetic transfers. As proposed previously [6] [9] [10] [28], desiccation could be the key mechanism shaping the genomes of bdelloids by mediating the introduction of new genetic material horizontally and by homogenizing the genome through frequent gene conversion events associated to DNA repair. As already suggested in Flot et al. [6], the homogenizing and diversifying roles of sex seems replaced in bdelloids by gene conversion and horizontal DNA transfer, in an unexpected (and possibly unique) convergence of evolutionary strategy with bacteria. Indeed, simulations suggest that unidirectional horizontal transfers are almost as efficient as bidirectional sexual recombination in preventing the accumulation of deleterious mutations and promoting the fixation of beneficial ones [29] [30]. We propose here to designate 'sapphomixis' (from "Sappho" the greek lesbian poetess and "mixis") this ameiotic strategy of allelic exchange and recombination among asexual, morphologically female organisms.

Our data presented here added one more piece of evidence to the asexuality of bdelloid rotifers; other pieces of evidence for their asexuality include the apparent absence of males or male organs in all individuals studied (whereas the PTH model would probably require the presence of males), the fact that meiosis was never observed in bdelloids so far, and the finding that *A. vaga*'s genome structure is incompatible with meiotic pairing and allelic segregation. Although none of these arguments is decisive on its own, their accumulation makes in our view bdelloid sexuality much less likely than horizontal genetic exchange.

Experimental procedures

Samples collection and DNA extraction

Thirty-six patches (25 lichen and 11 soil patches) were collected from five trees (of the genera *Acer* and *Platanus*) spread over less than 300 m² in Parc Louise-Marie, Namur, Belgium. The thirty-six collected patches were hydrated with spring water in separate Petri dishes, and all active bdelloid individuals morphologically identified as *Adineta vaga* that recovered from anhydrobiosis within 48 hours were isolated (following the protocol of [23]). DNA was extracted from each *A. vaga*

individual separately using the QIAamp DNA Micro kit (Qiagen) according to the manufacturer's instructions.

Markers development, DNA amplification and sequencing for each isolated individual are described in the supplemental data.

Phasing of Nuclear Markers from Direct Sanger Sequencing and Species Delimitation

For nuclear markers, the haplotypes of heterozygotes were directly reconstructed from the patterns of double peaks in the forward and reverse chromatograms for each nuclear marker [31] by using Champuru 1.0 [32], SeqPHASE [33] and PHASE [34].

Species delimitation was performed using two DNA taxonomy methods, one based on shared alleles [16] and the other one based on variation in branching rates in phylogenetic trees [17][18]. Haplotype webs (haplowebs in short) are haplotype networks or trees on which curves are added connecting haplotypes found co-occurring in heterozygous individuals [16]. A group of haplotypes linked together by heterozygotes forms an allele pool and the corresponding group of individuals is called a field for recombination (FFR), i.e., a putative species [17]. In contrast, the GMYC (generalized mixed Yule-coalescent) approach to species delimitation rests on the assumption that intraspecific branching follows a different model than interspecific branching [18].

To investigate allele sharing, median-joining haplotype networks [35] were constructed using the program Network, exported into PDF using Network Publisher (Fluxus Technology) and turned into haplowebs using Inkscape [36]. The allele sharing information from all our markers was then integrated into one consensus species delimitation using a "conspecificity matrix" approach. In this matrix, the conspecificity score of each pair of individuals is the number of markers for which these individuals belong to the same FFR. After computing this matrix, we reordered the rows and columns of the resulting sum to maximize the scores along the diagonal using the hierarchical clustering method implemented in the R package "heatmap3" [37]. Using this graphical, intuitive approach, species appear as blocks along the diagonal of the matrix (characterized by high conspecificity scores within blocks and low scores among them).

For the GMYC approach, our COI dataset was combined with other published COI sequences including 110 *Adineta* haplotypes from Fontaneto et al. [23] and 6 *Adineta* haplotypes from Birky Jr [38]. As outgroup in our tree, we used two sequences of the monogonont rotifer species *Brachionus plicatilis* downloaded from GenBank (accession numbers AF266895.1 and AF266853.1; [39]). We constructed an ultrametric Bayesian tree using the program BEAST v1.6.2 [40] with a single sequence for each haplotype as recommended by Tang et al. [41]. We chose the GTR+Γ4+I substitution model selected by jModelTest 3.8 [42] following the Bayesian Information Criterion [43]. The Markov chain Monte Carlo (MCMC) was run for10^8 generations with sampling every 10,000 generations. The tree with maximal clade credibility among the last 1,000 trees sampled by BEAST was determined using TreeAnnotator v 1.6.2 as implemented in the BEAST package [40]. This ultrametric tree was used as input for the GMYC analysis using the SPLITS R package (http://r-forge.r-project.org/projects/splits/). This method uses a maximum-likelihood approach to detect the shift in branching rate from interspecific branching (expected to follow a Yule model) to intraspecific branching (modelled as a neutral coalescent) [18][19].

Genomic Sequencing, Assembly and Analyses

We used the remaining gDNA (10-12ng) of three individuals (Ind31, Ind 42 and Ind51) that shared alleles of marker EPIC25 for whole-genome sequencing using the Illumina HiSeq2500 platform (Genomics core, UZ Leuven, Belgium). Because of the very low amount of input material, we started by performing 10 whole-genome amplification cycles before library preparation. Paired-end sequencing yielded in total approximately 100 million 101-bp read pairs for each individual.

Additional sequencing was performed for Ind42 (up to a total of 167 million 101-bp read pairs), as this library appeared more contaminated with non-bdelloid DNA compared to Ind31 and Ind51.

As *de novo* assemblies of the reads were extremely fragmented (data not shown), we resorted to assemble targeted genome regions using the MITObim package [44] that runs MIRA [45] iteratively, using our Sanger-sequenced genetic markers as baits. Each assembled contig was then scrutinized for SNPs using SAMtools [46] and Tablet [47]. The contigs were aligned in MAFFT (E-INS-i method [48]) and phased using SeqPHASE [33] and PHASE [34].

The phased sequences were then analysed with different methods (RDP, GENECONV, Chimaera, MaxChi and Bootscan) implemented in RDP3 [49] using default settings except that the general options “linear sequences”, “Bonferroni corrections” and “window size of 10” were selected to detect recombination events. Those methods measure the relatedness (pairwise genetic distances, phylogenies or substitution distributions *e.g.* chi square value, Pearson’s regression) of sequences using a window-based scanning approach. As our dataset was small (six sequences), RDP3 could not infer which sequences were the parental ones or the recombinant, and we therefore did not consider this information. Regions for which more than three methods detected recombination events were considered as recombinant and the different DNA fragments delimited were used to build maximum-likelihood trees in MEGA5 [50] following the GTR+Γ4+I model chosen by jModelTest [51] following the Bayesian Information Criterion [43].

AUTHOR’S CONTRIBUTIONS

N.D., X.L., J.F.F. and K.V.D. designed the experiment

N.D., X.L. and B.H sampled rotifer individuals

N.D. and X.L. prepared the DNA

N.D., C.Q.T., and I.E. developed the genetic markers

N.D. and J.F.F delimited the species

N.D., D.F. and J.F.F did the ecological analyses

N.D., J.F.F. and K.V.D wrote the core of the manuscript

K.V.D. acquired the funds necessary for this study

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SUPPLEMENTARY INFORMATION

Supplementary information, figures and texts are associated to this document.

Supplemental information: Genetic Exchange among Bdelloid Rotifers Is More Likely Due to Horizontal Gene Transfer Than to Meiotic Sex.

Experimental procedures

COI PCR Amplification and Sequencing

A fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene was PCR-amplified using universal primers [S1] (Suppl. Data, Table 1). Amplifications were performed in 25 µL reaction mixtures containing 1X GoTaq reaction buffer (1.5 mM MgCl2), 0.2 mM of each dNTP, 0.5 µM of each primer, 0.5 U of GoTaq DNA Polymerase (Promega), and 2 µL (ca. 10 ng) of genomic DNA. PCR conditions comprised an initial denaturation at 94°C for 4 min; followed by 30 cycles of 45 s denaturation at 94°C, 45 s annealing at 40°C, and 50 s elongation at 72°C; and a final elongation step of 10 min at 72°C. PCR products were sequenced in both directions with the same primers used for amplification (Genoscreen, Lille, France). Sequences were assembled and edited using Sequencher4 (Gene Codes).

28S rDNA Marker Development, Amplification and Sequencing

A large fragment of the 28S rDNA gene was PCR-amplified using primers designed from a set of full-length rotifer sequences (~2800bp) obtained from GenBank (*Adineta vaga* [DQ089739]; *Epiphanes senta* [DQ089742]; *Brachionus patulus* [AY829084] and *Rotaria rotatoria* [DQ089743]). These sequences were aligned in Geneious 5.4.2 [S2] using the MAFFT [48] plugin with the default settings. Four overlapping primer pairs (Suppl. Data, Table 1) were designed to provide a broad amplification success across both bdelloid and monogonont rotifers by binding to conservative regions; amplicon sizes ranged from 500 to 700bp. PCR conditions were the same as for COI except that the annealing temperature was in this case 60°C.

EPIC25 and EPIC63 Marker Design and Amplification

We adapted the pipeline for designing exon-primed, intron-crossing (EPIC) markers developed by Li et al. [S3]. Here, we used the coding sequences (CDS) of the *Adineta vaga* genome [6] from which we selected introns of 350-450 bp surrounded by exons longer than 50bp. To check the copy number of each candidate EPIC marker retrieved from the pipeline, we blasted each exon against the genome scaffolds using BioEdit 2.2.10 [S4] and selected only genes with four copies present in the genome (because of its degenerate tetraploid structure [6]) with a threshold e-value of 10^{-5}. We aligned the four copies using MAFFT ([19]; E-INS-i method) then designed the PCR primer pair EPIC25+63F and EPIC25+63R (Suppl. Data, Table 1) using Primer3 (http://primer3.ut.ee/ [S5]). To obtain chromatograms with double peaks but no triple or quadruple peaks, we designed ohnologue-specific sequencing primers EPIC25F, EPIC25R, EPIC63F and EPIC63R (Suppl. Data, Table 1).

The designed EPIC markers were PCR-amplified using the same protocol as for COI but with an annealing temperature of 54°C.

Nu1054 Marker Development and Amplification

Orthologs from transcriptomes of the bdelloid species *Rotaria socialis, R. magnacalcarata, R. sordida,* and *R. tardigrada* present in the *Adineta ricciae* transcriptome [8] were aligned using MAFFT

([48]; E-INS-i method). Primers were designed using Primer3 [S8] with a minimum and optimal annealing temperature of 45°C and 50°C, respectively, but using default settings for all the other parameters. Twenty-four primer pairs were screened by PCR using four genomic DNA extracts from lab cultures of *Adineta riccae, Adineta vaga, Habrotrocha bidens*, and *Habrotrocha elusa elusa*. Of these 24 primer pairs, Nu1054F and Nu1054R (Table 1) consistently amplified a 418-bp fragment. This marker, called Nu1054, is present in two copies in the *A. vaga* reference genome [6] and is of non-metazoan origin.

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Supplemental figures and legends

Figure S1: Haploweb of the nuclear markers a) 28S rDNA, b) Nu1054, c) EPIC63 and d) EPIC25 amplified in 82 *Adineta* individuals. Haplowebs consist in median-joining haplotype networks on top of which links (here shown in blue) are added between haplotypes (alleles) that co-occur within heterozygous individuals. The number of mutations (SNPs) separating two haplotypes is indicated in red when higher than one. The frequency of each haplotype is not represented as all circles are of the same size. Each allele pool (group of haplotypes linked together by heterozygous individuals) are represented in grey.

Figure S2: Pruned ultrametric tree showing the six species delimited by the GMYC method (Pons et al, 2006). The original tree built from the total COI dataset (our 40 haplotypes, 110 haplotypes from Fontaneto et al (2011), 6 haplotypes from Birky Jr et al (2010) and two *Brachionus* sequences used as outgroup (Gomez et al, 2000) for the GMYC analysis and the statistics of the test are available in the Excel file. The pattern of intra-specific branching (neutral coalescence) is represented in red whereas inter-specific branching (Yule process) is in black. The six species (A-F) delimited by the congruence matrix method are indicated.

Figure S3: Representation of the contigs assembled around the *hsp82* and *his3* markers (Hur et al, 2009; Signorovitch et al, 2015) for the three individuals we sequenced. The size of each aligned region is indicated. The Maximum-Likelihood (GTR+G+I, MEGA5) trees indicate the evolutionary pattern for each portion of the contigs. Coloured tree labels correspond to the recombination patterns highlighted in the alignments. The two allelic pairs (A1/A2 and B1/B2) of each individual

were represented for the *his3* locus. The tree built from the *his3* region is characteristic of asexual evolution (for the allelic pair B) followed by a gene conversion event in ind42 (for the allelic pair A). In contrast, the tree built from the *hsp82* region is representative of allele-sharing among individuals. The trees were rooted with the *Adineta vaga* homologous sequences from Flot et al (2013) but were not displayed.

Figure S4: Distribution of the horizontally acquired genes along the ten longest scaffolds of the draft genome of *Adineta vaga* (Flot et al, 2013). In total, 195 genes harboured by those ten scaffolds had an Alien Index superior to 45 (AI>45). The blue dots indicate the location of each HGT for each scaffold.

Table S1: Sequences of the primer pairs used to amplify the five genetic markers.

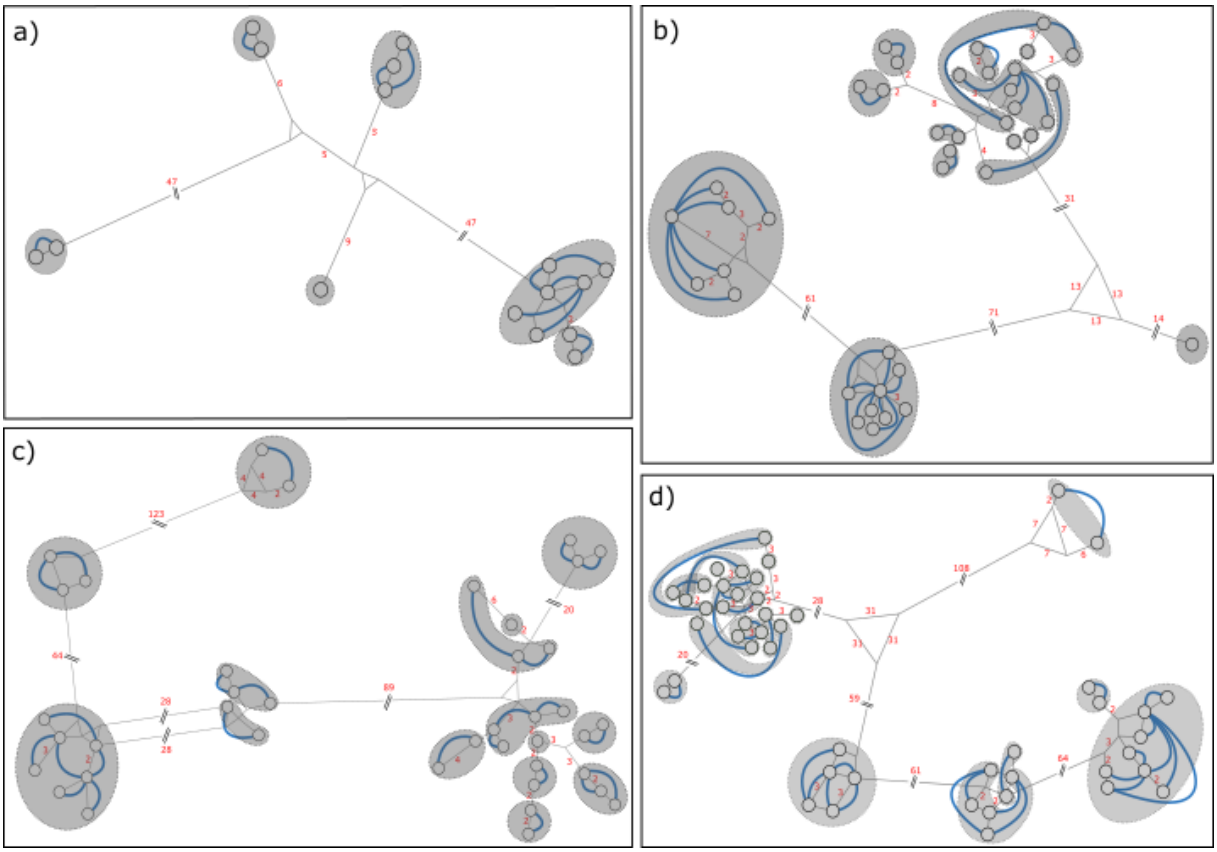

Figure S1

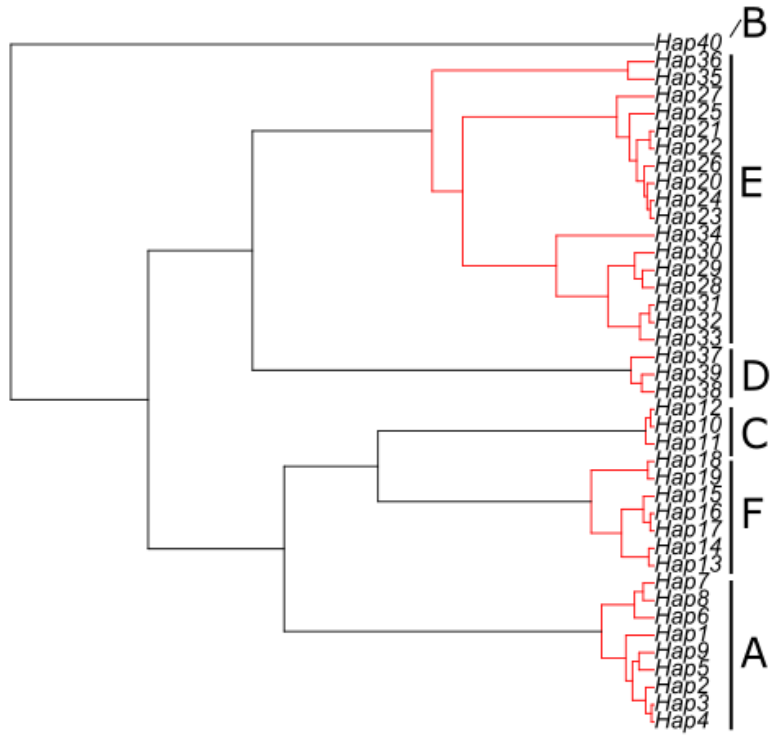
B
Hap40
Hap36
Hap35
Hap27
Hap25
Hap21
Hap22
Hap26
Hap20
Hap24
Hap23
Hap34
Hap30
Hap29
Hap28
Hap31
Hap32
Hap33
E
Hap37
Hap39
Hap38
D
Hap12
Hap10
Hap11
C
Hap18
Hap19
Hap15
Hap16
Hap17
Hap14
Hap13
F
Hap7
Hap8
Hap6
Hap1
Hap9
Hap5
Hap2
Hap3
Hap4
A

Figure S2

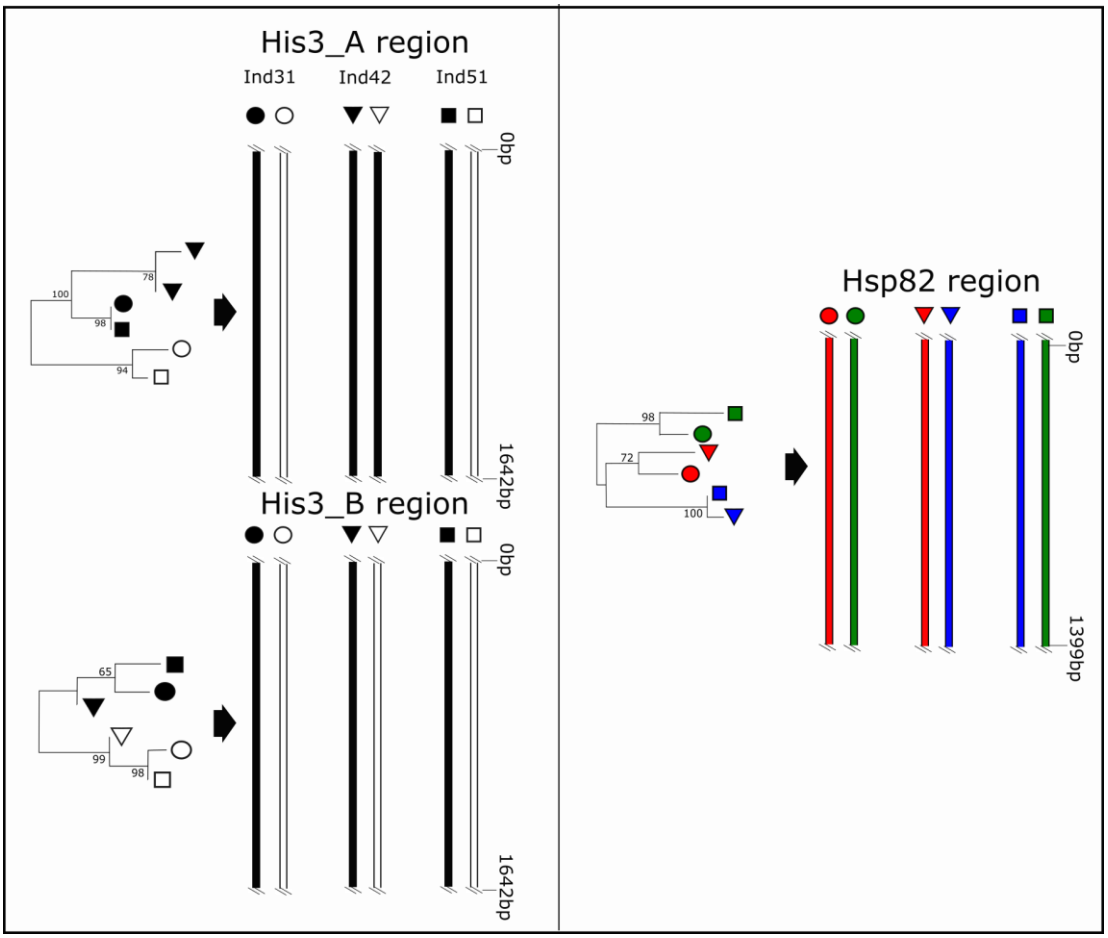

Figure S3

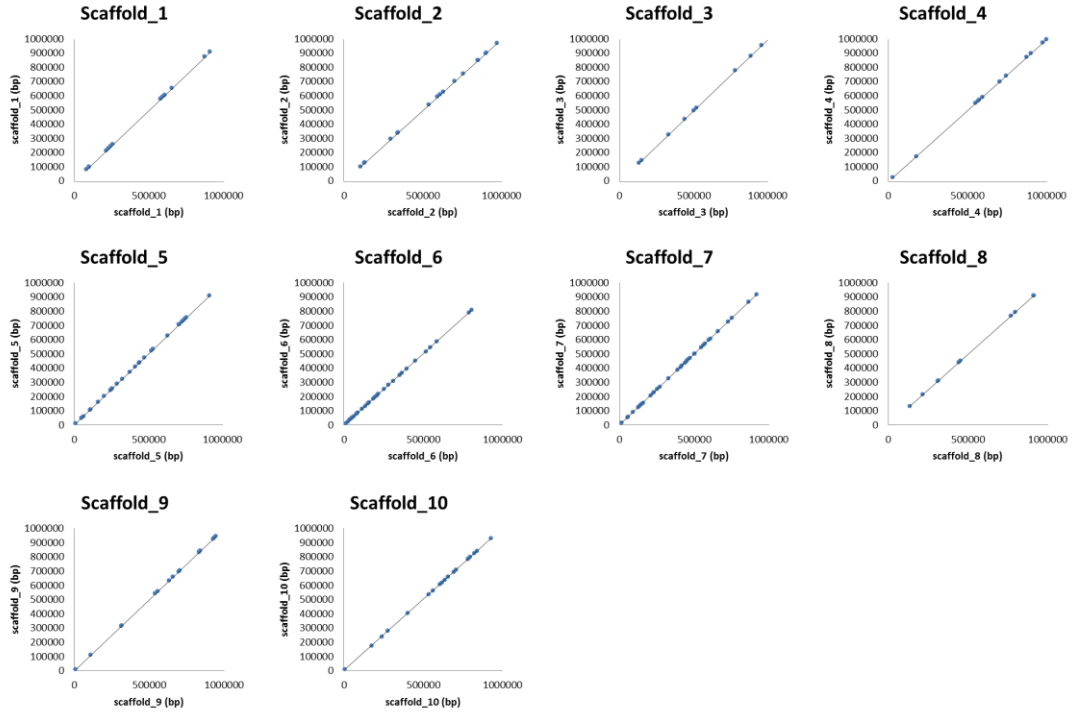

Figure S4

Primer name	Primer sequence
LCO-1490	5′ - GGT CAA CAA ATC ATA AAG ATA TTG G - 3′
HCO-2198	5′ - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3′
28S0FCT	5′-ACG AAT GGC CGC ATT CAT CAG AT-3′
28S1RCT	5′-GTT TGA CGA TCG ATT TGC ACG TC -3′
28S2FCT	5′-GAC CCG AAA GAT GGT GAA CT-3′
28S2RCT	5′-CGT CAG TCT TCA AAG TTC TCA TTT GA-3′
28S3FCT	5′-GCG TCG AAG GCT AAC ACG TGA-3′
28S3RCT	5′-TGT TTT AAT TAG ACA GTC GGA TTC C-3′
28S4FCT	5′-CTT CGG GAT AAC GAT TGG CTC TAA G-3′
28S5RCT	5′-GAG TCA AGC TCA ACA GGG TCT TCT T-3′
EPIC25+63F	5′-TGA AGA TGC ATT TTG TTT GCT T-3′
EPIC25+63R	5′-GCT GAA AAA GAA TGT GCC GTA-3′
EPIC25F	5′-GCA TTT TGT TTG CTT TGG ATG T-3′
EPIC25R	5′-AAA AAG AAT GTG CCG TAC TTG GT-3′
EPIC63F	5′-GGA TAT GCA TTT TGT TGG CTT T-3′
EPIC63R	5′-CTC GGC TCG TTA TTT CAA GG-3′
Nu1054F	5′-AGT ACG TGG ACC TAT GGG TAT TGG-3′
Nu1054R	5′- CCT GGT GGA GTA TCA TCT ACT TTG ACA-3′

Table S1

Chapter 2: Response and thoughts on Wilson and colleagues comments

Soon after the publication of our results (see chapter 1) in *Current Biology*, various comments from the scientific community emerged. The first comment was made by Matthew Meselson and colleagues who reported allele sharing among three individuals from the same *Macrotrachela quadricornifera* mitochondrial clade (Signorovitch et al, 2015). Their approach was similar to our experimental setup, a genetic study using four unlinked genomic regions (2.8-9.7kb) corresponding to the *his* (A and B) and *hsp* (A and B) genes, and sampling only bdelloid individuals identified as *M. quadricornifera*, but isolated from different geographic areas. Interestingly, the authors observed one pattern of allele sharing across all the loci studied between three individuals, a hallmark of sex. Given the ameiotic genome structure of bdelloid rotifers, they concluded that their results could still be explained by sexual reproduction with a specific *Oenothera*-like meiosis without chromosome pairing (except at the telomeric regions). In our publication, we refuted this hypothesis by arguing that such meiotic mechanisms should result in the exchange of complete haplotypes sets whereas we observed a patchwork of signatures (recombination, gene conversion and asexual divergence) within a specific genomic region, giving more weight to the possibility for horizontal gene transfer among bdelloids. Although their results and interpretation were slightly different than ours, both studies led to the main conclusion that DNA transfers were possible among bdelloid rotifers but that further investigations were needed (Signorovitch et al, 2016; Flot et al, 2016; see also comment of Schwander, 2016).

Later, Chris Wilson and colleagues (in prep.) argued that our results could be explained by cross-contaminations between individuals. In their paper, the authors raised concerns about the possibility for DNA transfers between individuals with a genetic divergence of approximately 30% (mtCOI similarity between species C and E is 86.4%, see chapter 1). Moreover, they invoked that the use of whole-genome amplification kits on single bdelloid individuals may easily amplify contaminant DNA and bias the results of our Current Biology paper. More specifically, samples contaminated by the presence of a second individual could produce the same pattern we observed if WGA unequally amplifies each allele present depending on the GC-content. The authors presented different conditions in which WGA could indeed induce amplification biases. They used the mtCOI chromatograms from our study to check the presence of more than one individual per sample because each animal should harbor only one mtCOI haplotype and this was the only marker sequenced prior WGA. The statistical method Wilson et al. (in prep.) designed to detect contaminant sequences (ConTAMPR) was manually applied to some of our samples presenting signatures for interspecific transfers (Debortoli et al, 2016), revealing contamination signals. The method could not be applied to our samples with signatures of intraspecific transfers. In this chapter 2 we discuss their results and make some clarifications about our own results. Furthermore, we automatized their ConTAMPR method, which appears an interesting tool to verify contaminations in WGA samples, to make analyses reproducible as we showed that it was sensitive to errors when implemented manually.

<u>The response to Meselson's comment was published in Current Biology:</u>

Flot, J. F., Debortoli, N., Hallet, B., & Van Doninck, K. (2016). Response to Signorovitch et al. *Current Biology*, *26*(16), R755.

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Schwander, T. (2016). Evolution: the end of an ancient asexual scandal. *Current Biology*, *26*(6), R233-R235.

Signorovitch, A., Hur, J., Gladyshev, E., & Meselson, M. (2015). Allele sharing and evidence for sexuality in a mitochondrial clade of bdelloid rotifers. *Genetics*, *200*(2), 581-590.

Signorovitch, A., Hur, J., Gladyshev, E., & Meselson, M. (2016). Evidence for meiotic sex in bdelloid rotifers. *Current Biology*, *26*(16), R754-R755.

Wilson, C. G., Nowell, R. W., & Barraclough, T. G. (2017). Evidence for" inter-and intraspecific horizontal genetic transfers" between anciently asexual bdelloid rotifers is explained by cross-contamination. *bioRxiv*, 150490.

Published paper: Response to Signorovitch *et al.*

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Summary

Signorovitch *et al.* [1] comment that an *Oenothera*-like meiosis [2] could produce a pattern similar to what we observed in our study of natural isolates of the bdelloid rotifer *Adineta vaga*, which we attributed to horizontal gene transfers (HGTs) [3]. Indeed, our HGT hypothesis appears at first sight difficult to conciliate with their observation of a congruent pattern of allele sharing at four large loci possibly located on different chromosomes [4]. However, one might imagine conditions under which massive horizontal gene transfer between bdelloid individuals could produce such a pattern, notably if the individuals involved had previously lost most of their heterozygosity because of their exposure to frequent desiccation (which produces DNA double-strand breaks [5]). In the published *A. vaga* genome the loss of heterozygosity due to large-scale gene conversion events or break-induced replication covers only about 10% of the genome [6], but this percentage may be much higher in environmental isolates that often experience desiccation. Besides, if an *Oenothera*-like mode of meiosis occurs in bdelloids frequently enough to be detected in a single sampling of 29 individuals (as in [4]), one would expect males and meiosis to be observed at least occasionally, and instances of congruent allele sharing across loci should turn up frequently in genetic surveys. This was not the case in [3]: among the 82 *A. vaga* individuals sequenced for four nuclear markers, no trio of individuals presented congruent patterns of shared sequences at different loci. For these reasons, and in the absence of any direct evidence for an *Oenothera*-like meiosis in bdelloids, we still consider inter-bdelloid HGTs a more parsimonious explanation for our results.

Main Text

Clearly, additional multilocus surveys of allele sharing in bdelloid populations will be required to solve the puzzling discrepancy between our observations and those of Signorovitch *et al.*, as well as to determine the mechanism and frequency of genetic exchange among bdelloids. A completely assembled bdelloid genome sequence would also allow us to test the hypothesis of an *Oenothera*-like mode of meiosis: one prediction of this model is that pairs of colinear telomeric regions should be detected (see Figure 6 in [3]). Besides, one might imagine that several distinct mechanisms mediate genetic exchange in bdelloids, possibly in a genus or clade-specific fashion (notably, the uneven chromosome numbers of *Philodina rosela* and *Habrotrocha tridens* mentioned in [4] do not seem compatible with the *Oenothera* model, in which there should be an equal number of α and β chromosomes [2]). Whatever the outcome, this is an exciting time for rotiferology, as upcoming population genomic surveys will allow us to unravel and quantify how bdelloid rotifers, which were until recently dubbed “ancient asexual scandals” [7], actually exchange genetic information.

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Submitted paper: Response to Wilson *et al.*

Debortoli N., Hallet B., Flot J.-F. & K. Van Doninck

Introduction

A group of microscopic metazoans, namely the bdelloid rotifers, have reproduced asexually for over 60 million years and, as such, have been considered as "evolutionary scandals" (Maynard Smith, 1978). The most direct evidence of bdelloid asexuality was provided by the whole-genome sequencing of the first lineage, *Adineta vaga*, which contains alleles organized as palindromes on the same chromosome, a structure incompatible with conventional meiosis (Flot et al., 2013). However, two recent publications based on population genetic analyses showed signatures of DNA transfers within and between bdelloid species (Signorovitch et al., 2015; Debortoli et al., 2016). These results did start some debates among researchers in this field, commenting on those results (Umen 2015; Signorovitch et al., 2016; Flot et al., 2016; Schwander 2016; Wilson et al., pers. comm.).

It is however not so surprising to observe signatures of DNA exchanges in animal species with unusually high number of horizontal gene transfers (HGTs) from non-metazoan sources (Gladyshev et al., 2008; Flot et al., 2013; Eyres et al., 2015). Even though no precise mechanism underlying those massive HGTs have ever been determined, different characteristics of bdelloid rotifers may promote such transfers. First, most bdelloid species desiccate frequently in their temporarily semi-terrestrial habitats, a phenomenon known to induce multiple genomic double-strand breaks. During desiccation fragmented DNA could enter more easily through the leaky membranes and subsequently be integrated in the genome following DNA DSB repair (Hespeels et al., 2014; Eyres et al., 2015). Second, the presences of previously undescribed giant transposable elements, called terminons, in telomeric regions that can exceed 40 kb in length and in-between which host genes are captured. Those elements are strongly co-located with foreign genes in *A. vaga*, eventually participating in intra- and intergenomic transfers (Arkhipova et al., 2017).

In a recent comment on the paper of Debortoli et al. (2016), Wilson et al. however postulated that "Evidence for inter- and intraspecific horizontal genetic transfers among anciently asexual bdelloid rotifers **is explained** by cross-contamination" after a detailed examination of our raw data (Wilson et al., in prep). The authors have developed a simple, yet interesting, statistical method (ConTAMPR) to evaluate if the pattern of double peaks observed in the recombining individuals were due to sample contaminations. This comment results from a year-long discussion and collaboration during which we tested several alternative hypotheses taking into account their cross-contamination concerns. We are aware that contaminations have been at the origin of several incorrect claims of HGTs (Tardigrades, photosynthetic slugs). We therefore started a new experiment based on a more adapted, experimental setup already before the release of Debortoli et al (2016). Yet, it is important to note here that the results of our CB paper (Debortoli et al. 2016) are strong enough to discard their contamination hypothesis, which we outline in the present chapter.

Precisions regarding the sampling and bdelloid rotifer isolation protocol

The first argument raised by Wilson et al. in favor of the contamination hypothesis is that "In every case where incongruence was reported, the inferred *donor species* was recovered from the same maple or plane tree as the *recipient individual* at the time of sampling […]. If genetic exchange occurs so promiscuously among such diverse and mobile animals, why should every *donor species* happen to be sampled in the same small area as the recipients at the same time? Even more striking,

every case of incongruence involves a haplotype whose sequence is identical to a haplotype found 'natively' in one of the other 81 rotifers sampled". This is actually not the case. As an example, Ind1 (with signatures of interspecific recombination) harbored the allele hap9 for marker EPIC25, which was never observed in any other individual. Besides, in the cases of interspecific DNA exchanges, the "recipient" and the "donor" could be found on the same tree, but never within the exact same lichen or soil patch. For example, Ind1 (named B14) harbored markers from species A, C and E and was sampled in lichen B1 whereas the only individuals from the same tree B attributed to species C were found in lichen B2, more than a meter away and similarly, individuals from tree B belonging to species E were present in lichen B3 near the roots. The same observation can be made for the other cases of interspecific transfers. This was probably a misunderstanding of our results that deserved additional precision here.

In addition, we want to clarify here our isolation protocol because of the main criticism by Wilson et al. suggesting that our methodology was not rigorous enough to avoid contaminations. We isolated single bdelloid individuals in individual Eppendorf tubes using the same commonly accepted method (Ricci and Melone, 2000; Fontaneto et al., 2008; Wilson and Sherman, 2010; Iakovenko et al., 2013). More specifically, each individual was washed by pipetting it into several clean Spa® water drops and transferred to one tube. To avoid any contamination, we used new pipette tips and distinct water drops for each transferred individual. We then centrifuged each tube to pellet the individual and inspected its presence under binoculars. If no individual was visible at this step, the tube was discarded. We are aware of the difficulties to manipulate and isolate active bdelloid rotifers, especially *Adineta* species, which are particularly sticky. However, we think that this commonly used protocol should have prevented contamination by a second individual, and should certainly not have resulted in more than 10 contaminations (the number of individuals with inter- and intra-specific signatures). We were surprised that our isolation protocol was challenged by the authors as their own isolation method was summarized by "individual rotifers were transferred singly by pipette from a source population" (Wilson and Sherman, 2010) and did not differ from the general protocol used by several rotiferologists. Moreover, Wilson et al. did not provide an alternative protocol for *Adineta* species isolation, even upon request. To the best of our understanding, the only difference between both protocols is the use of glass tips instead of plastic ones.

Testing for whole-genome amplification skewness

After our first discussions with Wilson et al. in October 2016, raising doubts about the results of DNA transfers among bdelloid species (Debortoli et al., 2016), we checked for the skewness induced by the Illustra GenomiPhi V2 DNA amplification kit (GE Healthcare). We used two distinct *A. vaga* species kept in culture, initially named AD008 and 42.1D5. The percentage identity between those two species at the mtCOI marker is 84.7%, similarly to the species A and E (86.1%) or species C and E (86.4%) for which we found interspecific recombinants (Debortoli et al., 2016). We isolated single animals from each species in individual Eppendorf tubes using the same method than in our original study. We extracted the gDNA of each individual independently and quantified it using the Qubit fluorometric quantitation protocol (Thermo Fisher Scientific). We then mixed gDNA of the two species in triplicates according to three distinct ratios, 1:1, 1:10 and 1:50, strain AD008 being underrepresented for the last two dilutions. The nine tubes were then submitted to WGA and the resulting amplicons were used as template for PCR-amplification of the nuclear Nu1054 marker, as in Debortoli et al. (2016).

We could retrieve signals corresponding to the distinct alleles of both AD008 and 42.1D5 in the chromatograms of eight out of the nine samples; even the ones that were diluted 1:50 (Figure 1). This shows evidence that there is a skew linked to the WGA and/or PCR reactions. Those results indicate that a low amount of DNA from one individual can become majority when amplified together with the DNA of a second individual. However in one of the 1:1 dilution replicate, only the alleles of one individual were retrieved suggesting possible strong amplification biases. Wilson et al. reported that this could be due to higher affinity of the Illustra GenomiPhi V2 DNA amplification kit for templates with lower GC content (Han et al., 2012). Here, the amplification bias was in favour of the template with the higher GC content, although the difference between the two rotifer species was limited in Nu1054 region (35.6-37.7% in strain AD008 whereas clone 42.1D5 had a GC content of 34.2-35.1%). It is not possible at this stage to know if the bias results from differences in GC content or simply from clone-specific mutations in the priming sites.

Finally, a preoccupying pattern resulting from WGA was observed at the red star position (Figure 1). At this site, the minority peak expected “G” (black) was missing, indicating the absence of one (AD008_allele_a) of the four alleles on the chromatogram. In our study Debortoli et al. (2016) we never had one allele of one individual combined with the allele of a second individual, resulting in a diploid “hybrid” individual. Moreover, in our study, if contamination by a second individual occurred in the same tube, it should have been in a 1:1 dilution ratio. We are however aware here that a lot of caution should been taken when using multiple amplification steps.

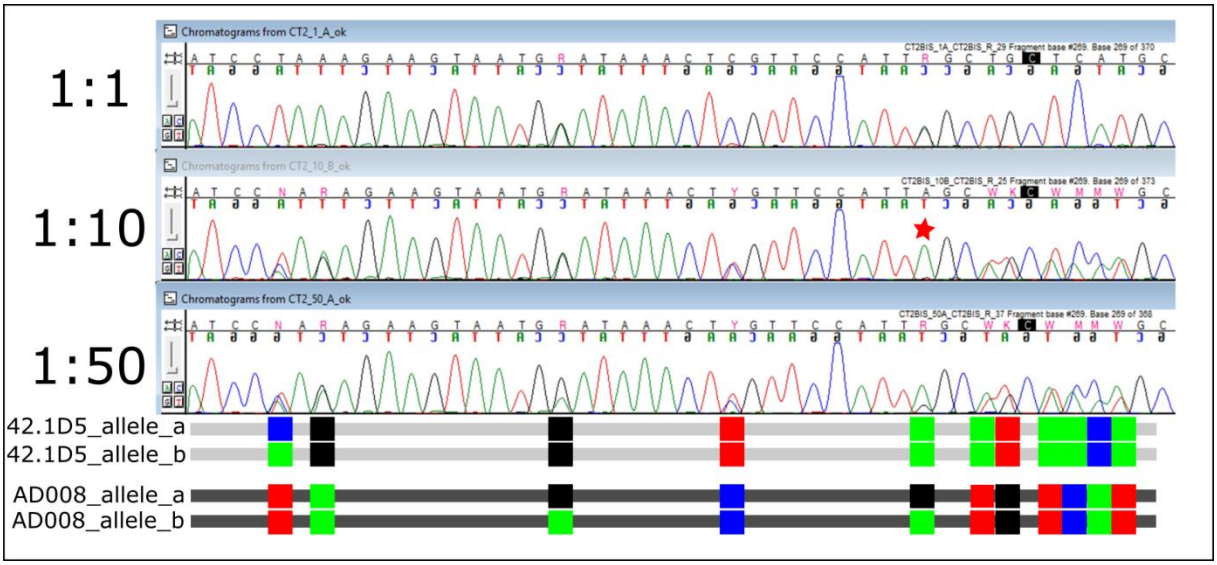

Figure 1 : Chromatograms of the Nu1054 marker amplified from WGA amplicons. The gDNA of individual 42.1D5 and AD008 were mixed in 1:1, 1:10 and 1:50 ratios prior WGA respectively. The chromatogram of each dilution was aligned for minority peak analysis. The varying sites of the expected four alleles are presented below the Sanger chromatograms. The red star highlights the site for which an expected secondary peak is missing.

The ConTAMPR method to determine the presence of contaminant sequences

Wilson and co-authors presented a simple method to statistically distinguish random sequencing noise from contamination. The method proposed by Wilson et al. consists in aligning the chromatograms obtained through Sanger sequencing in both directions with a potentially contaminant sequence and, for each polymorphic site, record the rank of the base fitting the contaminant haplotype. Contingency tables are then used to determine if the overall distribution of the peak ranks significantly differed from a null distribution as expected under the random noise

often observed in Sanger chromatograms. Wilson et al. applied this simple quantitative method, named ConTAMPR, to their own dataset showing that uncontaminated samples presented minority peaks distribution suiting the null model. In contrast, samples that were deliberately contaminated by putting two individuals of distinct species (*Adineta sp.* AD006 and *A. vaga* AD008, 87.5% identity for mtCOI) in a same tube provided results that were significantly deviating from the null expectation.

We think that this method is interesting as it reduces the chances to cherry-pick accommodating peaks. The application of this method is however restricted to a dataset for which the potential contaminant, or closely related sequences, are known. In addition, this χ^2-based method is highly sensitive to sample size, i.e. the number of observations or SNPs between the compared haplotypes. It may thus be irrelevant to apply it to compare closely related sequences.

Intraspecific DNA transfers

The main conclusions of Debortoli et al. (2016) stemmed from the observation of allele-sharing patterns within the EPIC25 and Nu1054 haplowebs that were interpreted as evidence for intraspecific DNA transfers (see Figure 3b, d and Figure 5 from Debortoli et al., 2016). On those haplowebs, individuals from a same species harboured genotypes a||b, b||c and a||c in a cyclic fashion that can hardly be explained other than intraspecific DNA transfers. This pattern was observed at two loci (EPIC25 and Nu1054) in species A and at one locus (EPIC25) in species C (Table 1). In the case of intraspecific exchanges it is more complicated to check for DNA contamination by other individuals as the number of SNPs is reduced in the mtCOI (0-21 SNPs within species A and 0-3 SNPs within species C). We had a closer look at the mtCOI sequences of all the individuals involved in the cycles and could not retrieve minority peaks testifying the presence of a second haplotype. To rule out all doubts, we applied the ConTAMPR method to those individuals, even though this may be irrelevant when haplotypes are separated by a really low genetic distance, leading to a restricted number of observations to which χ^2 is sensitive. We could not find any significant correlation between the distribution of minority peaks rank and the presence of a potential contaminant, even when the chromatograms were aligned with the most distantly related haplotype (hap1|hap9 = 15 SNPs) that would be statistically less sensitive.

Table 1 Individuals from Debortoli et al. (2016) dataset that belonged to species A and C for which patterns of allele sharing were observed. Each genotype contributing to the allele sharing cycle is highlighted in a different colour.

		ID_individual	COI (species)	28S (species)	EPIC25 (species)	EPIC63 (species)	Nu1054 (species)
Species A	IND_2	B15	Hap6 (A)	Hap1 (A)	Hap1 (A)	Hap1 (A)	Hap2 (A)
	IND_3	C1T1	Hap3 (A)	Hap1 (A)	Hap1 (A)	Hap1 (A)	
	IND_4	C3T3	Hap3 (A)	Hap1 (A)	Hap1 (A)	Hap1 (A)	Hap1-Hap5 (A)
	IND_6	D12	Hap3 (A)	Hap1 (A)	Hap1-Hap4 (A)	Hap1 (A)	Hap1-Hap6 (A)
	IND_7	D13	Hap3 (A)	Hap1 (A)	Hap1-Hap4 (A)	Hap1 (A)	Hap1-Hap7 (A)
	IND_8	E1B1	Hap4 (A)	Hap1 (A)	Hap4 (A)	Hap1 (A)	
	IND_9	E1B3	Hap4 (A)	Hap1 (A)	Hap1-Hap4 (A)	Hap1 (A)	Hap1-Hap4 (A)
	IND_10	H001	Hap1 (A)	Hap1 (A)	Hap1 (A)	Hap1 (A)	Hap1-Hap8 (A)
	IND_11	H3-03	Hap1 (A)	Hap1 (A)	Hap1-Hap2 (A)	Hap1 (A)	Hap1-Hap2 (A)
	IND_12	H3-04	Hap2 (A)	Hap1 (A)	Hap2-Hap4 (A)	Hap1-Hap3 (A)	Hap2-Hap9 (A)
	IND_13	HB01	Hap1 (A)	Hap1 (A)	Hap1-Hap2 (A)	Hap1 (A)	Hap1-Hap2 (A)
	IND_14	C28	Hap3 (A)	Hap1 (A)	Hap4 (A)	Hap1 (A)	Hap2 (A)
	IND_15	C33	Hap9 (A)	Hap1 (A)	Hap1-Hap4 (A)	Hap1-Hap3 (A)	Hap2-Hap3 (A)
	IND_16	C31	Hap3 (A)	Hap1 (A)	Hap4 (A)	Hap3 (A)	Hap2 (A)
	IND_17	C1T2	Hap4 (A)	Hap1 (A)	Hap2-Hap4 (A)	Hap2-Hap3 (A)	
	IND_18	D11	Hap3 (A)	Hap1 (A)	Hap2-Hap4 (A)	Hap1 (A)	Hap2 (A)
	IND_19	E11	Hap3 (A)	Hap1 (A)	Hap4 (A)	Hap3 (A)	
	IND_20	E31	Hap5 (A)	Hap1 (A)	Hap3-Hap5 (A)	Hap1 (A)	Hap1-Hap3 (A)
	IND_22	B33	Hap7 (A)	Hap1 (A)	Hap1-Hap5 (A)	Hap1 (A)	
Species C	IND_30	H004	Hap10 (C)	Hap5-Hap6 (C)	Hap6 (C)	Hap4-Hap7 (C)	Hap10-Hap15 (C)
	IND_31	H4-28	Hap10 (C)	Hap5-Hap6 (C)	Hap6-Hap14 (C)	Hap7-Hap8 (C)	Hap10-Hap16 (C)
	IND_32	Hprim34	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	
	IND_33	Hprim53	Hap11 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap11 (C)
	IND_34	HPRIME1	Hap12 (C)	Hap5-Hap6 (C)	Hap6 (C)	Hap4 (C)	Hap10 (C)
	IND_35	HPRIME21	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap7 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_36	HPRIME22	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_37	HPRIME36	Hap12 (C)	Hap5-Hap6 (C)	Hap10 (C)	Hap4 (C)	Hap10 (C)
	IND_38	A110	Hap12 (C)	Hap5-Hap7 (C)	Hap6-Hap10 (C)	Hap4-Hap5 (C)	Hap10-Hap11 (C)
	IND_39	A12	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_40	A11	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_41	A111	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_42	A112	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_43	A13	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_44	A14	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_45	A15	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_46	A18	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_47	A19	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_48	C29	Hap10 (C)	Hap5-Hap6 (C)	Hap10-Hap15 (C)	Hap4-Hap6 (C)	Hap10-Hap13 (C)
	IND_50	D22	Hap10 (C)	Hap5-Hap6 (C)	Hap10-Hap14 (C)	Hap4-Hap9 (C)	Hap10-Hap12 (C)
	IND_51	D23	Hap10 (C)	Hap5-Hap6 (C)	Hap10-Hap14 (C)	Hap4-Hap9 (C)	Hap10-Hap12 (C)
	IND_52	D21	Hap10 (C)	Hap6 (C)	Hap10-Hap15 (C)	Hap4 (C)	Hap10-Hap12 (C)
	IND_53	A16	Hap12 (C)	Hap6 (C)	Hap6-Hap10 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_54	A17	Hap12 (C)	Hap6 (C)	Hap6-Hap11 (C)	Hap4 (C)	Hap10-Hap11 (C)
	IND_56	A3B1	Hap12 (C)	Hap5-Hap6 (C)	Hap6-Hap8 (C)	Hap4 (C)	

Since the mtCOI chromatograms of the individuals involved in the intraspecific transfers are not showing any evidence of contamination by a second individual, we had a closer look at the DNA fragment transferred, the EPIC25 chromatograms. Here, we show a more detailed example of one highly variable region (6 SNPs over 37bp) that highlights the cyclic pattern observed in Debortoli et al (2016) by providing the chromatograms of three individuals described as recombinant (Ind31, Ind42 and Ind51, Figure 2). The stars on those clean chromatograms indicate the SNPs considered in the study, the colors of the stars represent the sites that were variable across each pair of individuals. In figure 2 we only considered those variable sites and reconstructed the alleles according to the peak rank (1st or 2nd), giving sequences that corresponded to the alleles reconstructed through statistical methods (PHASE) in Debortoli et al. (2016). Those sequences were used for the haploweb reconstruction on which the three alleles retrieved (white, grey and black) were linked when found co-occurring in a heterozygous individual. While this is not providing additional results than our original paper, we think that this more detailed explanation could help readers to better understand our haploweb method used and rule out all doubts about those intraspecific DNA exchange patterns.

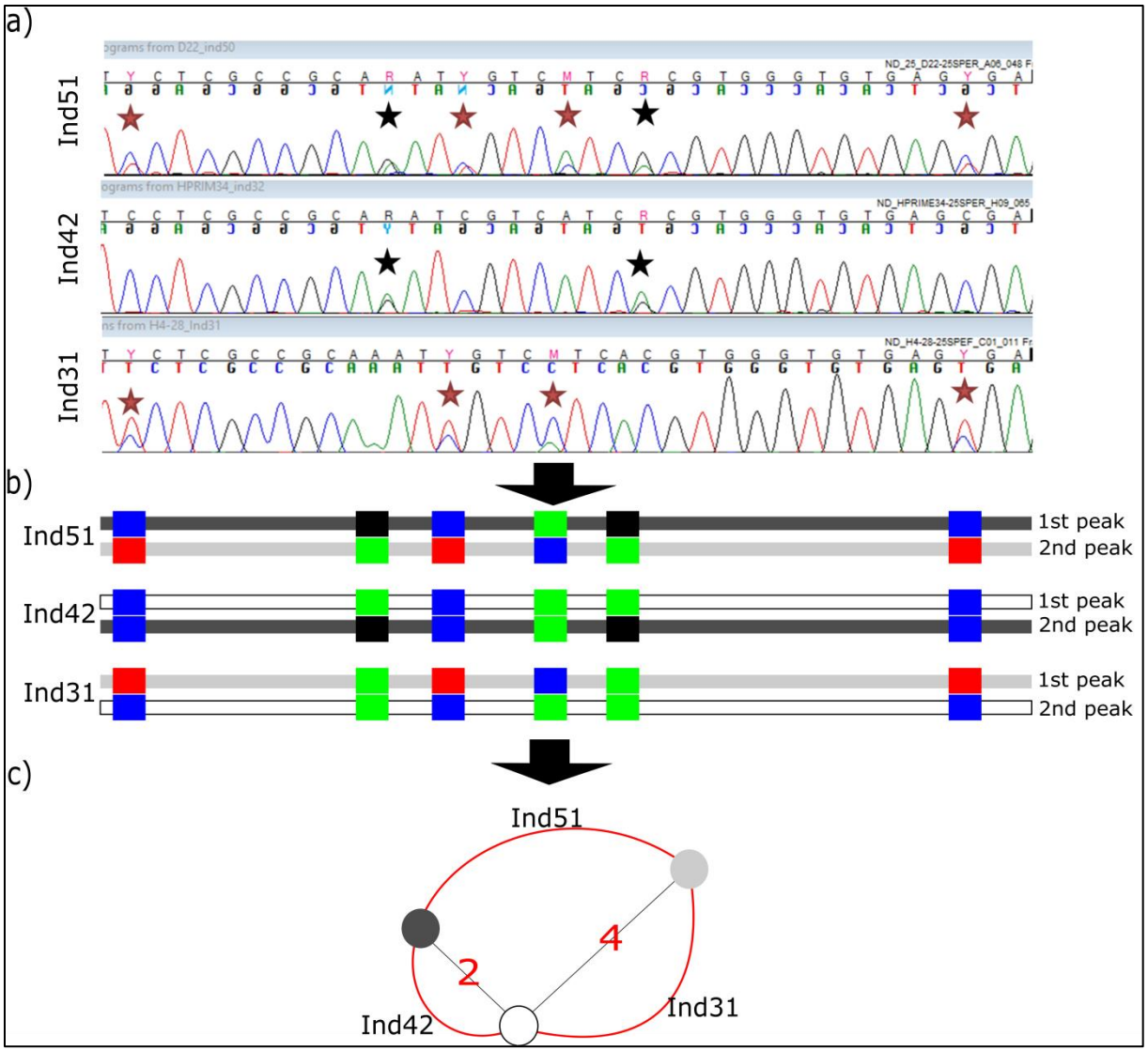

Figure 2 Evidence for intraspecific recombination for the nuclear marker EPIC25. a) Chromatograms of three *Adineta vaga* individuals (Ind31, 42 and 51) involved in allele sharing patterns. The secondary peaks considered in Debortoli et al. (2016) are highlighted by stars: the pattern of double-peaks in Ind51 is a heterozygous mixture of an allele from Ind31 (red stars) and the other allele from Ind42 (black stars). b) Phased alleles inferred from the chromatograms with one allele or haplotype represented by the 1st peaks and the second haplotype by the 2nd peaks. This reconstruction was congruent with the one obtained through the statistical method (PHASE) used in Debortoli et al. (2016). c) Simplified haploweb built from the phased alleles of Ind31, 42 and 51. The red numbers indicate the number of SNPs separating two alleles. Each allele is linked to another by a red curve when they co-occur in heterozygous individuals.

At this point, one can still hypothesize that this pattern of double peaks in the chromatograms may be the result of a bias caused by WGA (although the GC-content of the alleles of interest is almost identical, 48.6-49.5%). Indeed, if the gDNA of two individuals (Ind42 with genotype hap6-10 and Ind51 with genotype hap10-14) was present in the same tube and WGA mostly amplified hap6 and hap14, an artefactual individual with a genotype identical to Ind31 would be produced. If this was the case, and since we had to perform ten cycles of WGA to generate enough DNA for Illumina® library preparation and sequencing, one would expect to find reads corresponding to the four variants, even at very low coverage. This was never the case in our dataset.

Whole-genome sequencing of the three individuals (Ind31, 42 and 51) involved in the intraspecific allele sharing pattern for EPIC25 marker (see species C, Figure 3b and 5 from Debortoli

et al., 2016), it would be an undeniable evidence to show reads that overlap the recombination breakpoints we found (orange reads on Figure 3). Hopelessly, we could not find such reads as the region in-between two SNPs at the breakpoint locus was larger (X+Y) than the read size being 250bp (X) (Figure 3). Thus, no single read could physically overlap the entire breakpoint region. This is important to point out as the same problem could apply to future studies on recombination among bdelloid rotifers, requiring therefore long-reads technology (PacBio, Oxford Nanopore).

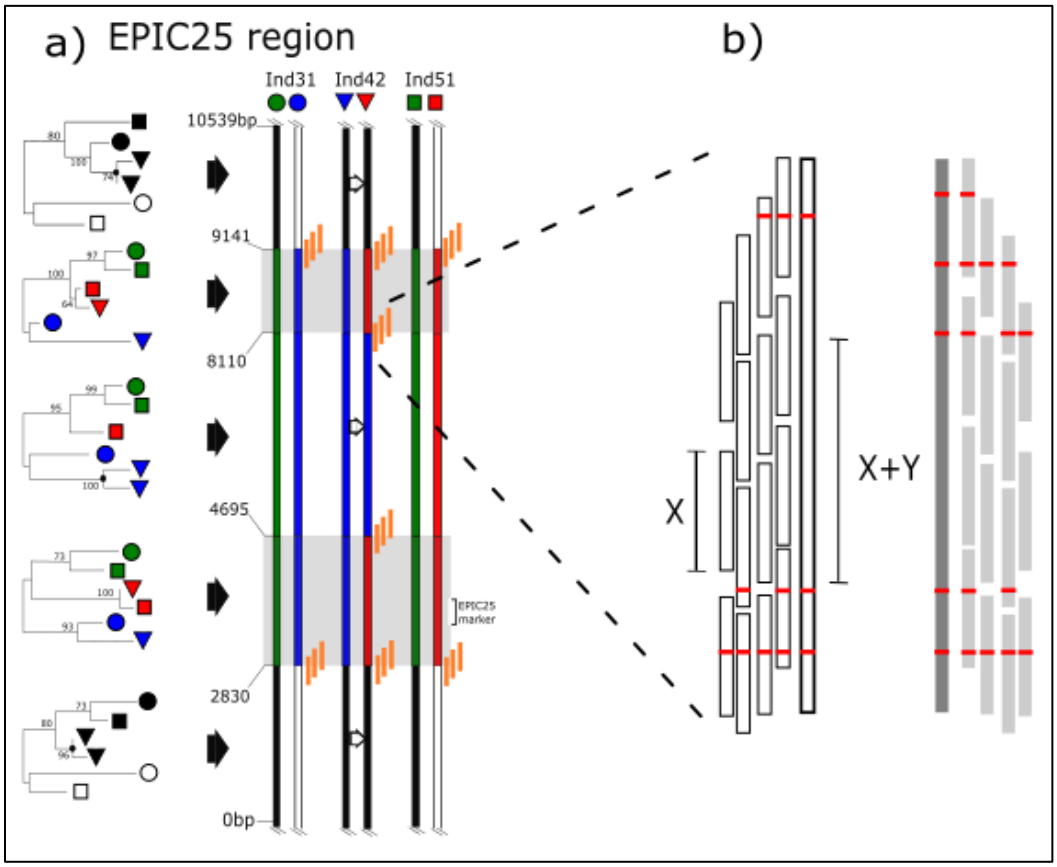

Figure 3 Contig assembled around the EPIC25 marker (adapted from Debortoli et al., 2016). a) Sites at which Illumina® reads (orange) would irrevocably support breakpoints and thus recombination. b) Representation of the assembly at one of the breakpoint region. The white and grey alleles (SNPs in red) are supported by multiple reads but the distance between the breakpoint informative SNPs (X+Y) is longer than the reads size (X).

First, on Fig3b and d from Debortoli *et al.* (2016), several individuals of species A show patterns of allele sharing in a cyclic fashion (genotypes a|b, a|c and b|c). If those patterns were due to the presence of two individuals in a tube, one would expect that the individuals implicated in the allele-sharing trio for marker Nu1054 (Ind 11, 13, 15 and 20) would be the same ones for marker EPIC25 (Ind 6, 7, 9, 11, 12, 13, 15, 17 and 18). Second, the fact that each of the three different genotypes taking part in the cycle for marker EPIC25 are supported by at least two individuals reduces the chances to explain this pattern by cross-contaminations.

Discussions and thoughts about the contamination risks pointed out by Chris Wilson

As often seen in Sanger sequencing output, there was a baseline noise in the chromatograms from Debortoli et al. (2016). Most of the time, this noise was low in comparison with the clear majority peaks or resulted from polymerase slippage in large poly-A regions. However, finding secondary peaks that would indubitably be congruent with the presence of a second haplotype in the

mtCOI chromatograms would deserve special attention especially when it comes to horizontal gene transfers. Indeed, except in some peculiar cases of DNA exchanges, such as described in parasexuality where cytoplasmic mixing occurs or HGT of mtCOI itself, individuals should not harbor more than one mtCOI haplotype. With this concern, Dr. Chris Wilson (in prep) took a closer look at our dataset and pointed out some minority peaks that he suggested as being the evidence for a second *A. vaga* species haplotype in the chromatograms representing interspecific recombinants. According to Wilson "the most eye-catching case is sample B14 (Ind1)", with several secondary peaks present that could represent a contamination by haplotypes hap12 (species C) and hap31 (species E). Although we admitted that this chromatogram was the noisiest and that several minority peaks were congruent with additional *A. vaga* haplotypes, it is impossible to highlight these distinct haplotypes from the random smaller peaks (see Figure 4). It is moreover not surprising to find several minority peaks when working with specimens directly isolated from nature, where plant and metazoan debris are present. DNA fragments could have been amplified, even after several washing steps, because we did not get rid of the gut content or the outside cuticle. It is therefore necessary to remain careful here and not to cherry-pick subsets of peaks (colored arrows on Figure 4) to refute a result.

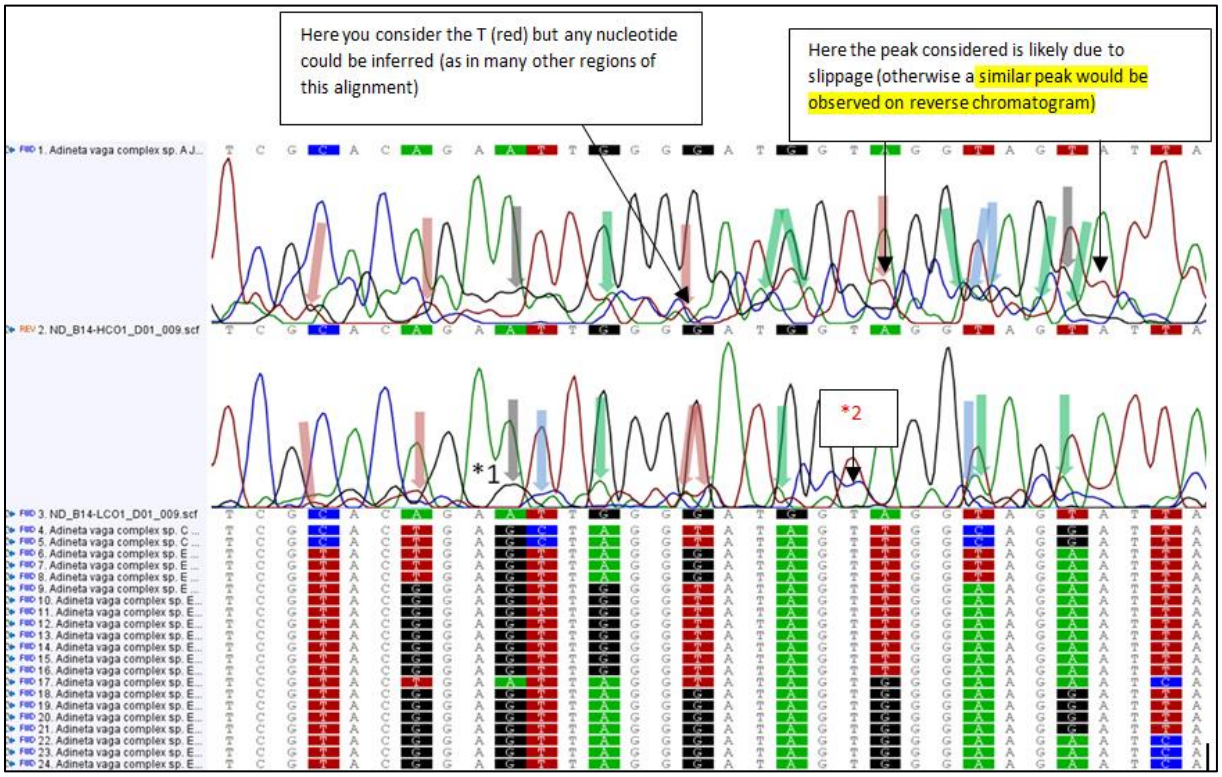

Figure 4 Chromatograms from Ind1 (B14) mtCOI sequence from Debortoli et al. (2016) pointed out by Wilson et al. The signal was increased to observe the minority peaks and find patterns corresponding to contaminant haplotypes. The variable sites are pointed out by colored nucleotides in the alignment below. The colored arrows are the minority peaks taken into account by Wilson et al. Asterisk 1 was considered by Wilson and co-authors as particularly informative as the signal was "strong". Asterisk 2 highlight a site with even stronger signal which turns out to be irrelevant.

Even in *A. vaga* individuals that were not identified as putative recombinants for the studied markers, one can observe several minority peaks and find a subset that would depict the presence of a second haplotype. As an example figure 5 depicts a region of the mtCOI marker of Ind16 (species A), showing the secondary peaks due to slippage: some sites may reveal the presence of a second individual from species C in the same tube (highlighted by 1), but there are peaks unrelated to

slippage or a second species (highlighted by 2). Therefore, such small secondary peaks cannot be considered as informative. We have discussed and rechecked all the other interspecific recombinants from Debortoli et al. (2016) and could not distinguish a clear signal of contamination by the presence of a second haplotype (see below).

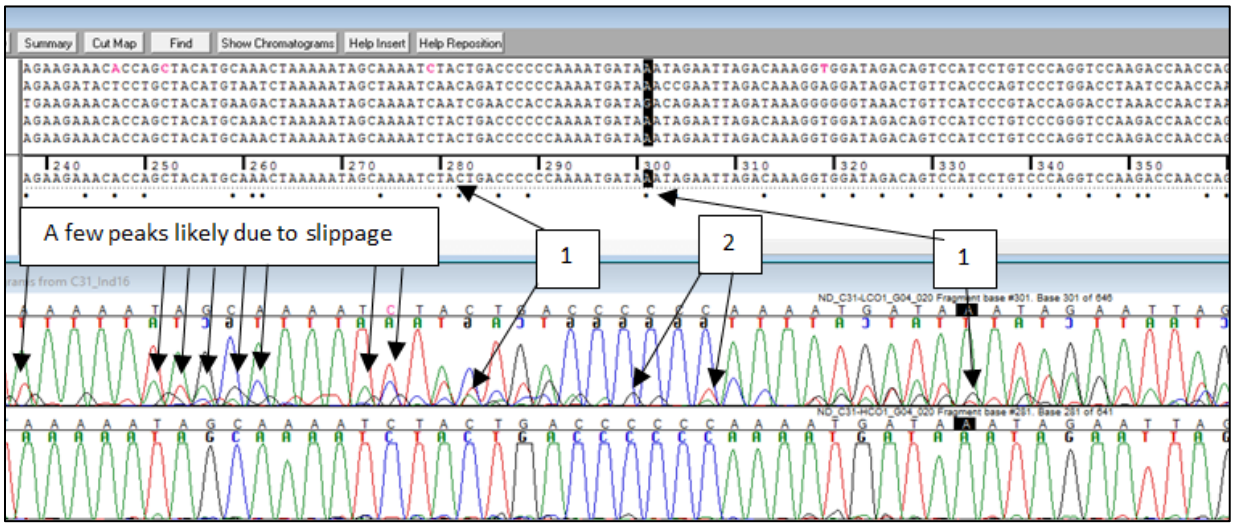

Figure 5 Chromatograms from Ind16 (C31) mtCOI sequence from Debortoli et al. (2016). It is possible to call for the presence of a second haplotype by selecting a subset of accommodating secondary peaks (1) without considering the minority peaks on the whole sequence (2).

Checking the impact of WGA on the interspecific recombinants

A puzzling case to analyze is Ind21 for which the mtCOI marker and the nuclear 28S rDNA marker belonged to species A whereas the additional three nuclear markers EPIC25, EPIC63 and Nu1054 belonged to species E. The alleles sequenced for those three last markers were all found within the single individual Ind81 from species E (Table 2). Here, the hypothesis of a WGA bias due to differences in GC content seems more plausible as EPIC25, EPIC63 and Nu1054 had a higher GC% in species E than in A, whereas mtCOI and 28S markers had similar GC content across both species (Table 3). In that case, one alternative hypothesis could be that the multiple copies of mtCOI and 28S of Ind21 (corresponding to species A) were exponentially amplified by WGA and masked those markers of species E while amplification of other nuclear regions was strongly skewed towards species E, eventually masking the species A alleles. In general, the 28S chromatograms were very clean, with almost no noise except for a couple of individuals. Even when increasing the signal, secondary peaks were still low for Ind21 and likely to be due to polymerase slippage (Figure 6a). In order to compare with the quality of an individual without any interspecific exchange, Figure 6b shows the 28S chromatogram of Ind18 attributed to species A. The black dots under the alignment indicate the differences between sequences from species A and sequences from species E. On the top chromatogram, we see some small secondary-peaks similar to the ones observed for Ind21 (Figure 6b).

Table 2 Summary of the genotypes for individuals attributed to species A and E (simplified from Debortoli et al., 2016). Ind21 presented mtCOI and 28S rDNA from species A, as in Ind2, but EPIC25, EPIC63 and Nu1054 from species E, as in Ind81.

		ID_ind.	COI (species)	28S (species)	EPIC25 (species)	EPIC63 (species)	Nu1054 (species)
Species A	IND_1	B14	Hap6 (A)	Hap1 (A)	Hap9-Hap10 (C)	Hap16-Hap20 (E)	
	IND_2	B15	Hap6 (A)	Hap1 (A)	Hap1 (A)	Hap1 (A)	Hap2 (A)
	IND_3	C1T1	Hap3 (A)	Hap1 (A)	Hap1 (A)	Hap1 (A)	
	IND_4	C3T3	Hap3 (A)	Hap1 (A)	Hap1 (A)	Hap1 (A)	Hap1-Hap5 (A)
	IND_5	D14	Hap3 (A)	Hap1 (A)	Hap10 (C)	Hap1 (A)	Hap1 (A)
	IND_6	D12	Hap3 (A)	Hap1 (A)	Hap1-Hap4 (A)	Hap1 (A)	Hap1-Hap6 (A)
	IND_7	D13	Hap3 (A)	Hap1 (A)	Hap1-Hap4 (A)	Hap1 (A)	Hap1-Hap7 (A)
	IND_8	E1B1	Hap4 (A)	Hap1 (A)	Hap4 (A)	Hap1 (A)	
	IND_9	E1B3	Hap4 (A)	Hap1 (A)	Hap1-Hap4 (A)	Hap1 (A)	Hap1-Hap4 (A)
	IND_10	H001	Hap1 (A)	Hap1 (A)	Hap1 (A)	Hap1 (A)	Hap1-Hap8 (A)
	IND_11	H3-03	Hap1 (A)	Hap1 (A)	Hap1-Hap2 (A)	Hap1 (A)	Hap1-Hap2 (A)
	IND_12	H3-04	Hap2 (A)	Hap1 (A)	Hap2-Hap4 (A)	Hap1-Hap3 (A)	Hap2-Hap9 (A)
	IND_13	HB01	Hap1 (A)	Hap1 (A)	Hap1-Hap2 (A)	Hap1 (A)	Hap1-Hap2 (A)
	IND_14	C28	Hap3 (A)	Hap1 (A)	Hap4 (A)	Hap1 (A)	Hap2 (A)
	IND_15	C33	Hap9 (A)	Hap1 (A)	Hap1-Hap4 (A)	Hap1-Hap3 (A)	Hap2-Hap3 (A)
	IND_16	C31	Hap3 (A)	Hap1 (A)	Hap4 (A)	Hap3 (A)	Hap2 (A)
	IND_17	C1T2	Hap4 (A)	Hap1 (A)	Hap2-Hap4 (A)	Hap2-Hap3 (A)	
	IND_18	D11	Hap3 (A)	Hap1 (A)	Hap2-Hap4 (A)	Hap1 (A)	Hap2 (A)
	IND_19	E11	Hap3 (A)	Hap1 (A)	Hap4 (A)	Hap3 (A)	
	IND_20	E31	Hap5 (A)	Hap1 (A)	Hap3-Hap5 (A)	Hap1 (A)	Hap1-Hap3 (A)
	IND_21	B11	Hap6 (A)	Hap1 (A)	Hap35 (E)	Hap16 (E)	Hap22 (E)
	IND_22	B33	Hap7 (A)	Hap1 (A)	Hap1-Hap5 (A)	Hap1 (A)	
	IND_23	B3B1	Hap1 (A)	Hap1-Hap2 (A)	Hap35 (E)	Hap1 (A)	
Species E	IND_62	H4-02	Hap21 (E)	Hap11-Hap16 (E)	Hap25-Hap29 (E)	Hap17-Hap21 (E)	Hap17-Hap31 (E)
	IND_63	H4-04	Hap28 (E)	Hap11-Hap16 (E)	Hap23-Hap32 (E)	Hap33-Hap34 (E)	Hap19-Hap21 (E)
	IND_64	HPRIME15	Hap20 (E)	Hap11-Hap16 (E)	Hap28-Hap29 (E)	Hap25 (E)	Hap28 (E)
	IND_65	HPRIME19	Hap22 (E)	Hap11-Hap16 (E)	Hap38-Hap41 (E)	Hap32 (E)	Hap17-Hap30 (E)
	IND_66	B39	Hap10 (C)	Hap11-Hap16 (E)	Hap30-Hap36 (E)	Hap16 (E)	Hap19 (E)
	IND_67	C3B1	Hap35 (E)	Hap11-Hap12 (E)	Hap26 (E)	Hap19-Hap23 (E)	Hap32-Hap33 (E)
	IND_68	C3T1	Hap36 (E)	Hap11-Hap12 (E)	Hap26 (E)	Hap19-Hap35 (E)	Hap34-Hap35 (E)
	IND_69	HPRIME16	Hap33 (E)	Hap16 (E)	Hap31 (E)	Hap28-Hap29 (E)	Hap18 (E)
	IND_70	A21	Hap23 (E)	Hap16 (E)	Hap39-Hap43 (E)	Hap27 (E)	Hap17-Hap27 (E)
	IND_71	A22	Hap26 (E)	Hap16 (E)	Hap39-Hap44 (E)	Hap27 (E)	Hap17-Hap27 (E)
	IND_72	A32	Hap28 (E)	Hap16 (E)	Hap33-Hap37 (E)	Hap32-Hap34 (E)	Hap19-Hap21 (E)
	IND_73	A33	Hap33 (E)	Hap16 (E)	Hap42 (E)	Hap17-Hap18 (E)	Hap17-Hap26 (E)
	IND_74	A24	Hap29 (E)	Hap16 (E)	Hap37 (E)	Hap34 (E)	Hap21 (E)
	IND_75	A25	Hap25 (E)	Hap16 (E)	Hap29-Hap40 (E)	Hap26-Hap27 (E)	Hap17-Hap27 (E)
	IND_76	A23	Hap24 (E)	Hap16 (E)		Hap27 (E)	Hap29 (E)
	IND_77	B34	Hap27 (E)	Hap16 (E)		Hap30-Hap31 (E)	Hap17-Hap31 (E)
	IND_78	B38	Hap34 (E)	Hap14-Hap16 (E)	Hap24-Hap36 (E)	Hap26-Hap27 (E)	Hap17 (E)
	IND_79	E3B2	Hap33 (E)	Hap12-Hap15 (E)	Hap27-Hap34 (E)	Hap18-Hap22 (E)	Hap20-Hap23 (E)
	IND_80	E3T1	Hap36 (E)	Hap12 (E)	Hap26 (E)	Hap24 (E)	Hap24-Hap25 (E)
	IND_81	HPRIME14	Hap31 (E)	Hap13-Hap16 (E)	Hap30-Hap35 (E)	Hap16 (E)	Hap19-Hap22 (E)

Table 3 GC content (%) at for each marker in the trio presented in Table 2.

	mtCOI	28S rDNA	EPIC25	EPIC63	Nu1054
Ind2	31.9	44.6	44.7	41.9	56.7
Ind21	31.9	44.6	27	28	35.5
Ind81	30.3	42.7-42.7	26.8-27	28	35.5-36.3

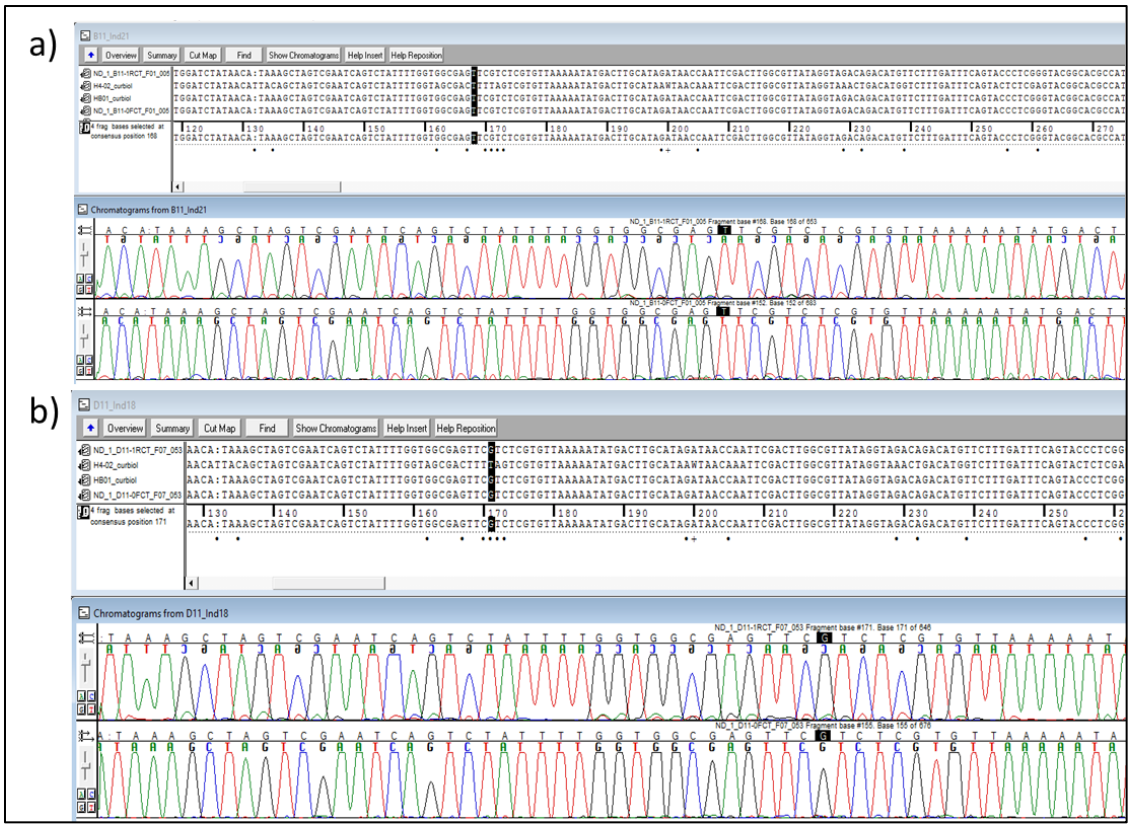

Figure 6 Chromatograms from the 28S rDNA marker of a) Ind21 and b) Ind18. Even when the signal was increased to show the minority peaks, those remained extremely low.

We could not find clear patterns of minority peaks that would reveal the presence of DNA from a second individual on the chromatograms obtained by Sanger-sequencing of the mtCOI marker amplified directly from gDNA. However, if WGA can be biased towards underrepresented DNA templates we would eventually be able to retrieve such signal by PCR-amplifying the WGA products instead of the gDNA. We used the WGA products of the individuals identified as interspecific recombinants in Debortoli et al. (2016), when there was some DNA left (Ind5, 23, 58 and 66) for mtCOI amplification and sequencing in both directions. The profile of minority peaks was then carefully inspected but no relevant signal could be detected in at least three of the four individuals (Ind5, 23 and 58). The chromatograms corresponding to Ind66 presented a few secondary peaks that matched the SNPs expected if a contamination by mtCOI haplotype hap31, as harbored by Ind81. This observation indicated that rare DNA templates can be made visible by successive biased amplifications steps.

Using ConTAMPR on Debortoli et al (2016) mtCOI dataset corresponding to the interspecific exchanges

Besides testing their ConTAMPR method on deliberately contaminated samples, Wilson et al. also applied their statistical method to the chromatograms from individuals showing interspecific exchanges in our paper Debortoli et al. 2016 and postulated that our conclusions resulted from contaminations. As it is often tricky to assess the rank of the expected nucleotides correctly due to little shifts between the major peak and the corresponding minority peaks, or due to polymerase slippage or weak signals, we used the same ConTAMPR method independently on the COI chromatograms of the same six individuals and had contrasting results. To eliminate the chance of

cherry-picking the peaks, we also asked a naïve observer to perform the same task in parallel. The analysis conducted by both observers gave congruent outputs that differed from the ones obtained by Wilson et al (2018). Because ConTAMPR results are hardly reproducible, error prone and time consuming, we automatized the ConTAMPR method with Dr. Jitendra Narayan from the LEGE research group in order to distinguish contaminations from background noise (https://github.com/jnarayan81/ConTAMPR) without bias. The determination of minor peaks is slightly different between the manual and the automatized method (Figure 7). In the manual method, we systematically determined the highest minor peak within of close-by the major peak as secondary peak. In contrast, the minor peak with the largest surface within the major peak was ranked as the secondary peak in the automatized method.

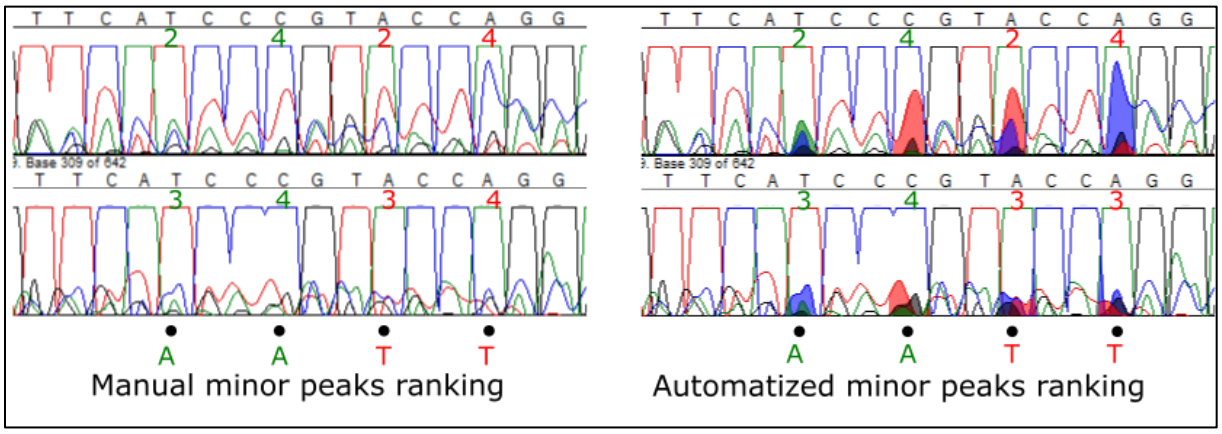

Figure 7 Implementation differences in the ConTAMPR method. In the manual method, the height of each minor peak determines its rank. In the automatized method, the surface under each minor peak is calculated and the rank of each minor peak is determined according to the surface it delimits.

The automatized version of ConTAMPR allowed us to analyse our whole dataset of 82 individuals instead of focusing solely on the individuals showing signal of recombinations and the expected contaminants. Overall, the results obtained through ConTAMPR when applied manually or automatized were most of the time congruent for the six individuals pointed out by Wilson et al (2018). However, the automatized version found three additional cases of contamination (Figure 8). In those three cases, the differences between the manual method and the automatized method were low, indicating that the Chi^2-test p-value oscillated around 0.05. Nonetheless, the automatized ConTAMPR confirmed the observation of Wilson et al, (2018) that Ind1 and Ind21 could be contaminated by sequences found in species E and species C, respectively. However, when using autoConTAMPR to cross-compare each COI chromatogram pair from the whole dataset, instead of focusing solely on individuals pointed out by Wilson et al (2018), we retrieved significant hits (P<1%) for 49 of the 80 individuals (61%) for which both chromatograms were available (two individuals were not taken into account because of low quality). We ran the autoConTAMPR method on an amphipod dataset which resulted in 42/67 individuals (63%) with contamination signals. This indicates that the frequent background contamination detected by autoConTAMPR in our Current Biology dataset is not unusual for COI chromatograms and can be parsimoniously explained by minute amounts of carry-over or post-PCR contamination.

In addition, we analysed the forward and reverse chromatograms separately instead of combining information from both as in Wilson *et al* (2018). For our Current Biology dataset we found 41 (51%) significant hits in the forward chromatograms and 18 (22.5%) signiticant hits in reverse

chromatograms, whereas only four individuals (B11, A3B1, Hprim12 and H4-04; 5%) had P<1% hits on the same sequence for both chromatograms. Only one of the six rotifers for which we inferred putative interspecific gene transfers (B11) had identical P<1% hits on both chromatograms: these were matches with species D and E, whereas the inferred horizontal transfer was from E to A. Regarding the other five individuals: B39 (inferred mitochondrial capture from C to E) had no P<1% hit; B22 (inferred transfers from E to C) had a P<1% hit with species F (for the forward chromatogram) but none with species E; B3B1 (inferred transfer from E to A) had P<1% matches with species E and D for the forward chromatogram; B14 (inferred transfers from C and E to A) had P<1% matches with species D and E (for the reverse chromatogram) but none with species C; and D14 (inferred transfer from C to A) had P<1% matches with species B, D, E and F (for the forward chromatogram) but none with species C. Hence, it appears difficult to draw any definitive conclusion regarding interspecific recombination at this stage.

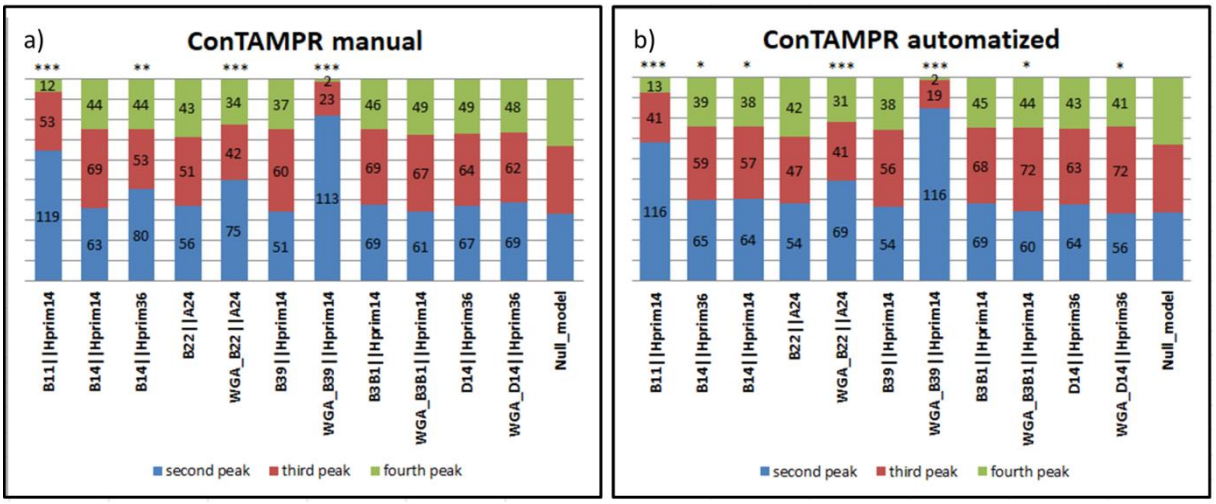

Figure 8 ConTAMPR output. The mtCOI chromatograms for each of the six interspecific recombinant individuals Ind1, 5 21, 23, 58 and 66 (B14, D14, B11, B3B1, B22 and B39, respectively) were aligned with the putative contaminant haplotypes. When possible, the mtCOI chromatograms amplified from WGA amplicons were also used. The distribution of minority peaks rank is indicated by the figures and the Chi-square test p-value is indicated when significantly different than the null model (*). a) The ConTAMPR method was applied manually and b) implemented through our automatized version.

Another interesting observation is that for Ind5 (D14), Ind23 (B3B1), Ind58 (B22) and Ind66 (B39) we could not detect any pattern of minority peaks that deviated from the null model when using the original chromatograms obtained from the direct mtCOI amplification of fresh gDNA (χ^2 p-value = 0.21, 0.56, 0.423 and 0.066, respectively) (Figure 8), both in the manual and automatized method. In contrast, we had significant deviation from the null hypothesis when using the automatized method on the chromatograms if WGA products were used as template for mtCOI amplification. Those results are less evident to interpret; the amount of "contaminating" DNA was probably so low that it does not appear on the chromatograms originating from gDNA but could become visible when WGA products were used. This highlights the hypothesis presented by Wilson et al. that Illustra GenomiPhi V2 DNA amplification kit (GE Healthcare) may have resulted in a skewed amplification and traces of contaminant DNA become amplified.

In our sense, those results show that it is tricky to clearly tell real contamination by a second individual from random sequencing noise or contamination by other taxa (bacteria, plants,…), even when using the ConTAMPR method of Wilson et al. Yet, we thank the authors for their fruitful critics

and discussions that certainly raised concerns about the use of WGA kits when it comes to the HGTs question.

Mechanistic issue

The last concern of Wilson et al. was related to our hypothesis that "DNA double-strand break repair through homologous recombination" could maybe enable horizontal genetic exchanges in bdelloid rotifers. First, we would like to emphasize here that this hypothesis indeed requires additional data to unravel the mechanisms responsible for DNA transfers in bdelloid rotifers. Yet, homologous recombination is a possible explanation, especially with regards to the intraspecific recombination patterns observed on Figure 5 from Debortoli et al (2016). It is still not clear how interspecific recombination is mediated and it would not be surprising that different mechanisms could be involved. Although Wilson et al. argue that the genetic distance separating the individuals for which we detected interspecific HGTs is beyond the limits observed in other organisms; we think that it is tricky to compare the mechanisms potentially involved in bdelloid rotifers with the ones known in some model organisms knowing that bdelloid rotifers have integrated non-metazoan genes within their genome.

In addition, we want to add here that within this discussion, parasexuality should still be considered as a mechanism for genetic exchange that may result in a similar pattern than the one observed here taking into account the WGA biases. In some basidiomycete fungi, successful mating between two haploid individuals produces short-term diploid cells with cytoplasmic mixing that carries the possibility for multiple mitochondrial haplotypes in the progeny (Anderson and Kohn, 1998). Parasexuality does not require chromosome pairing during meiosis which is compatible with bdelloids genome structure.

Conclusions

In conclusion, we do not think that Wilson et al. arguments are strong enough to refute the results of Debortoli et al. (2016). None of their concerns, neither their analyses, focused on intraspecific horizontal genetic transfers for which we provided the largest piece of evidence (Fig 1, 2, 3 and 5). The results of interspecific recombination are indeed more puzzling and we are continuing studying this in more detail. We have added in our new protocols triplicates analysis of each sample. We are confident that our isolation protocol did not enable the presence of more than a single individual per tube (and certainly not repetitively), we will although never be able to eliminate foreign DNA from the gut or cuticle completely.

We thought about potential contaminations and the possibility to perform population genetics/genomics experiments in order to tackle irrevocably the question of DNA exchanges among species of bdelloids back in 2015 when a specific procedure was designed and started, this is discussed more in detail in chapter 3.

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Chapter 3: More evidence for allele sharing among bdelloid rotifers of the genus *Adineta*.

When gathering the first data supposing patterns of allele sharing (as presented in Debortoli et al, 2016), we were aware that the amount of DNA retrieved from a single *Adineta* individual sampled in the wild would be a limiting factor impeding our full understanding of this mystery of genetic exchanges within this asexual clade. Indeed, a single *A. vaga* contains ≈0.2ng of DNA (as one nucleus contains 0.21pg and one individual is composed of approximately 1000 cells) making whole-genome sequencing of one individual difficult to study at the genomic level the patterns of genetic exchanges. In addition, while being cautious with contaminations when working with specimens isolated from nature, we can never exclude the possibility for the presence of foreign DNA enclosed in the gut or stuck to the cuticle of individuals being amplified when using whole-genome amplification kits. As previously discussed, some signatures of DNA transfers, as pointed out by Wilson et al (2017) (see Chapter 2), should be verified even if we were very cautious during the isolation of single individuals per tube. Moreover, it appeared obvious that employing whole-genome amplifications could lead to biases, eventually resulting in the overrepresentation of initially rare, foreign DNA.

In 2015, we designed a new experiment relying on clonal cultures of each individual collected from moss patches. The whole-genome amplification step was not required since we would work with bdelloid clones that were dense enough. In addition, we wanted to study at which taxonomic level DNA transfers occurred (intraspecific, interspecific, intergenera, interfamilies). We were indeed surprised by the concern of Wilson et al (2017) about interspecific genetic transfers within bdelloid rotifers while different cases of inter-kingdom transfers are commonly accepted (Gladyshev et al, 2008; Boschetti et al, 2012; Flot et al, 2013; Eyres et al, 2015).

We isolated bdelloid specimens from fifty nearby moss patches, representing a total of 301 individuals that were cultured. Debortoli et al (2016) and additional preliminary studies revealed the importance to genotype a large number of individuals to detect genetic transfers, highlighting the rarity of such events. Moreover, enough variability is required among sequences to detect such transfers (which will be undetectable between clones). However, this experimental setup turned out to be unsuitable as we could only genotype a limited number of individuals per genetic species (Table 1). We retrieved indeed a large number of individuals, but many of them could not be cultured stably reducing the final number of specimens per species in our dataset.

In this chapter, I will first briefly go through the main results obtained for the entire dataset comprising individuals from all bdelloid families and partly conducted by my master student B. Delcomenne. Even though those results are still preliminary, we observed several patterns of DNA transfers. Then, I will provide the preliminary results of a refined study focusing on Adinetidae.

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Preliminary results on DNA transfers between bdelloid rotifers individuals from distinct families

A wide sampling campaign was carried out in order to isolate and culture as many bdelloid rotifers individuals as possible, from all families encountered. We sampled four times (June, October and December 2015, as well as March 2016) around the Park d'Haugimont site, representing a total of fourty patches (22 lichens, 14 mosses, 1 piece of tree bark and 2 sediments from a dry pond) and a single time (August 2016) on a slag heap where ten mosses patches were retrieved. We managed to isolate and culture 344 individuals from the 452 observed among the fifty patches. We identified morphologically 310 individuals to family level (133 Adinetidae, 72 Habrotrochidae and 105 Philodinidae), whereas thirty-four specimens could not be confidently identified. When each clonal culture reached >10 individuals we started genotyping using one mitochondrial marker (COI) and four nuclear markers (HisB, Nu1054, Scaff1 and 28S rDNA). The success rate for each marker varied across cultures (especially for marker HisB) and some cultures went extinct while we were genotyping, resulting in partial data for most cultures (see Supplemental Table 1).

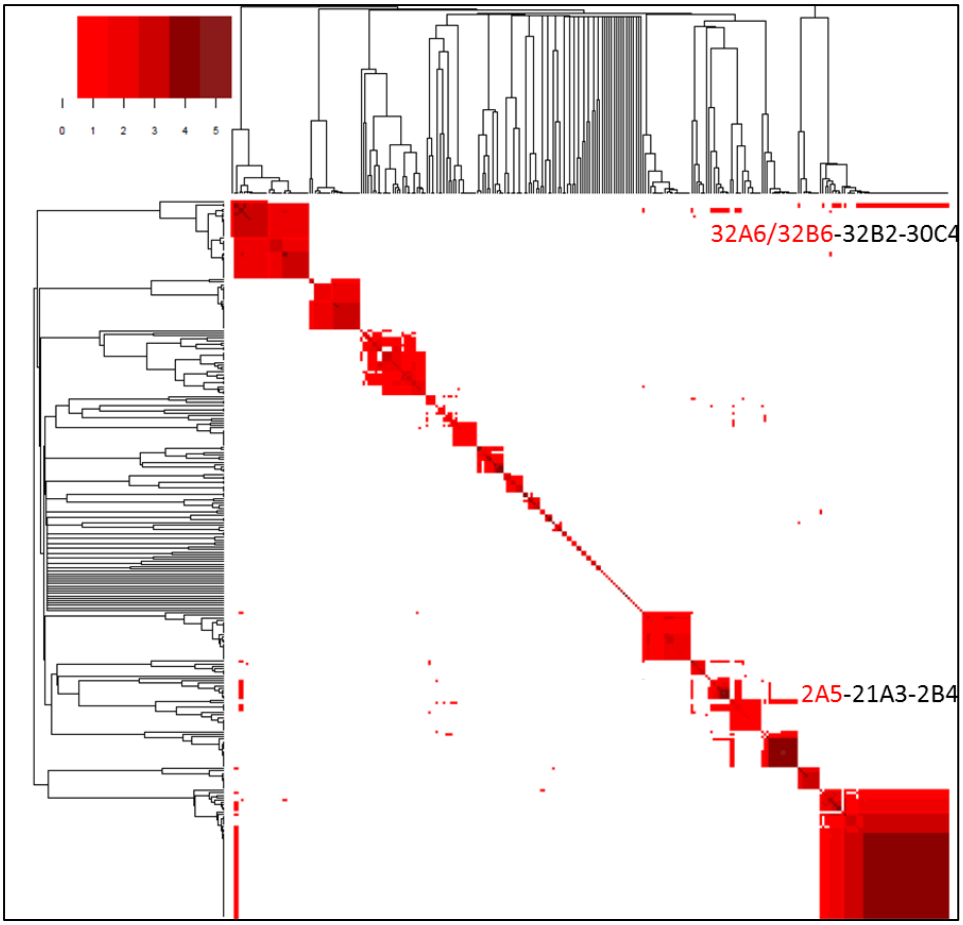

Figure 1 : Conspecificity matrix built from data retrieved from all bdelloid individuals genotyped. Red blocks along the diagonal represent individuals sharing 1 to 5 alleles (corresponding to the five regions sequenced), the darker the more alleles are in common. As a result, blocks along the diagonal represent species. Blocks outside the diagonal indicate individuals belonging to one block, but also sharing one allele with another species, *i.e.* considered as interspecific DNA exchange.

Even though this preliminary dataset (Supplemental Table 1) was partial, we used the information retrieved from the alleles co-occurring in heterozygous individuals to build a conspecificity matrix (see Chapter 1 and next part of Chapter 3; Figure 1). Fourty-three species were delineated by the matrix, with nineteen of them being represented by only one individual. Interestingly, three individuals (2A5, 32A6 and 32B6) harboured alleles from distinct species, highlighted by signals outside the diagonal (Figure 1) as observed in Debortoli et al (2016). Individual

2A5 was morphologically identified as Habrotrochidae and harboured alleles identical to 2B4 (Habrotrochidae) for marker COI, 28S and Scaff1, one of the two alleles found at locus Nu1054 was identical to the allele observed in 21A3 (Philodinidae). Similarly, individuals 32A6 and 32B6 (morphologically undetermined) presented the same genotype as individual 32B2 (morphologically undetermined) except for one Nu1054 allele identical to 30C4 (*Pleuretra sp.*, Philodinidae).

We designed specific primer pairs in order to amplify each allele independently to screen for more such cases in individuals from the same clonal cultures than 2A5, 32A6 and 32B6. In example, one primer pair could only amplify the Nu1054 sequences observed in 2B4 (Nu1054_2B4) while a second primer pair could only amplify the Nu1054 sequences found in 21A3 (Nu1054_21A3). Our specific primers Nu1054_2B4 produced amplicons when gDNA from individual 2B4 (Habrotrochidae) was used as template, but did not work on individual 21A3 (Philodinidae). In contrast, our specific primers Nu1054_21A3 amplified the gDNA from individual 21A3 but not 2B4, showing specificity of the designed primers. When screening for more recombinant individuals in the 2A5 clonal culture using those specific primers, we retrieved three clones for which amplification worked with both specific primers pairs out of 20 clones screened. The success rate was even lower after a second screening of the same 2A5 culture, with only one individual presenting amplicons for both specific primers pairs out of 50 clones screened. We used the gDNA extracted from this same individual to amplify the Nu1054 marker with the original Nu1054 primers (not allele specific) and directly Sanger-sequenced half of the amplicons obtained while the other half was used for cloning into plasmids to sequence each allele separately. With this method, we were able to retrieve four alleles in 2A5; two alleles identical to 2B4 (1 and 3) and two alleles identical to 21A3 (47 and 53). Unfortunately, we have developed allele specific primers for the 32A6 and 32B6 cases but we never obtained amplicons while screening those cultures for additional recombinant individuals. As a result, we decided to focus on the trio composed by individuals 2A5, 2B4 and 21A3, taking particular care to those cultures in order to avoid cross-contamination. When the cultures reached a density of 30 000 individuals, we collected the individuals for DNA extraction using the PureGene extraction kit® and sent the samples for whole-genome sequencing (Illumina®, HiSeq2500, 2x250bp; Genoscope, Evry, France).

The Illumina® libraries obtained were used to retrieve the five genetic markers used for genotyping (mtCOI, 28S rDNA, HisB, Scaff1 and Nu1054 for which recombinant signals were observed). The baiting and elongation around the markers was done using MITObim, a package that runs MIRA iteratively to reconstruct contigs. Although we could reconstruct short contigs around our five markers when baiting with the 2B4 sequences, we could not retrieve one single read in the library corresponding to individual 2A5 that corresponded to the COI, 28S, HisB, Scaff1 and even Nu1054 sequences observed in individual 21A3. This observation suggests that cross-contaminations between 2A5 and 21A3 gDNA could be responsible for the pattern observed in Table 1 or that the recombining region was rapidly lost throughout generations. One puzzling result is the fact that we could observe signals of recombination five times independently for this case after our screening, eventually eluding the contamination hypothesis. Similarly, individual 32A6 and 32B6 presented patterns of possible recombination and were identical for the regions sequenced. This suggest either that we cross-contaminated those two samples in exactly the same way or that those two individuals are daughters of an uncultured recombinant. Because improved assembly methods need to be developed (ongoing) in order to further investigate the sequenced genomes and because screening more individuals is expensive and time-consuming, we focused on Adinetidae populations which are easier to maintain under laboratory conditions. According to our experience, a large genetic diversity

within species is important to delineate species (GMYC, ABGD, conspecificity matrix) and to observe genetic exchanges. In the next part of this chapter, I will describe in details the methods and results of a study targeting the intraspecific genetic exchanges in Adinetidae.

Table 1 : Table showing the genotype of individuals involved in potential interspecific exchanges for each of the five markers sequenced.

Clone_ID	Moss_patch	Family	COI_haplotype	HisB_alleles	Nu1054_alleles	Scaff1_alleles	28S_alleles
2A5	2	Habrotrochidae	49	NA	Hap1-53(3-47)	Hap47-47	Hap9-9
2B4	2	Habrotrochidae	49	NA	Hap1-3	Hap47-47	Hap9-9
21A3	21	Philodinidae	71	NA	Hap47-53	Hap12-15	Hap5-5
30C4	30	Philodinidae_pleuretra	56	NA	Hap123-127	Hap80-83	Hap34-35
32A6	32	undertemined	10	NA	Hap127-94	Hap59-59	Hap24-24
32B2*	32	undertemined	10	NA	Hap94-94	Hap59-59	Hap24-24
32B6*	32	undertemined	10	NA	Hap127-94	Hap59-59	Hap24-24

DNA transfers among conspecific individuals from the genus *Adineta* confirmed

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Introduction

The asexual evolution of bdelloid rotifers has been admitted since a wide range of studies, from genomics to morphology, have reported the absence of conventional meiosis (*i.e*. the pairing, segregation and re-assortment of chromosomes) (Hsu, 1956 a and b; Mark Welch *et al*, 2008; Flot *et al*, 2013; Fontaneto and Barraclough, 2015) and the absence of males (Mark Welch *et al,* 2009). The most compelling evidence, being recently confirmed by new genomic studies, is the presence of palindromic alleles on single chromosomes (Flot *et al*, 2013; Karine Van Doninck, pers. comm. of ongoing research).

Yet, even though this peculiar structure is not compatible with conventional meiotic processes, alternative mechanisms for the exchange of DNA material remain possible. Recent studies have indeed reported signatures of DNA transfers among bdelloid rotifers (Signorovitch *et al*, 2015; Debortoli *et al*, 2016; Vakhrusheva *et al*, poster at SMBE congress). Those results have been widely debated, the mechanisms for such transfers and the taxonomic range at which they occur (intra-species, inter-species, inter-genera or inter-families) still being obscure (Signorovitch *et al*, 2016; Schwander *et al*, 2016; Flot *et al*, 2016; Wilson *et al*, in review). However, a massive number of horizontal gene transfers (HGTs) from non-metazoan origin have been detected in bdelloid rotifers, constituting up to 8-10% of the predicted genes (Gladyshev *et al*, 2008; Flot *et al*, 2013; Eyres *et al*, 2015). Those observations seem to highlight the absence of a taxonomic barrier for HGT in bdelloid rotifers; DNA from other kingdoms being integrated within their genome and expressed. Moreover, the transcriptomic study of Eyres *et al* (2015) demonstrated that around half of the HGTs were shared among bdelloids of distinct families suggesting their acquisition prior to bdelloid radiation. In addition, HGTs appear ongoing in bdelloid rotifers and significantly correlated with resistance to desiccation, the number of such events being on average 12.8 transfers per million years (Eyres *et al*, 2015). Yet, non-desiccating species keep accumulating foreign genes too, at a slower rate though, suggesting the possibility for mechanisms alternative to desiccation to integrate DNA. In addition, the constraints (genetic distance, GC content, presence of introns …) for the acquisition of genes from non-metazoan origin are probably more important than the ones for DNA transfers among bdelloids, eventually relying on other molecular processes.

Sex-related mechanisms that do not require chromosome pairing have been put forward to explain DNA exchanges (Flot *et al*, 2013; Signorovitch *et al*, 2015). The *Oenothera*-like meiosis proposed by Signorovitch *et al* (2015) would theoretically be possible as no chromosomes pairing or segregation is necessary. In this system, the chromosomes of each parent form distinct groups (linkage group α and β) that pair and recombine only at telomeric regions (Cleland, 1972; Golczyk *et al*, 2008). Since only crosses between α and β gametes are possible, heterozygosity is preserved within populations. As recombination is restricted to telomeres, haplotypes are maintained through

generations and rearrangements occurring within haplotypes also (Dietrich *et al*, 1997). Alternatively, parasexuality as described in *Candida albicans,* could also explain allele sharing patterns across multiple loci. In such reproductive system, diploid cells of each mating type a and α (determined by a single gene *MTL*, mating-type-like) fuse to form a tetraploid zygote (Hull and Johnson, 1999). The tetraploid daughter cells can divide by mitosis but they will eventually return to a diploid state through several unequal divisions with non-disjunction of chromosomes, a process called "concerted chromosome loss" (Bennett and Johnson, 2003; Hickman *et al*, 2015). Parasexuality or *Oenothera*-like meiosis would fit the observation of allele sharing across multiple loci between individuals (Signorovitch *et al*, 2015). Yet, no unusual chromosome pairing have been reported neither aneuploid bdelloid individuals suggesting extremely rare events, if any.

Confirming DNA exchange in bdelloid rotifers, understanding the mechanisms underlying these transfers and the frequency at which they occur, in comparison to HGTs from non-metazoan origin, would bring new insights on the evolutionary success of bdelloid rotifers. Here, we designed an experiment to study DNA exchanges in which we maintained clonal cultures of each bdelloid individual isolated from natural moss patches. We focused on the genus *Adineta* for which a reference genome is available (Flot *et al*, 2013) and because it can be easily cultured in lab conditions. Frequent cleaning of the clonal cultures and re-isolation of individuals was performed to limit cross-contaminations. Furthermore, clonal cultures enabled genotyping without whole-genome amplification step, reducing the chances for experimental artifacts.

Material and Methods

Sampling, culturing and individual isolation

We collected nearby moss patches from the ground located around an old slag heap in Courcelles, Belgium [50°26'50.57"N; 4°21'30.98"E] where we have identified several *Adineta* species in a preliminary study. The sampling period took place in August 2016. Dry substrate patches (>5 cm^2) were collected and re-hydrated in mineral water (Spa®) overnight in separate Petri dishes. Each active individual morphologically identified as *Adineta sp.* (Donner, 1965) was then carefully pipetted in successive Spa® water drops and isolated in an individual well (24-wells plates). Particular care was taken to avoid more than a single individual per well. Each individual was then monitored during a few weeks, fed with autoclaved lettuce extract and washed by transfer into a clean well if necessary. The genotyping was only applied to clones that reached a population of at least 10 individuals, which we considered as a proxy to limit culture crash and loss. We isolated one individual of each stable culture into a new tube in which 35 µL of Chelex® and 1µL of Proteinase K were added for genomic DNA extraction. This mix was then heated for 20' at 56°C followed by 10' at 95°C and centrifuged for 5' at 14000rpm. For each individual, the extracted DNA present in the supernatant was carefully transferred to a new tube for genotyping.

Individual genotyping

We first genotyped all the stable cultures by PCR-amplifying a fragment of the mitochondrial cytochrome oxidase subunit I (COI) marker using the universal HCOI and LCOI primers (Table 2; Folmer *et al*, 1994). The PCR mixes contained 1X GoTaq reaction buffer (1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 0.5 µM of both primers, 0.5 U of GoTaq DNA Polymerase (Promega), 5µL (≈10 ng) of gDNA and pure PCR reaction water for a total volume of 25 µL. The amplification conditions started with an initial denaturation at 94°C for 4'; followed by 60 cycles of 45" denaturation at 94°C, 45"

annealing at 40°C and 50" elongation at 72°C; a final elongation step was conducted for 10' at 72°C. The resulting amplicons were Sanger-sequenced in both directions using the same primers as for the PCR (Genewiz UK Ltd, Stortford, UK) and the chromatograms were assembled with Sequencher4 (Gene Codes).

We further genotyped with four additional nuclear markers the clones that were identified using the mitochondrial COI marker. Two of them, the 28S rDNA and Nu1054 markers, consistently amplified across all bdelloid families and were used as in Debortoli *et al* (2016). The only exception is that we performed the PCR amplification directly on the gDNA of single individuals, *i.e.* no whole-genome amplification (WGA) step, and, for the 28S marker, we used the Phusion polymerase (NEB) to amplify the whole fragment length (≈2800bp). The PCR mixes contained 5X HF buffer, 0.2 mM of each dNTP, 0.5 µM of both primers, 0.2µL of GoTaq DNA Polymerase (Promega), 5µL (≈10 ng) of gDNA and pure PCR reaction water for a total volume of 25 µL. PCR conditions consisted in an initial denaturation of 30" at 98°C; 35 cycles of 10" denaturation at 98°C and annealing/elongation 1'35" at 72°C; a final 10' elongation at 72°C. The 28S amplicons were then sequenced using two pairs of nested primers (Table 2), each fragment being approximately 700bp-long while the Nu1054 amplicons were sequenced using the same primers than for amplification (≈450bp).

Table 2 : List of primers used for each marker and the expected size of the corresponding amplicons.

Marker_name	Size_.bp.	Polymerase	Primer_name	Primer_sequence
COI (mt)	700	Taq	HCOI	5' – TAA ACT TCA GGG TGA CCA AAA AAT CA – 3'
			LCOI	5' – GGT CAA CAA ATC ATA AAG ATA TTG G – 3'
28S	2800	Phusion	28S1FCT	5' – CGA GAC CGA TAG CGA ACA AGT ACC GTG – 3'
			28S5RCT	5' – GAG TCA AGC TCA ACA GGG TCT TCT T – 3'
	700		28S2FCT*	5' – GAC CCG AAA GAT GGT GAA CT – 3'
			28S2RCT*	5' – CGT CAG TCT TCA AAG TTC TCA TTT GA – 3'
	700		28S3FCT*	5' – GCG TCG AAG GCT AAC ACG TGA – 3'
			28S3RCT*	5' – TGT TTT AAT TAG ACA GTC GGA TTC C – 3'
Nu1054	450	Taq	Nu1054F	5' – AGT ACG TGG ACC TAT GGG TAT TGG – 3'
			Nu1054R	5' – CCT GGT GGA GTA TCA TCT ACT TTG ACA – 3'
Scaff1	500	Taq	Scaff1F	5' – GTG CTG CTG GAC TCA TAC TTG – 3'
			Scaff1R	5' – GAA CAA GCA AAA CAT GTT CGT – 3'
HisB	530	Taq	HisB_F	5' – TTT CGT TTC TAT ATT CGT CAT CG – 3'
			HisB_R	5' – AAT TGA ATG TCC TTT GGC ATA A – 3'

* primers used for sequencing; all the other primers were used for both amplification and sequencing

Two additional markers (Scaff1 and HisB) were developed by aligning the *Adineta vaga* genome to the available bdelloid transcriptomes *Rotaria socialis*, *R. magnacalcarata*, *R. sordida*, *R. tardigrade* and *A. ricciae* (Boschetti *et al*, 2012; Flot *et al*, 2013; Eyres *et al*, 2015), and with the published *his* sequences for HisB (Van Doninck *et al*, 2009). We checked the number of copies for both markers into the *Adineta vaga* reference genome using tblastx in order to design primers specific to a single allelic pair. Primers were designed with Primer3 using default settings and each primer pair was tested on clones from available lab cultures representing at least one species of the Adinetidae, Habrotrochidae and Philodinidae families. For both the Scaff1 (≈500bp) and HisB (≈520bp) markers the PCR mix was composed of 1X GoTaq reaction buffer (1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 0.8 µM of both primers (Table 2), 1 U of GoTaq DNA Polymerase (Promega), 5µL (≈10 ng) of gDNA and pure PCR reaction water for a total volume of 25 µL. The reaction programs were identical to the one used for the COI marker except for the annealing temperature which was set to 54°C and 58°C for Scaff1 and HisB, respectively.

As further genotyping with four nuclear markers (28S, HisB, Nu1054 and Scaff1) required more gDNA than extracted from a single individual and as whole-genome amplifications was shown to induce biases (Wilson *et al*, in review), we considered that all individuals from a given clonal culture were identical and used several clones isolated in distinct tubes here.

Phasing sequences and species delimitation

For the four nuclear markers, the forward and reverse chromatograms of each clone were used to phase the two alleles of heterozygotes directly from the patterns of double-peaks observed (Stephens *et al*, 2001; Flot *et al*, 2006; Flot, 2007; Flot, 2010). The sequences obtained for each marker were aligned in MAFFT (E-INS-i method; Katoh *et al*, 2013) and used as input to generate haplowebs (Flot *et al*, 2010). Connections between alleles that co-occur in heterozygous individuals were added to median-joining networks (Fluxus Technology; Bandelt *et al*, 1999) to form distinct allele pools, called fields for recombination (FFRs), i.e. putative species (Doyle, 1995). The allele-sharing information present in each haploweb was then used to compute the conspecificity matrix in which a score is attributed to each pair of individual corresponding to the number of markers assigning this pair to a same FFR (Debortoli *et al*, 2016). The resulting square matrix was then reordered to maximize scores along the diagonal using hierarchical clustering in R package "heatmap3" (Zhao *et al*, 2014). The blocks formed correspond to species within which scores are higher than scores between species.

In addition, we used the General-mixed Yule Coalescent (GMYC) method on our COI dataset that relies on a maximum likelihood approach to detect a transition from speciation to coalescence in the branching rate pattern (Pons *et al*, 2006; Monaghan *et al*, 2009). As the GMYC can be sensitive to undersampling and to the frequency of singletons (Talavera *et al*, 2013; Tang *et al*, 2014), we aligned our COI sequences with those obtained in other projects and with available public data (GenBank). One unique sequence was then selected for each distinct haplotype and this dataset was used to generate an ultrametric Bayesian tree (BEAST v 1.6.2; Drummond and Rambaut, 2007) using the GTR+Γ4+I substitution model. For each group, the Markov chain Monte Carlo (MCMC) was run three times independently for 10^8 generations, sampling every 10^4 generations, and combined into a single consensus maximal clade credibility tree (LogCombiner and TreeAnnotator packages). The consensus tree was submitted to the General-mixed Yule coalescent (GMYC) analysis in R, using SPLITS (http://r-forge.r-project.org/projects/splits/).

Finally, we used a third approach, namely the Automatic Barcode Gap Discovery (ABGD), based on the distribution of pairwise genetic distance among haplotypes to delimit species. A "barcode-gap" can often be observed between the distribution of distances among haplotypes belonging to a same species and the distance between haplotypes from distinct species (Puillandre *et al*, 2011). Our aligned COI dataset was submitted to ABGD in command line mode using default parameters (software download at http://wwwabi.snv.jussieu.fr/public/abgd/).

Confirming the phasing sequences

We checked the sequences obtained from direct phasing using the forward and reverse chromatograms by cloning the same PCR-products into plasmids using the pGEM®-T Easy Vector kit according to the provided protocol (Promega). This enabled us to check that no chimeric sequences were created during allelic reconstruction using the statistical method PHASE or through manual editing during the length-variant heterozygote phasing with Champuru v1.0 (Stephens *et al*, 2001; Flot *et al*, 2006; Flot, 2007; Flot, 2010). In addition, sequencing cloned PCR-products may reveal additional rare alleles (large screening eventually required) that were not visible on the chromatograms retrieved from direct sequencing.

Culture preparation for whole-genome sequencing

The clonal cultures for which the conspecificity matrix highlighted incongruences or for which the haplowebs presented patterns of allele-sharing were boosted by feeding and cleaning them more regularly in order to reach a population of ≈30 000 individuals for whole-genome sequencing (WGS). The rotifers of each clonal culture were pooled in respective individual Falcon tubes (50mL), centrifuged for 10' at 4000 rpm (4°C) and stored at -80°C in separated cryotubes. We extracted the genomic DNA of each sample using the PureGene kit according to the supplied protocol and sequenced on an Illumina HiSeq2500 platform (Genoscope, Evry, France).

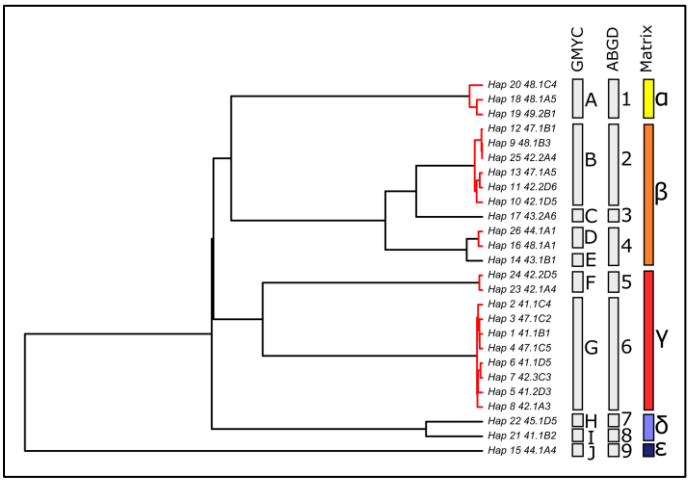

Figure 2 : Pruned phylogenetic tree presenting the COI haplotypes retrieved in this study. For the original tree see chapter 4. Species delimitations based on three methods (GMYC, ABGD and conspecificity matrix) are indicated.

Results

Sampling and species delimitation

A total of 130 individuals morphologically identified as *Adineta* sp. were isolated from ten moss patches around the slag heap (Courcelles, Belgium). Overall, 104 individuals (80%) led to stable cultures that were further used for genotyping (Table 3) and among which we could retrieve 26 distinct mtCOI haplotypes (haplotype diversity: Hd = 0.926 and nucleotide diversity: π= 0.085). The GMYC approach significantly delimited 117 species (confidence interval: 109-126) across the whole dataset built-up from the present study and the COI sequences available on GenBank, ten of which corresponded to species sampled throughout the present study (species A-J, Figure 2). Interestingly, the ABGD method congruently delimited nine species, regrouping species D and E of the GMYC within one single cluster (Figure 2).

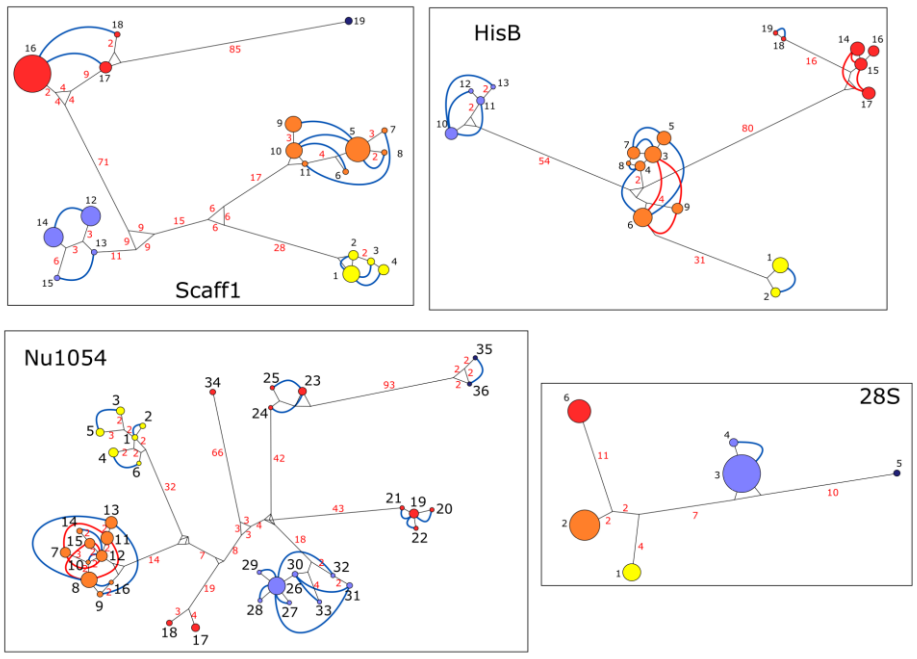

Figure 3 : Haplowebs built for each of the four nuclear markers sequenced (HisB, Nu1054, Scaff1 and 28S). Each circle represents a sequenced allele and its frequency in the population (size is proportional). When alleles are co-occurring in one heterozygous individual, the corresponding circles are linked on the haploweb (blue lines). Each color corresponds to one of the five (α-ε) species delimited in Figure 2. Red figures indicate the number of mutations separating two alleles. The red links highlight pattern of intraspecific DNA exchanges.

Further genotyping using the 28S rDNA and the Scaff1 nuclear markers worked consistently across

all *Adineta* species whereas the HisB and Nu1054 markers provided contrasting results working only on a subset of individuals (Table 3). The four haplowebs built by using the information of heterozygous individuals delimited 5 to 11 FFRs depending on the marker considered (Figure 3). When gathered in the conspecificity matrix, the information retrieved from those four nuclear markers and the mitochondrial COI marker delimited a total of five distinct species (species α-ε), merging sister clusters delimited by the GMYC and ABGD methods (Figure 2 and Figure 4).

Table 3 : Summary of the alleles found in each *Adineta* for each of the five markers sequenced.

	ID_individual	Moss_patch	COI_haplotype	HisB_alleles	Nu1054_alleles	Scaff1_alleles	28S_alleles	Matrix	GMYC_species	ABGD_species
1	48.1A5	48	18	Hap1-1	Hap1-2	Hap1-3	Hap1-1	Alpha	A	1
2	48.1C1	48	18	Hap1-1	Hap1-2	Hap1-3	Hap1-1		A	1
3	49.1A4	49	19	Hap1-2	Hap4-6	Hap1-2	Hap1-1		A	1
4	49.2B1	49	19	Hap1-2	Hap4-4	Hap1-2	Hap1-1		A	1
5	49.2B2	49	19	Hap1-2	Hap4-4	Hap1-2	Hap1-1		A	1
6	48.1C4	48	20	Hap1-1	Hap5-3	Hap1-4	Hap1-1		A	1
7	49.1B6	49	20	Hap1-1	Hap5-3	Hap1-4	Hap1-1		A	1
8	49.1D1	49	20	Hap1-1	Hap5-3	Hap1-4	Hap1-1		A	1
9	49.1C4	49	NA	Hap1-1	Hap5-3	Hap1-4	NA		NA	NA
10	48.1B3	48	9	Hap3-3	Hap8-15	Hap11-7	Hap2-2	Beta	B	2
11	42.1D5	42	10	NA	Hap11-8	Hap5-9	Hap2-2		B	2
12	42.3A1	42	10	Hap5-6	Hap11-8	Hap5-9	Hap2-2		B	2
13	42.3B2	42	10	Hap5-6	Hap11-8	Hap5-9	Hap2-2		B	2
14	43.1B3	43	10	Hap5-6	Hap11-8	Hap5-9	Hap2-2		B	2
15	43.2A3	43	10	Hap5-5	Hap11-8	Hap5-9	Hap2-2		B	2
16	41.1C3	41	11	Hap3-6	Hap7-11	Hap5-5	Hap2-2		B	2
17	41.2C6	41	11	Hap3-6	Hap14-12	Hap5-9	Hap2-2		B	2
18	41.2D6	41	11	Hap3-7	Hap12-15	Hap5-9	Hap2-2		B	2
19	42.2D6	42	11	Hap6-6	Hap12-15	Hap5-5	Hap2-2		B	2
20	42.3A5	42	11	Hap6-6	Hap12-15	Hap5-5	Hap2-2		B	2
21	43.1C3	43	11	Hap3-6	Hap8-15	Hap10-5	Hap2-2		B	2
22	43.1D4	43	11	Hap3-7	Hap7-11	Hap5-9	Hap2-2		B	2
23	43.2A1	43	11	Hap3-6	Hap7-11	Hap5-9	Hap2-2		B	2
24	45.1D6	45	11	Hap6-6	Hap13-13	Hap5-8	Hap2-2		B	2
25	48.1B5	48	11	Hap3-6	Hap7-11	Hap5-9	Hap2-2		B	2
26	47.1D1	47	12	Hap5-7	Hap12-10	NA	NA		B	2
27	47.1A5	47	13	Hap4-6	Hap7-12	Hap10-5	Hap2-2		B	2
28	43.1B1	43	14	Hap3-6	Hap8-15	Hap10-6	Hap2-2		E	4
29	48.1A1	48	16	Hap3-3	Hap8-16	Hap10-5	Hap2-2		D	4
30	43.2A6	43	17	Hap4-6	Hap13-9	Hap10-5	Hap2-2		C	3
31	43.2B2	43	17	Hap4-8	Hap13-9	Hap10-5	Hap2-2		C	3
32	42.2A4	42	25	Hap6-9	Hap14-12	Hap5-5	Hap2-2		B	2
33	44.1A1	44	26	Hap3-9	Hap13-8	Hap10-5	Hap2-2		D	4
34	48.1A2	48	26	Hap3-9	Hap13-8	Hap10-5	Hap2-2		D	4
35	48.1B2	48	26	Hap3-9	Hap13-8	Hap10-5	Hap2-2		D	4
36	48.1C2	48	26	Hap3-9	Hap13-8	Hap10-5	Hap2-2		D	4
37	48.1C3	48	26	Hap3-9	Hap13-8	Hap10-5	Hap2-2		D	4
38	43.1B2	43	NA	Hap5-6	Hap11-8	Hap5-9	Hap2-2		NA	NA
39	41.1A3	41	21	NA	Hap26-27	Hap12-14	Hap6-6	Delta	I	8
40	41.1B2	41	21	NA	Hap26-28	Hap12-14	Hap6-6		I	8
41	41.1C2	41	21	Hap10-11	Hap26-26	Hap12-14	Hap6-6		I	8
42	41.1D3	41	21	NA	Hap26-26	Hap12-14	Hap6-6		I	8
43	41.2A3	41	21	NA	Hap31-30	Hap12-14	Hap6-6		I	8
44	41.2B1	41	21	Hap10-12	Hap26-26	Hap12-14	Hap6-6		I	8
45	41.2D5	41	21	NA	NA	NA	NA		I	8
46	42.1D3	42	21	NA	NA	Hap12-14	Hap6-6		I	8
47	42.2A3	42	21	NA	Hap26-26	Hap12-14	Hap6-6		I	8
48	42.2B6	42	21	NA	NA	Hap12-14	Hap6-6		I	8
49	42.3A2	42	21	Hap10-13	Hap26-26	NA	NA		I	8
50	42.3A3	42	21	NA	Hap26-26	NA	NA		I	8
51	43.1C2	43	21	NA	NA	Hap12-14	NA		I	8
52	43.2B6	43	21	NA	Hap26-26	Hap12-14	Hap6-6		I	8
53	46.1A4	46	21	Hap10-11	Hap26-32	Hap12-14	Hap6-6		I	8
54	46.1B3	46	21	Hap10-10	Hap26-26	Hap12-14	Hap6-6		I	8
55	46.1D1	46	21	Hap11-11	Hap29-26	Hap12-14	Hap6-6		I	8
56	46.1D2	46	21	Hap10-10	Hap31-26	Hap12-14	Hap6-6		I	8
57	50.1A4	50	21	NA	NA	Hap12-14	Hap6-6		I	8
58	45.1D5	45	22	NA	Hap30-33	Hap13-15	Hap6-6		H	7
59	44.1A4	44	15	NA	Hap35-36	Hap19-19	Hap5-5	Epsilon	J	9
60	41.1B1	41	1	NA	Hap19-19	Hap16-16	Hap3-3	Gamma	G	6
61	41.2A2	41	1	NA	NA	Hap16-16	Hap3-3		G	6
62	41.2B2	41	1	Hap15-17	NA	Hap16-16	Hap3-3		G	6
63	41.2C3	41	1	NA	NA	NA	NA		G	6
64	42.1B3	42	1	NA	Hap19-20	Hap16-16	Hap3-3		G	6
65	42.1B5	42	1	Hap17-17	Hap23-25	Hap16-16	Hap3-3		G	6
66	42.1C5	42	1	NA	NA	Hap16-16	Hap3-3		G	6
67	42.2A5	42	1	NA	NA	Hap16-16	Hap3-3		G	6
68	43.2A2	43	1	NA	NA	NA	NA		G	6
69	43.2A4	43	1	NA	Hap19-21	Hap16-16	Hap3-3		G	6
70	43.2B3	43	1	NA	NA	Hap16-16	Hap3-3		G	6
71	47.1B2	47	1	NA	NA	Hap16-17	Hap3-3		G	6
72	47.1B6	47	1	NA	Hap17-17	Hap16-16	Hap3-3		G	6
73	47.1D4	47	1	NA	Hap18-18	Hap16-17	Hap3-3		G	6
74	41.1C4	41	2	Hap14-17	NA	Hap16-16	Hap3-3		G	6
75	41.2B6	41	2	Hap14-14	NA	Hap16-16	Hap3-3		G	6
76	41.2C5	41	2	Hap14-17	Hap23-24	Hap16-16	Hap3-3		G	6
77	42.2A6	42	2	NA	NA	Hap16-16	Hap3-3		G	6
78	47.1A6	47	3	NA	Hap17-17	Hap16-16	Hap3-3		G	6
79	47.1B3	47	3	Hap14-14	NA	Hap16-16	Hap3-3		G	6
80	47.1C2	47	3	NA	NA	Hap16-16	Hap3-3		G	6
81	50.1A3	50	3	NA	NA	Hap16-16	Hap3-3		G	6
82	50.1B1	50	3	Hap14-17	Hap34-34	Hap16-16	Hap3-3		G	6
83	47.1C5	47	4	NA	Hap19-22	Hap16-16	Hap3-3		G	6
84	47.1B1	47	4	Hap17-17	NA	Hap16-17	Hap3-4		G	6
85	47.1D6	47	4	NA	NA	Hap16-16	Hap3-3		G	6
86	41.1A2	41	5	NA	Hap23-23	Hap16-16	Hap3-3		G	6
87	41.2B3	41	5	NA	NA	Hap16-16	Hap3-3		G	6
88	41.2C2	41	5	NA	NA	Hap16-16	Hap3-3		G	6
89	41.2D3	41	5	NA	NA	Hap16-16	Hap3-3		G	6
90	42.1B1	42	5	NA	NA	Hap16-16	Hap3-3		G	6
91	42.1B6	42	5	Hap14-17	NA	Hap16-16	Hap3-3		G	6
92	42.1C3	42	5	NA	NA	Hap16-16	Hap3-3		G	6
93	42.2B5	42	5	Hap17-17	NA	Hap16-16	Hap3-3		G	6
94	42.2D3	42	5	NA	NA	Hap16-16	Hap3-3		G	6
95	42.3A4	42	5	NA	NA	NA	NA		G	6
96	42.3A6	42	5	NA	NA	Hap16-16	Hap3-3		G	6
97	41.1D5	41	6	NA	NA	NA	NA		G	6
98	41.1D6	41	6	Hap16-16	NA	Hap16-16	Hap3-3		G	6
99	42.2C4	42	6	Hap14-15	NA	Hap16-16	Hap3-3		G	6
100	42.3C3	42	7	NA	NA	Hap16-16	Hap3-3		G	6
101	42.1A3	42	8	NA	NA	Hap16-16	Hap3-3		G	6
102	42.1A4	42	23	NA	NA	Hap16-17	Hap3-4		F	5
103	42.1C1	42	23	NA	NA	Hap16-17	Hap3-4		F	5
104	42.2D5	42	24	Hap18-19	NA	Hap16-18	Hap3-4		F	5

Intra-specific DNA transfers

In three cases, individuals belonging to a same species shared alleles in a cyclic fashion (Figure 3) as observed in Debortoli et al. (2016). In the first case, three genotypes within species β were connected in a cyclic fashion for marker HisB, with seven individuals harboring alleles 3||6, five individuals harboring alleles 3||9 and one individual presenting alleles 6||9 (Table 4 and Figure 5). However, the number of SNPs distinguishing each allele ranges from 3 to 8 mutations and only a single gene conversion event between position 335 and 379 could result in the transition from genotype 3||9 to genotype 6||9 (Figure 5, HisB). The five individuals presenting alleles 3||9 were identical for the five markers sequenced while the seven individuals harbouring alleles 3||6 for marker HisB were different for the other markers used. For ten out of twelve heterozygous individuals forming this cycle, allele phasing via *in silico* methods (Stephens *et al*, 2001; Flot *et al*, 2006; Flot, 2007; Flot, 2010; Flot *et al*, 2010) was confirmed by cloning the PCR-products into plasmids prior Sanger-sequencing. For the two other individuals (48.1C3 and 48.1B5), only one allele was retrieved

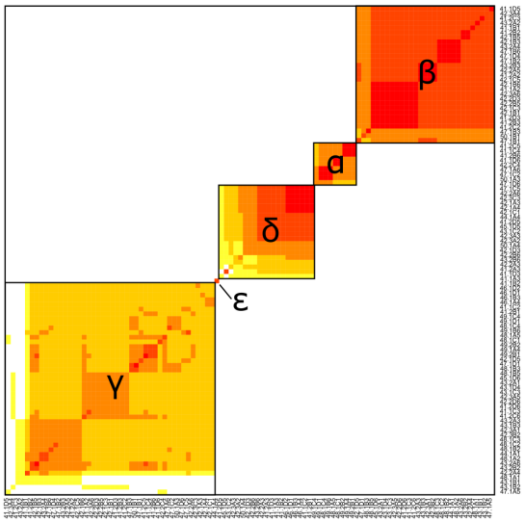

Figure 4 : Conspecificity matrix built from data retrieved from only *Adineta* individuals found in Courcelles (Belgium). Blocks along the diagonal represent individuals sharing 1 (yellow) to 5 (red) alleles (corresponding to the five regions sequenced). As a result, blocks along the diagonal represent cryptic species.

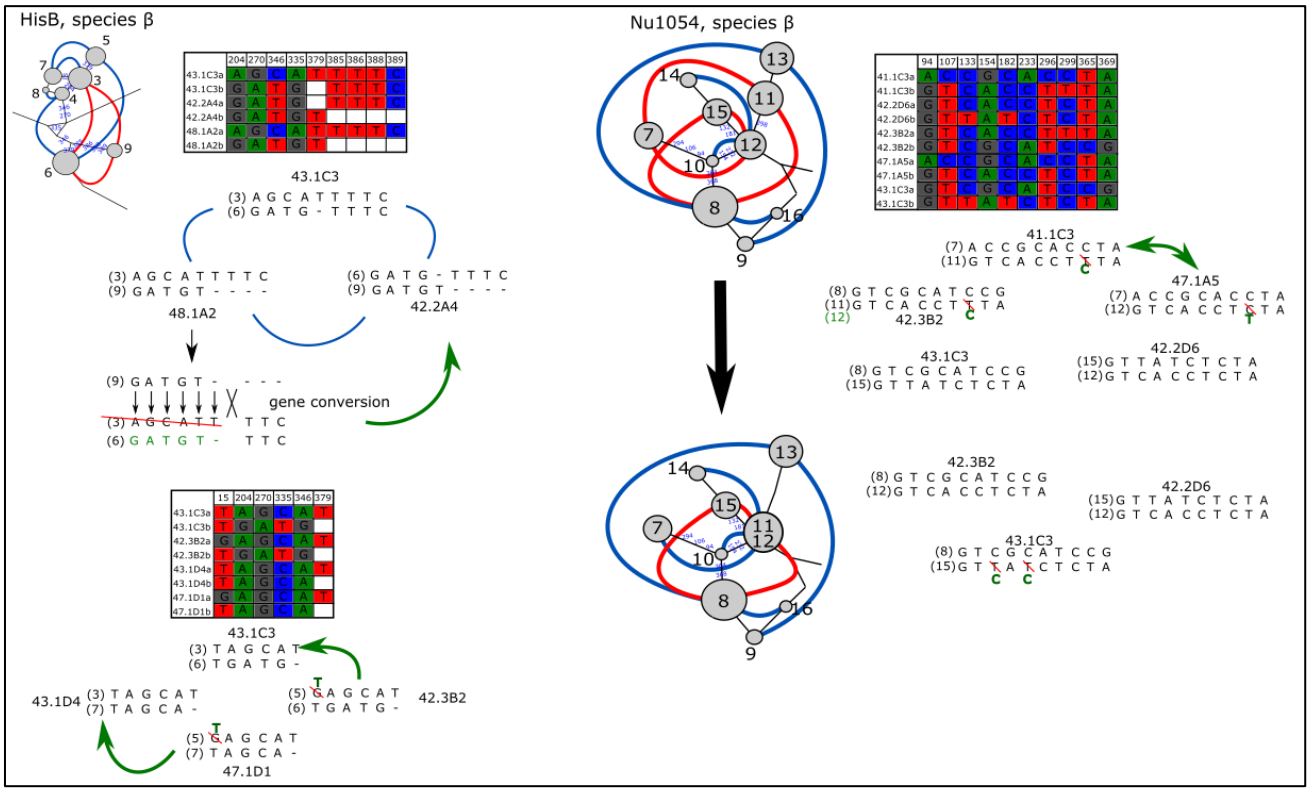

Figure 5 : Detailed analysis of the patterns of intraspecific DNA exchanges observed in Figure 3. Tables show the loci at which mutations occurred for individuals involved in the cycle. Gene conversions and SNPs theoretically explaining the transition from one allele to the other and resulting in the formation of a cycle without inter-individual exchange are indicated in green.

eventually due to under-sampling. In nine cases, additional sequences were found, but those additional copies were chimeras of the two alleles probably due to template switching during the final steps of the PCR reaction (Kanagawa, 2003). Additional PCR programs in which final elongation step was aborted were used to avoid this kind of artifacts, but no amplicons were obtained under the conditions tested. In addition, a more complex cycle composed of four distinct genotypes (3||6, 6||5, 5||7 and 7||3) was observed for this same species β on HisB. However, only one SNP distinguished alleles 5 and 7 from allele 3 (Figure 5, Nu1054). Another case of allele-sharing was observed for marker HisB, but among individuals belonging to species γ. Here again, only one mutation separated allele 14 and 15 causing transition from genotype 14||17 to 15||17 or inversely (Figure 3).

Finally, a last case of individuals sharing alleles in a cyclic fashion was observed in species β but for marker Nu1054 (Figure 3). This cycle was composed of five distinct genotypes (8||11, 11||7, 7||12, 12||15 and 15||8) in which alleles are separated by one to six mutations. Yet, a single mutation at position 299 would convert genotype 8||11 into genotype 8||12 simplifying the pattern into a cycle of three distinct genotypes (Figure 5). In this simplified cycle alleles are separated by two to six mutations but at least two mutations are necessary for the transition of one genotype to another. Here, the PCR-products corresponding to the individuals forming this cycle could not be cloned into plasmids as amplification did not work anymore, even though several PCR conditions as well as new primers and polymerase were tested.

Discussion

Patterns of allele sharing among individuals belonging to the same species were observed in this preliminary study with no whole-genome amplification step included, reducing the chances to generate genetic chimeras as recently debated (Debortoli *et al*, 2016, Wilson *et al*, 2018). Those patterns were similar to the ones observed in a previous study (Debortoli *et al*, 2016) and only three individuals were enough to close the cycle (Figure 3). However, after more detailed analyses of the sequences composing those cycles it appeared that only few mutations or a gene conversion event could explain the so-called allele sharing pattern for the markers sequenced (Figure 5). In another case, the cycle involved five individuals of species β but a DNA transfer among two individuals followed by a single point mutation would explain the exchange observed (Figure 5).

Another hypothesis parsimoniously explaining the patterns observed would be the presence of additional copies in the genomes, such as paralogs, with our genetic marker eventually amplifying different copies in distinct individuals. For the cycle observed in Nu1054 marker, alleles 8 and 15 were clearly distinguishable on the chromatograms of individual 48.1B3 but an additional allele 12 could have remained hidden due to PCR-biases. Similarly, the chromatograms obtained from individual 42.2D6 contained copies 12 and 15, allele 8 being eventually invisible. Overall, those biases would result in different sets of allele pairs being amplified and producing the cycle observed. This last hypothesis is very unlikely since we cloned and sequenced the amplicons of interest separately and could not detect the presence of such additional copies. Furthermore, one would expect to find at least a few cases in which more than two copies would be visible on the chromatograms obtained through direct amplicon sequencing (Figure 6). More specifically, additional alleles would not be visible on chromatograms from clone 48.1B3 (and the two others with same genotype) as a mix of alleles 8 and 15 results in a pattern theoretically containing the peaks expected under any combination of alleles 8, 12 and 15 (Figure 6). In contrast, chromatograms from clone 42.2D6 (and

the two other clones with the same genotype, see Table 4) should harbor minor peaks at position 365 and 369 testifying the presence of allele 8 in addition to alleles 12 and 15. Similarly, chromatograms from clone 42.3B2 (and the five other clones with identical genotype, see Table 4) should present minor peaks at position 133 and 182, indicating the presence of the unconsidered allele 15. However, species β appeared to be widely sampled as twenty-nine individuals were genotyped for this species and the amplification rate was high (96.5-100%) for each marker used; yet, we never observed any additional minor peak among our data. As a result, even though we could not verify through cloning the number of copies of Nu1054 for this allele sharing pattern, we are particularly confident that the cyclic patterns observed are not due to technical artifacts.

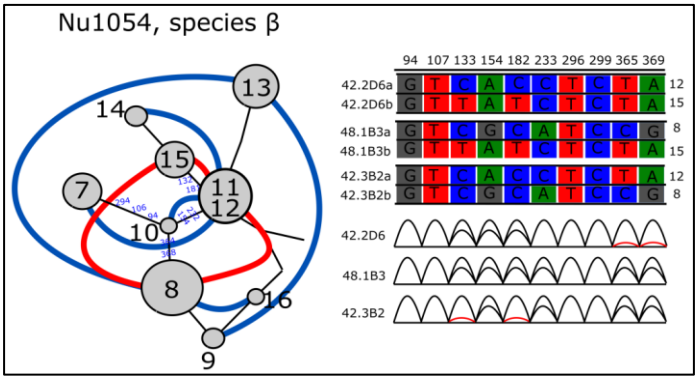

Figure 6 : Patterns of double-peaks expected in case of cross-contaminations between individual 42.2D6 and 42.3B2.

The patterns of allele sharing observed here may answer another question that was pointed out in the comments to Debortoli *et al* (2016) regarding the mechanisms responsible for such transfers. Indeed, distinct individuals are involved in the cycles observed for HisB and Nu1054, characterizing allele sharing among conspecific individuals (Table 4). Clones presenting genotype 3||9 and 6||9 for HisB harbor alleles that were not included in the cycle delimited for marker Nu1054 (genotypes 8||13 and 12||14). If a sex-related mechanism, such as *Oenothera*-like meiosis, was responsible for the patterns observed, one would expect to find similar patterns across multiple loci. Yet, the number of markers sequenced here is probably too small to infer the exact mechanisms. In the present case, two independent recombination events (sex-like or HGT) are more likely, the first event probably being a recombination between individuals harboring genotypes 8||15 and 12||15 at locus Nu1054 (*e.g.* between individuals 42.2D6 and individual 43.1C3) leading to the formation of a non-sampled recombinant. Then, a few mutations accumulated in the expanding population at different loci (*e.g.* Nu1054 cycles became more complex, with the formation of additional genotypes) resulting in the genotype observed in individual 42.3B2. Meanwhile, a second recombination among individuals similar to 43.1C3 and 42.2A4 resulted in individuals presenting a genotype similar to the one found in individual 48.1A2. Sequencing the genome of each clonal lineage involved in the allele sharing cycles described in species β would confirm those results since longer DNA regions, including the studied markers, and more DNA regions will be analyzed between the recombinants. If sharing of genetically near-identical alleles is observed throughout the genomes of the candidate individuals, the most parsimonious explanation would most likely be sex-related. If sharing is observed on a restricted number of loci, then horizontal gene transfers could be the key mechanism underlying the patterns observed. Dr. Matthieu Terwagne is working on the genome sequencing of the clones involved in the allele sharing cycles.

Table 4 : Detailed table focusing on species β for which two patterns of intraspecific exchanges were found. The genotypes described on Figure 5 are colored in orange.

	ID_individual	Moss_patch	COI	HisB	Nu1054	Scaff1	28S	Consp.Mat.	GMYC_species	ABGD_species
1	48.1B3	48	9	Hap3-3	Hap8-15	Hap7-11	Hap2-2	Beta	B	2
2	42.1D5	42	10	NA	Hap8-11	Hap5-9	Hap2-2		B	2
3	42.3A1	42	10	Hap5-6	Hap8-11	Hap5-9	Hap2-2		B	2
4	42.3B2	42	10	Hap5-6	Hap8-11	Hap5-9	Hap2-2		B	2
5	43.1B3	43	10	Hap5-6	Hap8-11	Hap5-9	Hap2-2		B	2
6	43.1B2	43	NA	Hap5-6	Hap8-11	Hap5-9	Hap2-2		NA	NA
7	43.2A3	43	10	Hap5-5	Hap8-11	Hap5-9	Hap2-2		B	2
8	41.1C3	41	11	Hap3-6	Hap7-11	Hap5-5	Hap2-2		B	2
9	41.2C6	41	11	Hap3-6	Hap12-14	Hap5-9	Hap2-2		B	2
10	41.2D6	41	11	Hap3-7	Hap12-15	Hap5-9	Hap2-2		B	2
11	42.2D6	42	11	Hap6-6	Hap12-15	Hap5-5	Hap2-2		B	2
12	42.3A5	42	11	Hap6-6	Hap12-15	Hap5-5	Hap2-2		B	2
13	43.1C3	43	11	Hap3-6	Hap8-15	Hap5-10	Hap2-2		B	2
14	43.1D4	43	11	Hap3-7	Hap7-11	Hap5-9	Hap2-2		B	2
15	43.2A1	43	11	Hap3-6	Hap7-11	Hap5-9	Hap2-2		B	2
16	45.1D6	45	11	Hap6-6	Hap13-13	Hap5-8	Hap2-2		B	2
17	48.1B5	48	11	Hap3-6	Hap7-11	Hap5-9	Hap2-2		B	2
18	47.1D1	47	12	Hap5-7	Hap10-12	NA	NA		B	2
19	47.1A5	47	13	Hap4-6	Hap7-12	Hap5-10	Hap2-2		B	2
20	42.2A4	42	25	Hap6-9	Hap12-14	Hap5-5	Hap2-2		B	2
21	43.2A6	43	17	Hap4-6	Hap9-13	Hap5-10	Hap2-2		C	3
22	43.2B2	43	17	Hap4-8	Hap9-13	Hap5-10	Hap2-2		C	3
23	44.1A1	44	26	Hap3-9	Hap8-13	Hap5-10	Hap2-2		D	4
24	48.1A2	48	26	Hap3-9	Hap8-13	Hap5-10	Hap2-2		D	4
25	48.1B2	48	26	Hap3-9	Hap8-13	Hap5-10	Hap2-2		D	4
26	48.1C2	48	26	Hap3-9	Hap8-13	Hap5-10	Hap2-2		D	4
27	48.1C3	48	26	Hap3-9	Hap8-13	Hap5-10	Hap2-2		D	4
28	48.1A1	48	16	Hap3-3	Hap8-16	Hap5-10	Hap2-2		D	4
29	43.1B1	43	14	Hap3-6	Hap8-15	Hap6-10	Hap2-2		E	4

One would expect that sex-related mechanisms may potentially be more frequent than horizontal gene transfers of DNA fragments. Bdelloids however go often through cycles of desiccation/rehydration in their semi-terrestrial habitats during which the genome and cell integrity is compromised and then repaired, enhancing the chances for such transfers. It is hard to determine this frequency based on the present study as our experimental procedure aimed at being qualitative instead of quantitative to validate/invalidate the observation of Signorovitch *et al* (2015) and Debortoli *et al* (2016). Indeed, we isolated all *Adineta* individuals observed in the dried patches collected, but we could not culture all of them. Yet, we may still be able to provide a rough idea of how long ago this event occurred based on the diversity among individuals involved in the cycles. Indeed, if DNA transfers among conspecific individuals are rare, one may expect that the different individuals involved in the allele sharing cycles are daughters of the individuals that originally recombined (*e.g.* 44.1A1, 48.1A2, 48.1B2, 48.1C2 and 48.1C3 are probably relatives). This explanation would be more parsimonious than multiple independent exchanges among closely related individuals. If daughters diverge, one may hypothesize that the DNA transfer occurred a few generations ago and that clones had time to accumulate mutations or even additional transfers. As an example, all the clones harboring genotype 8||11 for Nu1054, and thus involved in the cycle observed in species β, have exactly the same genotype for all the other markers sequenced, suggesting a recent clonal expansion (Table …). In contrast, individuals presenting alleles 12||15 for Nu1054 differed at locus HisB by a few mutations (six to seven SNPs; Figure 5) indicating longer divergence time. Similarly, individuals harboring alleles 8||15 for Nu1054 differed by a few mutations for marker COI, Scaff1 and HisB (Figure 3). A simple hypothesis for these observations is

that individuals reproducing asexually for several generations diverged at multiple loci, then a DNA transfer event occurs between two distinct individuals (still conspecific) followed by a clonal expansion of the recombinant. Interestingly, the two most frequent genotypes found in species β are indeed the recombinant ones, i.e. one locus is involved in a allele sharing cycle. The absence of divergence among the daughters of one recombinant individual suggests that this event is quite recent.

Finally, we could not identify in our study signatures of interspecific transfers as presented in Debortoli *et al* (2016) (Figure 4). This does not mean that such transfers among more genetically distant individuals are not possible. However, our results point out that the frequency of interspecies transfers is probably much lower, potentially indicating that the molecular mechanisms responsible for such events are different than the ones for intraspecific transfers. A RADsequencing study started by Dr. Marie Cariou will investigate interspecific transfers through phylogenomic.

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Chapter 4: Bdelloid rotifer communities shaped by seasons, inter-specific variations and geographical distance

This fourth chapter is in connection with the initial aim of the experiment conducted in chapter 1, *i.e.* the study of diversity and structure of bdelloid rotifers community at a very local scale. Even though we mainly discussed the patterns of potential genetic transfers observed in that chapter, the results on community structure *per se* were really interesting too. First of all, a total of six distinct cryptic *Adineta* species (labelled A-F) were retrieved, one of which (species C) was extremely abundant representing 68.75% of the individuals sampled (Debortoli et al, 2016). Interestingly, this species was present in 38.89% of the patches sampled but extremely rare in patches collected from the soil (Table 1) suggesting adaptation to the local conditions. Second, we did not test it statistically, but it seemed that other species were absent or rare in patches colonized by dominant species C. Yet, it is hard to tell if this pattern is due to high competition strength of species C or different habitat preferences as shown for distinct morphospecies (Fontaneto et al, 2011). Similarly, some species were frequent in patches located on tree trunks at higher heights (species A and C) whereas other species seemed specific to patches collected near or from the soil (species E and F). Soil was hypothesized to desiccate less frequently than patches more exposed to wind (*e.g*. on trunks) and, as a result, to harbor more desiccation-sensitive parasites infecting bdelloid rotifers (Wilson et al, 2013). However, it remains unclear if the species present at ground levels were permanently dwelling there or if frequent immigration from the metacommunity caused the species distribution observed. The same question applies to the two species that were extremely rare, being represented by only one or four individuals (species B and D, respectively). It would not be surprising that frequent immigration may play an important role in the community structure as bdelloid rotifers are known to be easily dispersed by wind (Fontenato et al, 2006a; Fontaneto and Ricci, 2006b; Wilson et al, 2013), but this has still to be tested.

Another important question that has only had little consideration concerns the temporal dynamics of bdelloid communities (Ricci et al, 1989). Several ecological processes could explain the abundance of the dominant species C, such as frequent immigration from a source population or rapid clonal reproduction of rare colonizers. In both cases, it would also be relevant to understand if processes responsible for those dynamics are constant over time or vary with environmental conditions. Bdelloid rotifers have been shown to follow distribution patterns similar to protists which also have wide dispersal ranges (Fontaneto et al, 2006a). Nonetheless, bdelloids life cycle is sensitively different from protists, probably leading to slightly distinct dynamics. Overall, answering those questions would bring more insights into the evolutionary success of bdelloid rotifers in spite of their asexual reproduction.

In this chapter, we developed a simple ecological model to simulate the colonization of bare habitat and the early development of bdelloid communities. The different parameters and biological traits presented above (i.e. immigration, reproduction, species interaction strength, survival and environmental conditions) were implemented into distinct ecological scenario in order to

discriminate the intrinsic effect of each. We controlled the accuracy of this model by comparing the simulations with field data collected every three months.

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Table 1 : Frequency of each species (A-F) isolated from the thirty-six patches collected in Park Louise-Marie (Namur, Belgium) and presented in Chapter 1. The location of each patch on/around each tree is indicated; "high" patches were sampled above 1.5m, "low" patches were collected below "0.5m" and "soil" represents patches located on the ground around tree roots.

Patch_ID	Tree	Location	Species_A	Species_B	Species_C	Species_D	Species_E	Species_F
A1_1	A	High			36			
A1_2	A	High			14	1		
A1_3	A	High			1			
A1_4	A	High			9			
A1_5	A	High						
A1_6	A	High			12	3		
A2_1	A	Low					6	
A2_2	A	Low					5	
A3_1	A	Soil					2	
A3_2	A	Soil			2			
B1_1	B	High	42					
B1_2	B	High	12					
B1_3	B	High	14	1	142	1		
B1_4	B	High	3					
B2_1	B	Low			20		3	
B2_2	B	Low			113			
B3_1	B	Soil	3		1		3	
B3_2	B	Soil	2					
B3_3	B	Soil			1			
C1_1	C	High	1					
C1_2	C	High	4					
C1_3	C	High	3					
C2_1	C	Low	1		2			9
C2_2	C	Low			8			
C3_1	C	Soil	2					
C3_2	C	Soil					1	
C3_3	C	Soil	1				2	
D1_1	D	High	22					
D2_1	D	Low			35			
E1_1	E	High	2					
E1_2	E	High	17					
E2_1	E	Low						1
E2_2	E	Low						10
E3_1	E	Soil	1					
E3_2	E	Soil					1	
E3_3	E	Soil					1	
			130	1	396	5	24	20

Submitted paper: Bdelloid rotifer communities shaped by seasons, inter-specific variations and geographical distance

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Abstract

Bdelloid rotifers communities have been postulated to follow the same dynamics than for other microscopic organisms due to their high capacity to be passively dispersed by wind when desiccated. Here, we used a simple competition model to test if habitat preferences solely could explain bdelloid distribution over time or if dispersal limitation also plays a role. In order to test if our model correctly fitted the colonization of new habitats, we compared the simulations with *Adineta* communities collected seasonally for two years on rooftops (Namur, Belgium). We tested five ecological scenarios based on five hypotheses possibly explaining the bdelloid rotifers dynamics and found that immigration was the most critical parameter under the conditions tested with one species rapidly colonizing habitat patches and overcompeting most others. In addition, we run the model under different levels of environmental conditions (permissive, intermediate and harsh) to simulate community variations across seasons and found that communities endure important bottlenecks yearly in winter. Overall, our study indicates distinct immigration rates and habitat preferences amongst cryptic *Adineta* species.

Introduction

For many micro-organisms, frequent and long-range dispersal often makes global diversity (gamma) similar to local diversity (alpha) (Finlay, 2002). Some studies even suggest that the distribution of microscopic species is a function of habitat preferences rather than of dispersal limitation or historical contingency (Bass and Cavalier-Smith, 2004; Costello and Chaudhary, 2017). This has also been suggested for bdelloid rotifers, a clade of microscopic animals (<2mm) showing distinct specificities for habitat type and strong dispersal capacity (Fontaneto et al, 2006a; Fontaneto et al, 2011).

Bdelloid rotifers are very abundant animals in semi-terrestrial habitats such as soil, moss and lichen patches (Fontaneto *et al*, 2006a; Fontaneto *et al*, 2007; Fontaneto *et al*, 2008) forming small propagules, called “tuns”, when entering a dormant stage during long periods of drought or freezing (Ricci, 1998; Ricci and Caprioli, 2005). Available data reveal patchy local distribution of bdelloids, with almost no overlap in species composition among different substratum (Fontaneto *et al*, 2006a; Fontaneto *et al*, 2007; Fontaneto *et al*, 2008), suggesting specificity to habitat type (Fontaneto *et al*, 2006a; Fontaneto and Ricci, 2006b; Fontaneto *et al*, 2011). However, “tun” formation provides them strong dispersal capacity: they can easily disperse with the wind or be attached to microscopic sediments that can be passively transported over large distances, enabling a large geographic distribution of bdelloid rotifer species (Fontaneto and Ricci, 2006b; Wilson, 2011).

While the life-span of bdelloid rotifers rarely exceeds 30 days, propagules can persist several years in desiccated state and eventually return to an active state upon rehydration (Guidetti and

Jönsson, 2002). Survival after desiccation has never been quantified under harsh environmental conditions, but laboratory experiments demonstrated that survival could be extremely high under controlled permissive conditions depending on the protocol used (Ricci, 1998; Hespeels *et al,* 2014). For example, survival could reach 60-100% when groups of 10-20 individuals of *Adineta vaga* or *Philodina vorax* were desiccated on filter paper respectively (Ricci, 1998). In contrast, single *A. vaga* individuals desiccated on agarose rarely survived (Hespeels *et al*, 2014). Ricci and Caprioli (2005) even described a reproductive boost following rehydration of the desiccated propagules. Desiccation therefore appears a critical component of the bdelloid rotifers life-cycle also providing a way out from parasites (Wilson *et al*, 2010; Wilson, 2011). It has indeed been shown that the association of bdelloid rotifers with their *Rotiferophthora* parasites could be disrupted when dry periods were coupled with wind dispersal, the parasites being less desiccation-tolerant. Besides their extreme desiccation tolerance, bdelloids are remarkable for their asexual mode of reproduction Maynard Smith, 1978). No males or meiosis have ever been observed in bdelloids and their genome appears incompatible with homologous chromosome pairing, ruling out the possibility for sexual reproduction (Flot *et al*, 2013; Fontenato and Barraclough, 2015). Each female is able to clone herself, which allows rapid colonization of new habitats starting from a single individual.

Here, we studied how those peculiar eco-evolutionary features could impact the spatial and temporal dynamics of bdelloid communities. Several studies have been published on the spatial distribution of bdelloid rotifers species showing large dispersal capacities and low but significant habitat preferences (Fontaneto *et al,* 2006a and 2011). To our knowledge, only one study included the temporal dynamics, describing no variation in species composition with seasonality (Ricci *et al*, 1989). However, this study focused on a single large moss patch from which subsamples were collected monthly. As a result, it is difficult to disentangle the different ecological parameters (dispersal, habitat preferences, species interactions, environmental variations…) and their respective impact on community structure, each subsample being inter-dependent. We carried out controlled, quantitative field experiments during two years, sampling every three months nine

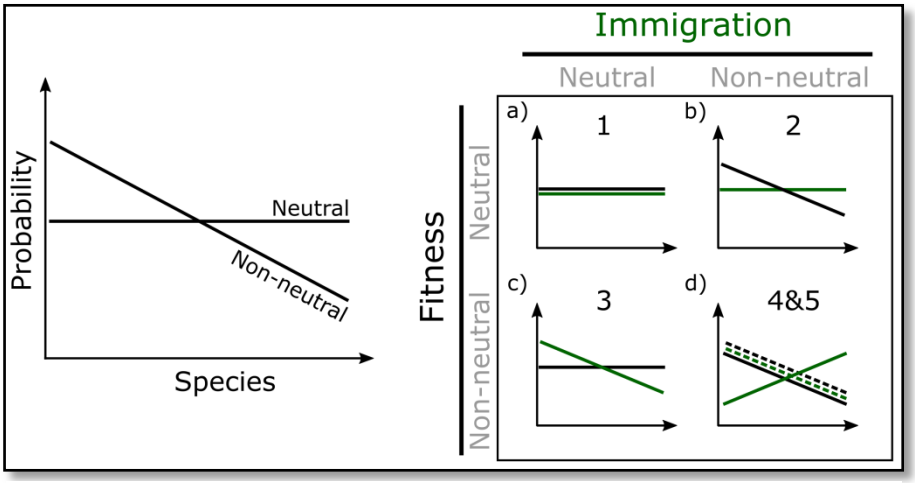

Figure 1 : Probability distribution tested for the model under five distinct scenarios. On the left panel: two scenarios are presented. In one scenario, the probabilities of a given parameters are identical across all species (neutral). In the other scenario, the probabilities vary across species (non-neutral). On the right panel: five scenarios build from distinct combinations (neutral and non-neutral) of two parameters (the species-specific probability to immigrate in the community (in green) and fitness (in black)) are shown. a) In scenario 1, all species have the same probabilities to immigrate, reproduce and survive at each time-step (linear distribution). b) In scenario 2, the probability to immigrate is identical across species, but the probability distributions to reproduce and survive vary. c) Scenario 3 simulates conditions opposite to scenario 2, reproduction and survival are equal for all species but the probability to immigrate varies across species. d) Solid lines represent scenario 4 which models a trade-off between the probabilities to immigrate and the fact to reproduce and survive. The dashed lines on d) represent scenario 5 in which probabilities to immigrate, reproduce and survive at each time-step co-vary.

communities located on three distinct roofs in Namur (Belgium) and analyzed the data with a simple competition model. Using the model, we simulated colonization and the subsequent community dynamics of bdelloid individuals of the genus *Adineta*. Our model included variation among individuals and species and relied on solely three main parameters: the immigration rate (*i.e.* the dispersal capacities of each species), the survival probability, and the reproductive output of each individual. The two latter parameters may be regarded to as indicative of species fitness. We ran the model under five distinct scenarios (Figure 1), making various assumptions on how fitness and dispersal contributed to local dynamics, and compared the resulting simulations to our experimental data.

In a first scenario, we assumed all species to have similar fitness (*Adineta* species have similar feeding capacities as they all graze on the substrate surface), similar dispersal rate (size of 'tuns' is comparable), and having closely related life-cycles resembling the assumptions underlying neutral models (Hubell, 2001). Species co-occurrence is only short-lived and due to stochastic events (birth-death-immigration). In a second scenario we also assumed similar dispersal rates, but distinct habitat preferences depicted by distinct probabilities to survive and reproduce (Fontaneto *et al,* 2006a and 2011). In a third scenario we assumed that dispersal rate varied across species. We define dispersal rate as the chance for one individual to be passively transported to the community from the metacommunity. This could be the case in the context of a source-sink dynamic in which extremely abundant species, at a given place, could immigrate more frequently in nearby habitat patches (Amarasekare and Nisbet, 2001; Lowe and McPeek, 2014). In a fourth scenario, we assumed that fitness and dispersal rate covaried negatively. This scenario reflects the observation of Ricci and Caprioli (2005) that life-history traits of bdelloid rotifers could vary with habitat preferences, providing evidence for a shorter life span, a higher fecundity and earlier age at first reproduction in bdelloid species that do not desiccate and inhabit permanent freshwater bodies. As a consequence, bdelloid species less tolerant to desiccation should have limited dispersal capacities but a higher reproductive output. Finally, in a fifth scenario all parameters covaried positively. We postulated that one or a few species are widely represented in the nearby area due to specific adaptation, favoring the chances for short-range dispersal and persistence. We included this scenario because Debortoli *et al* (2016) observed that the two most abundant species in the local community (named species A and C) were also the one present in most samples (39-44% of the lichens sampled) (see also Ricci et al, 1989). It has been shown in other microscopic species that community similarity decreased significantly with geographical distance, more than with environmental distance (Soininen et al, 2007).

Materials and methods

Sampling and species delimitation

We selected three flat roofs located across the UNamur campus (Belgium, Namur; 50°27'58.27"N; 4°51'37.76"E) on each of which we placed three Petri dishes (Ø = 10 cm) filled with 20mL of 3% solid agarose. To avoid any contamination the dishes were kept closed with Parafilm® until being placed on the respective roofs. The three Petri dishes from a same roof were located 8-25 meters from each other whereas 32-148 meters separated the dishes from distinct roofs. Each Petri dish was fixed on a block and maintained leaned for three months, being exposed to environmental conditions and to passive dispersal of micro-organisms (Figure 2a). This kind of setting resulted in a humid but not submerged community mimicking the limno-terrestrial habitat of rotifers with the

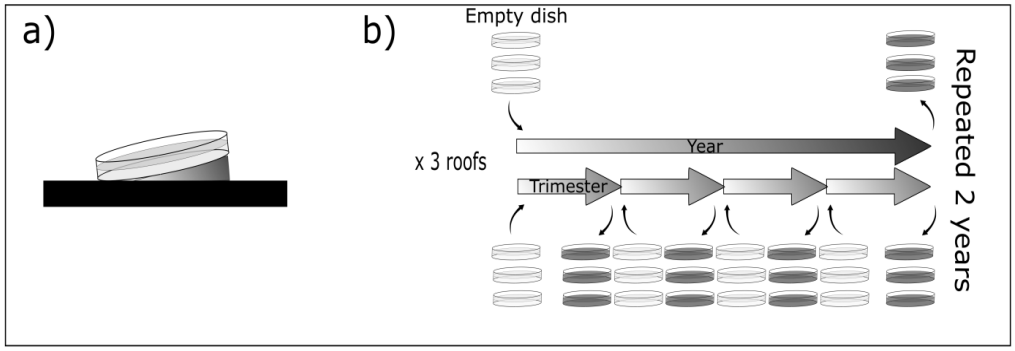

Figure 2 : Experimental design for *A. vaga* sampling on the roofs of UNamur campus (Belgium, Namur; 50°27'58.27"N; 4°51'37.76"E). a) Representation of the freshly placed 10cm² Petri dishes that were coated with 3% agarose and maintained inclined to enable moisture without bathing. b) On each of the three roofs, three distinct empty Petri dishes were set and replaced every three months with fresh dishes. The replaced dishes were brought back to the lab for community analyses. In parallel three additional dishes were placed on each roof for a period of one full year before being replaced. This experiment was conducted for two years.

layer of agarose buffering humidity variations and forming a suitable substrate for bdelloid rotifers (Wilson and Sherman, 2013). Every three months, each dish on each roof was carefully sealed with Parafilm® and brought back to the lab for morphological identification and isolation of each *Adineta sp.* individual morphologically determined following Donner (1965). Our isolation protocol consisted in washing each bdelloid rotifer by pipetting it into successive clean Spa® water drops and transferring it to an individual tube. Each tube was then briefly centrifuged to pellet the isolated animal and inspected under a binocular to make sure that only a single rotifer was present. If no individual was found in the tube, the tube was discarded (no second individual was added into the tube to avoid contamination).

In parallel, we did place one Petri dish, prepared with the same method, next to each of the trimestral dishes and left it one full year on the roof. It was then collected and analyzed like the others. The experiment took place from December 2013 to December 2015, in total 8 samplings of the trimestral dishes were done and 2 samplings of the yearly dishes (Figure 2b).

The genomic DNA of each isolated *Adineta sp.* individual was then extracted using the Chelex® protocol: each sample was mixed with 35µL of Chelex® solution [InstaGene™ Matrix, Bio-Rad, #7326030] and 1 µL of proteinase K [Qiagen, #19133], homogenized by vortexing, heated 20 min at 56°C and 10 min at 95°C. Then, the Chelex® beads were precipitated for 5 min at 14000 rpm and the supernatant containing the genomic DNA was transferred to a new tube and stored at -20°C. We used the data from Debortoli et al (2016) in which all individuals sampled were genotyped and calculated a rarefaction curve ('vegan' R package; Oksanen et al, 2007) to delimit the minimum subset of individuals to genotype in order to retrieve accurate species richness (Supplemental figure 1). As a result, for each combination of dish per sampling period, a subset of 24 individuals were randomly selected to amplify a portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene using Folmer's universal primers (HCOI: 5' - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3' and LCOI: 5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3'; Folmer *et al*, 1994). The PCR conditions were the same as in Debortoli *et al* (2016), except that the quantity of gDNA used was 5µL and that the number of cycles was set to 60. The amplicons were sent for Sanger sequencing to the GENEWIZ facilities (anciently Beckman-Coulter Genomics, UK).

The COI sequences obtained were aligned with 258 *Adineta sp.* sequences from GenBank and 378 *Adineta sp.* sequences from our own projects using MAFFT (E-INS-i method; Katoh and Standley, 2013) and visualized in MEGA5 (Tamura *et al*, 2011). One sequence was selected for each distinct

haplotype resulting in a final dataset of 364 COI unique sequences (Supplemental table 1) of 589bp which was used for the ultrametric Bayesian tree reconstruction (BEAST v1.6.2; Drummond and Rambaut, 2007). We used the same parameters than previously applied to bdelloid rotifers to generate the trees (Tang *et al*, 2014; Debortoli *et al*, 2016) except that we combined 3 independent analyses with the LogCombiner package to avoid MCMC to be stuck in a local optimum. The resulting tree was used as input for species delimitation by the General-Mixed Yule Coalescent method with single threshold (Pons *et al*, 2006; Fujisawa and Barraclough, 2013).

Community diversity across roofs and seasons

We represented our entire dataset on a species matrix clustered in order to group communities that present similar species assemblages using "heatmap3" package on R (Zhao *et al*, 2014). For each Petri dish, we calculated the total abundance, the number of species delimited by the GMYC method (referred to as "richness"), and Pielou's Index of evenness (Pielou, 1966). We also calculated the Bray-Curtis indices of dissimilarities for each pair of communities using the "vegan" R package (Bray and Curtis, 1957; Oksanen *et al*, 2007). Then, we computed a Mantel test to identify whether dissimilarities between communities was related to geographic distance and ANOVA tests to identify which parameters (seasons and roofs) affected the different metrics calculated for each community (abundance, species richness, Pielou's index of evenness, spatial dissimilarities). The meteorological data were provided by the Royal Meteorological Institute (IRM, see Supplemental table 2) which has a recording station located just outside Namur. Finally, we ran a factorial analysis (R, "ade4" package) to define species assemblages and community structures.

Model

We used a discrete time competition model (Beverton and Holt, 1957; Hart et al, 2016), but allowing overlapping generations. The model incorporates three processes which intuitively apply to bdelloid rotifers: clonal reproduction, passive immigration, and survival:

$$G_i = Binomial(N_i, g_i) \quad (1)$$

$$M_i = Binomial(1, m_i) \quad (2)$$

$$S_i = Binomial(N_i, s_i) \quad (3)$$

where N_i is the abundance of species i; g_i is its probability to reproduce; m_i is its probability to immigrate in the community; and s_i is its probability to survive. G_i is the number of individuals from this species that will reproduce. M_i is the number of immigrating individuals of species i (either 0 or 1). S_i is the number of individuals of species i that survive.

The actual reproductive output from the reproducing individuals is given by $G_i C_i$, where C_i measures the intensity of competition within and among species:

$$C_i = Poisson\left(\frac{Y_i}{1+\frac{\sum_j^n \alpha_{i,j} G_j}{K}}\right) \quad (4)$$

with Y_i the fecundity per individual of species i, K is the habitat size, and $\alpha_{i,j}$ is the intra- and inter-specific interaction strength.

Thus, the equation allows intraspecific variations and the overall dynamics of species i is given by:

$$N_{i,t+1} = G_i C_i + S_i + M_i \quad (5)$$

Parameters setting

1) Default parameters

To our knowledge, no studies measured survival or reproduction in field conditions for bdelloid species. Ricci et al (1983) observed that, in laboratory conditions (at 20°C), *Adineta vaga* lived 17 days on average with the main reproduction occurring during the early days of maturity and then slowly decreasing until death. They calculated that one *Adineta vaga* individual produced on average 1.2 eggs per day throughout its reproductive days, that the mean generation time was 7.7 days and that the intrinsic population increase was 0.344. As a result, we set Y equal to 1 for all species, corresponding to realistic numbers of eggs laid per individual at each time-step (*i.e.* one week or one generation)(Supplemental figure 2a). Although we observed one reproductive peak at day 6 during which fecundity reached 6 eggs, we estimated that this was due to controlled laboratory conditions and rarely the case in nature (see Supplemental data). Thus, we simplified the model by considering that fecundity was independent of the individual age and we implemented a stable fecundity throughout the simulations. We set the probability to reproduce (g_i) to 0.3 as Ricci (1983) observed a similar rate of natural increase in *A. vaga* (Supplemental figure 2b). We always ran the model for 12 time-steps, each representing one week, to simulate three months of community dynamics, which corresponded to our field experiment. For the parameters defined here, the maximum number of individuals for the species with the highest reproductive output would be 60 individuals after 12 time-steps on average.

We tested several survival probabilities (s_i) and obtained that 0.9 was the most accurate value in order to have around 40% of the initial population still alive after 12 time-steps, as observed in our preliminary studies on clone AD008 (Supplemental figure 2c and Supplemental data).The average number of immigrants per week was calculated in a preliminary study during which we used the same experimental design, but sampled dishes weekly. There were *Adineta* individuals in 13 out of 48 Petri dishes (13/48 = 0.271) after one week and 20 *Adineta* individuals in total (20/48 =0.417). As three dishes presented more than one individual, we could not determine if each rotifer immigrated independently or if the first colonizer reproduced; we therefore decided to set the probability to immigrate from the metacommunity (m_i) to 0.3 for *Adineta* species. Finally, we set the habitat size (K) to 150 as the maximum number of *Adineta* individuals found in a single lichen patch by Debortoli *et al* (2016) was 157.

2) Experimental factors

We ran the model according to the five scenarios explained in the introduction (Figure 1 and Supplemental figure 3). Furthermore, we considered that the indices of population growth calculated by Ricci et al (1983) in optimal laboratory conditions probably overestimated the reproductive output in harsher field conditions. We therefore ran each of the five scenarios three times: reproduction, survival and dispersal probabilities were 2 times lower in intermediate conditions (orange in Supplemental figure 3) and 10 times lower harsh conditions (red) than in permissive (green), respectively.

We ran all simulations using four different types of probability distributions for g_i, s_i and m_i: linear, exponential and normal, as well as a hybrid between the exponential and normal distribution

(Supplemental figure 3). Except for scenario 1, we predicate that probabilities to immigrate, reproduce and survive at each time-step are not equal across all species. The exponential and the normal distributions also represent differences among species, but one (exponential) or a few (normal) species are supposed to be particularly efficient at immigrating and well adapted in comparison with the other species (Ricci *et al*, 1989; Fontaneto *et al*, 2011; Debortoli *et al*, 2016). We also tested a hybrid between an exponential and a normal distribution that would represent the case in which one species adapted to the local conditions outcompetes a few other species that are able to colonize and persist in sub-optimal conditions, e.g. species from alike habitats located in the nearby area. For scenario 1, only the linear distribution is applicable.

Finally, because, to our knowledge, no information about the ecological interactions between bdelloid species are available, we ran the model under three arbitrary intensities of intra- and inter-species interaction strength (with $\alpha_{i,j}$ = 0, 0.1 and 0.2).

Comparison with field data

For each combination of scenario (5), conditions (3), distribution shape (5) and species interaction strength (3), we simulated 100 communities. We started all simulations with n species (delimited by the GMYC analysis) of null abundance to represent the empty communities at t_0. At each of the 12 time-steps, the frequency of each species was recorded.

In order to estimate the accuracy of the model, we compared the simulations with the experimental data as t the difference Δ between data and predictions (De Laender *et al*, 2014):

$$\Delta = -\sum_{i=1}^{r} |log(A_{pred} + 1) - log(A_{obs,i} + 1)|$$

Where r is the number of communities and A is the variable of interest (the relative abundance of each species across the whole simulation, the abundance of individuals in each patch, species richness, evenness, or spatial dissimilarities) at t=12. Values of Δ approximating zero indicate a better model fit. Then, the sum of all the Δ calculated for each simulation was calculated to compare global best model fit. The model and all statistics were run in R, using the "vegan" package for Pielou's index of species evenness and spatial β-diversity (Oksanen *et al*, 2007).

Results

Meta-community structure

We isolated bdelloid individuals morphologically identified as *Adineta sp.* from 81 out of the 90 roof patches sampled during two years, representing a total of 2663 *Adineta* individuals (median = 16.5 individuals per patch, range = 0-118). The sampling details and the subset of 1169 individuals for which we successfully sequenced the mtCOI marker are described in Table 1. This represented a total of 56 distinct mtCOI haplotypes that were combined with the published *Adineta* sequences to delimit genetic clusters (*i.e*. species) using the General-mixed Yule coalescent approach. This method significantly delimited 117 species within *Adineta* (confidence interval: 111-135; number of ML entities identified being 119 – 2 outgroup sequences; LR test: 0.0), 24 species were retrieved in our two-year study on the roofs (Supplemental figure 4).

Location	Timepoint	Season	Ind.Sampled	Ind.Genotyped	Species.Richness	Evenness	Spat.Dissim.
Roof_A_1	TP3	Winter	0	NA	NA	NA	NA
Roof_A_2	TP3	Winter	0	NA	NA	NA	NA
Roof_A_3	TP3	Winter	0	NA	NA	NA	NA
Roof_B_1	TP3	Winter	10	10	2	0.469	0.702
Roof_B_2	TP3	Winter	15	18	2	0.353	0.668
Roof_B_3	TP3	Winter	18	38	1	NA	0.665
Roof_C_1	TP3	Winter	22	25	1	NA	0.693
Roof_C_2	TP3	Winter	0	NA	NA	NA	NA
Roof_C_3	TP3	Winter	3	3	1	NA	0.835
Roof_A_1	TP4	Spring	1	4	1	NA	1.000
Roof_A_2	TP4	Spring	0	NA	NA	NA	NA
Roof_A_3	TP4	Spring	3	5	2	0.918	1.000
Roof_B_1	TP4	Spring	9	10	2	0.991	0.720
Roof_B_2	TP4	Spring	15	92	4	0.688	0.573
Roof_B_3	TP4	Spring	17	91	3	0.841	0.604
Roof_C_1	TP4	Spring	9	39	1	NA	0.570
Roof_C_2	TP4	Spring	15	38	1	NA	0.576
Roof_C_3	TP4	Spring	17	37	1	NA	0.597
Roof_A_1	TP5	Summer	20	21	4	0.628	0.974
Roof_A_2	TP5	Summer	10	11	3	0.582	0.617
Roof_A_3	TP5	Summer	22	24	4	0.671	0.967
Roof_B_1	TP5	Summer	23	83	1	NA	0.686
Roof_B_2	TP5	Summer	18	21	4	0.668	0.969
Roof_B_3	TP5	Summer	3	3	2	0.918	0.825
Roof_C_1	TP5	Summer	11	28	1	NA	0.595
Roof_C_2	TP5	Summer	21	47	3	0.710	0.641
Roof_C_3	TP5	Summer	10	12	2	0.469	0.614
Roof_A_1	TP6	Autumn	5	5	2	0.722	0.938
Roof_A_2	TP6	Autumn	4	4	1	NA	0.768
Roof_A_3	TP6	Autumn	15	15	3	0.442	0.888
Roof_B_1	TP6	Autumn	12	13	2	0.414	0.622
Roof_B_2	TP6	Autumn	1	1	1	NA	0.990
Roof_B_3	TP6	Autumn	24	118	4	0.798	0.776
Roof_C_1	TP6	Autumn	20	109	1	NA	0.584
Roof_C_2	TP6	Autumn	23	55	1	NA	0.608
Roof_C_3	TP6	Autumn	20	88	1	NA	0.584
Roof_A_1	Year1		20	60	5	0.970	0.939
Roof_A_2	Year1		16	18	2	0.337	0.684
Roof_A_3	Year1		20	64	4	0.798	0.939
Roof_B_1	Year1		24	61	3	0.819	0.709
Roof_B_2	Year1		13	17	2	0.619	0.929
Roof_B_3	Year1		1	2	1	NA	1.000
Roof_C_1	Year1		15	15	1	NA	0.692
Roof_C_2	Year1		9	14	2	0.764	0.781
Roof_C_3	Year1		0	NA	NA	NA	NA
Roof_A_1	TP7	Winter	0	NA	NA	NA	NA
Roof_A_2	TP7	Winter	8	8	1	NA	0.482
Roof_A_3	TP7	Winter	0	NA	NA	NA	NA
Roof_B_1	TP7	Winter	23	27	2	0.667	0.512
Roof_B_2	TP7	Winter	13	13	3	0.488	0.458
Roof_B_3	TP7	Winter	23	29	4	0.527	0.510
Roof_C_1	TP7	Winter	10	10	1	NA	0.434
Roof_C_2	TP7	Winter	9	9	1	NA	0.451
Roof_C_3	TP7	Winter	23	60	2	0.258	0.512
Roof_A_1	TP8	Spring	1	3	1	NA	0.969
Roof_A_2	TP8	Spring	1	3	1	NA	0.969
Roof_A_3	TP8	Spring	7	11	5	0.963	0.816
Roof_B_1	TP8	Spring	14	16	1	NA	0.495
Roof_B_2	TP8	Spring	22	36	3	0.872	0.596
Roof_B_3	TP8	Spring	24	69	1	NA	0.509
Roof_C_1	TP8	Spring	10	13	1	NA	0.558
Roof_C_2	TP8	Spring	23	68	1	NA	0.499
Roof_C_3	TP8	Spring	21	72	1	NA	0.490
Roof_A_1	TP9	Summer	23	93	2	0.667	0.888
Roof_A_2	TP9	Summer	5	5	5	1.000	0.914
Roof_A_3	TP9	Summer	23	54	2	0.426	0.825
Roof_B_1	TP9	Summer	19	61	1	NA	0.772
Roof_B_2	TP9	Summer	18	65	1	NA	1.000
Roof_B_3	TP9	Summer	7	8	1	NA	0.888
Roof_C_1	TP9	Summer	17	18	2	0.874	0.684
Roof_C_2	TP9	Summer	22	39	4	0.690	0.693
Roof_C_3	TP9	Summer	4	4	1	NA	0.824
Roof_A_1	TP10	Autumn	3	4	1	NA	0.689
Roof_A_2	TP10	Autumn	22	36	2	0.267	0.302
Roof_A_3	TP10	Autumn	22	98	3	0.721	0.407
Roof_B_1	TP10	Autumn	23	82	4	0.464	0.359
Roof_B_2	TP10	Autumn	21	34	3	0.662	0.413
Roof_B_3	TP10	Autumn	5	7	2	0.971	0.648
Roof_C_1	TP10	Autumn	21	26	1	NA	0.315
Roof_C_2	TP10	Autumn	23	55	2	0.426	0.301
Roof_C_3	TP10	Autumn	17	16	2	0.323	0.302
Roof_A_1	Year2		22	48	3	0.688	0.983
Roof_A_2	Year2		23	66	2	0.932	0.726
Roof_A_3	Year2		7	10	3	0.870	0.716
Roof_B_1	Year2		9	9	1	NA	0.634
Roof_B_2	Year2		8	9	1	NA	0.925
Roof_B_3	Year2		0	NA	NA	NA	NA
Roof_C_1	Year2		5	7	1	NA	0.663
Roof_C_2	Year2		12	12	2	1.000	0.640
Roof_C_3	Year2		22	31	2	0.267	0.719

Table 1: Sampling details and community statistics of the field data. The "location" column indicates the roof and the replicate from which each of the 90 community was sampled and analyzed, the time point and the corresponding season are also presented. For each community, the total number of *Adineta* individuals isolated is in the "Ind.Sampled" column and the subset of individuals genotyped in "Ind.Genotyped". Species richness, Pielou's index of species evenness (α-diversity) and spatial dissimilarities (β-diversity) are shown. NA corresponds to rows for which calculations were not applicable.

Interestingly, eight of the 24 species retrieved during our experiment (species 6, 7, 9, 13, 14, 15, 16, 19) have already been sampled from lichen patches throughout Belgium (SPEEDY project, unpublished, see chapter 5 in annex), four species (7, 9, 14 and 19) corresponded to species also previously detected in the park of Namur next to the University (referred to as Species A, B, C and F in Debortoli *et al*, 2016) (Figure 3). The dominant species 9 represented 788 out of the 1169 individuals (67.41%) genotyped and was retrieved in 64 roof patches (71.11%) during the two years (Figure 4). Two other species (species 8 and 13) were present in several roof patches sampled (22 and 18 patches respectively) but were much less abundant (93 and 97 individuals respectively) and not widespread within Belgium. The other species were less frequently found in our Petri dishes and were present at lower densities (see supplemental data for details and Figure 4).

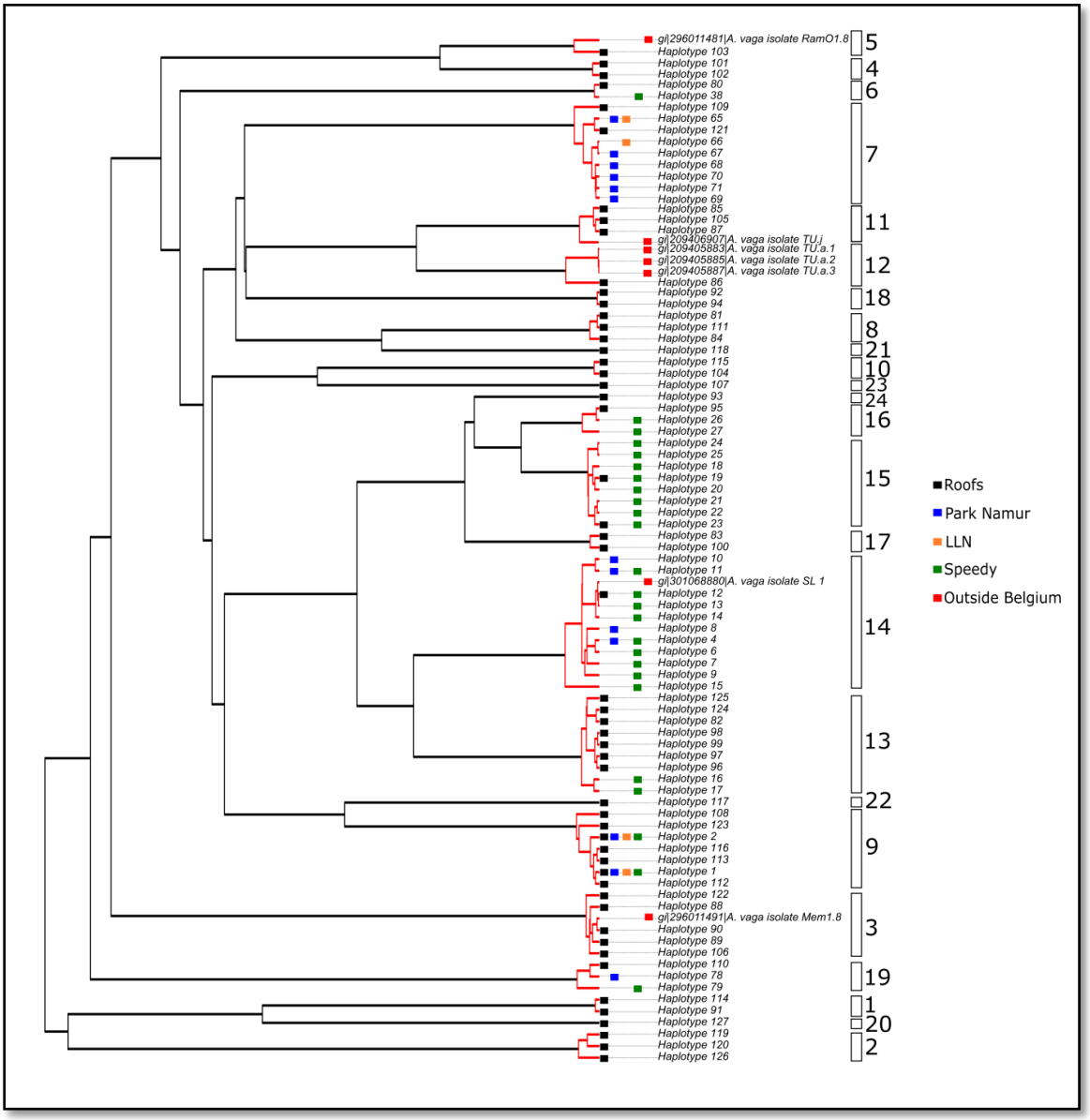

Figure 3 : Pruned ultrametric tree built from our local mtCOI dataset and *Adineta sp*. sequences available on GenBank. Only the 24 GMYC-defined species that were collected on the roof experiment are represented (boxes) in this simplified version of the ultrametric tree (Supplemental figure 4 and 5). Haplotypes that cluster within a same evolutionary entity are linked by red branches. Black squares indicate the haplotypes from the roofs dataset whereas blue, orange, green and red squares highlight the haplotypes that were retrieved from other studies and were sampled in Namur, Louvain-La-Neuve (LLN), flanders (Speedy) and outside Belgium, respectively.

Community diversity across roofs and seasons

The species richness in each roof patch ranged from 0 to 5 (median = 2) and Pielou's index of species evenness ranged from 0 to 1 (median = 0.66). The linear models revealed that roof location (A, B and C) did not influence significantly neither abundance of individual sampled nor species evenness and only slightly richness (p-value = 0.045, adjusted R^2 = 0.047) with a lower diversity on the BUMP roof (Table 2, Figure 4). In contrast, all metrics were significantly affected by seasons. Indeed, the number of individuals sampled was significantly higher in autumn (median = 30, range 1-118) and lower in winter (median = 9.5, range = 0-60) than in the other seasons (medians = 22.5-26, ranges = 0-93) as suggested by the ANOVA (Table 2, Figure 5). We also calculated which meteorological parameters (T°, relative humidity, rainfall and wind) varied the most across seasons. Autumn was characterized by slightly higher minimum humidity than other seasons whereas summer and spring were much drier (df = 3; R^2 = 0.424; p < $2e^{-16}$; Supplemental table 3, Supplemental figure 6). Temperatures were also significantly lower in winter than in other seasons (df = 3; R^2 = 0.604; p < $2e^{-16}$). Rainfall and wind intensity varied significantly among seasons but those variations were small (df = 3; R^2 = 0.015 and 0.056; p < 0.005 and $5.35e^{-09}$, respectively).

		ANOVA			Explained variability
	Variables	df	F stat	p value	adjusted R^2
Ind.Sampled	Roofs	2	1.404	0.251	0.009
	Seasons	4	2.496	0.049*	0.063
	Seasons:Roofs	6	2.185	0.052	0.074
Species.Richness	Roofs	2	3.213	0.045*	0.047
	Seasons	4	2.459	0.051	0.061
	Seasons:Roofs	6	2.883	0.013*	0.113
Evenness	Roofs	2	1.14	0.328	0.005
	Seasons	4	6.053	5.00E−04***	0.272
	Seasons:Roofs	6	5.208	3.39E−04***	0.319
Spat.Dissim.	Roofs	2	11.98	2.53E−05***	0.198
	Seasons	4	4.19	0.004**	0.125
	Seasons:Roofs	6	8.502	3.35E−07***	0.336

Table 2: Results of ANOVA analyzing the responses of each community to the roof location, the season and their interaction. The metrics significantly affected by one parameter are indicated by stars (* p-value <0.05; ** p-value <0.01; *** p-value <0.001). The adjusted R^2 indicates the fraction of the explained variability calculated from a linear model for each variable.

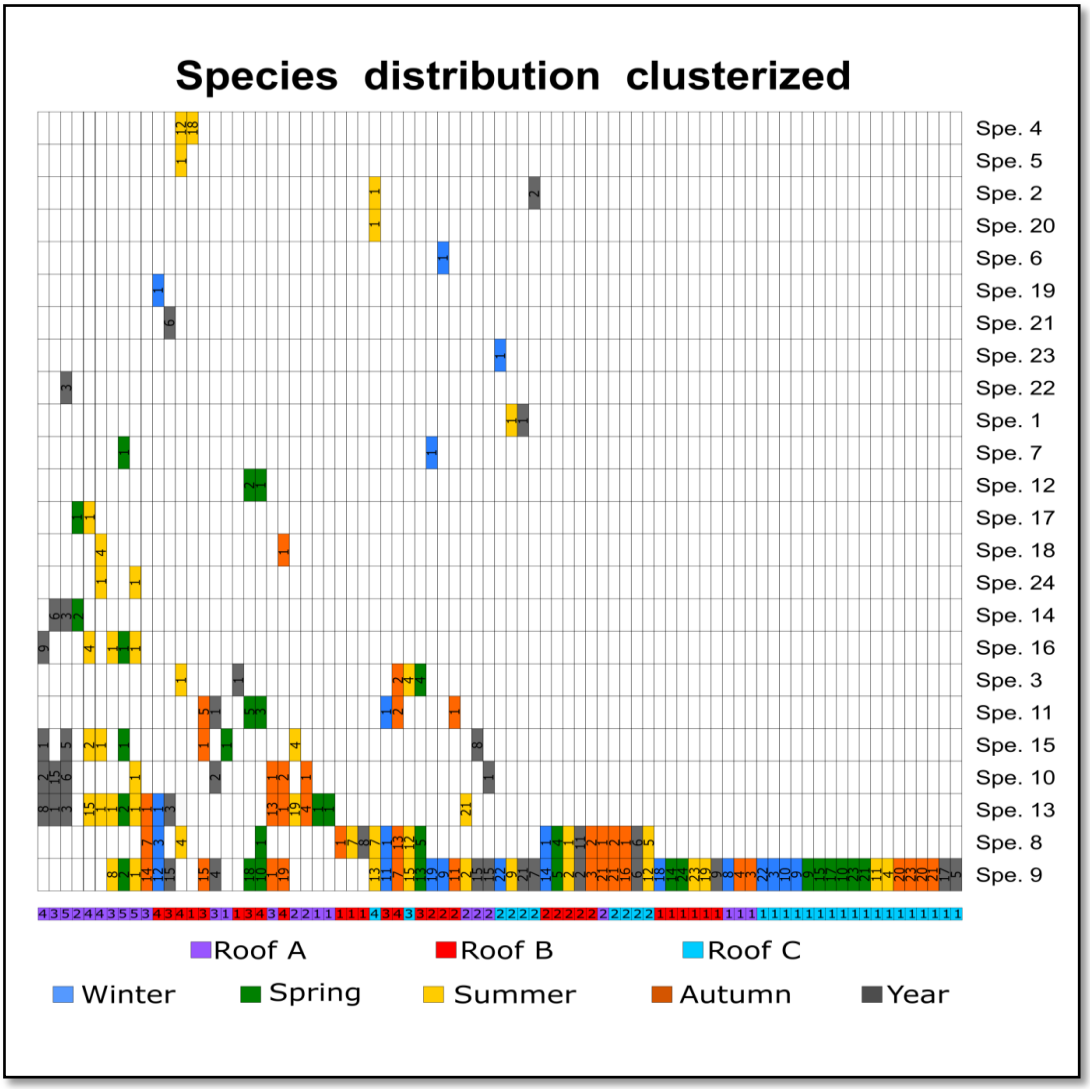

Figure 4 : Clustered matrix of species distribution. The matrix was built from the presence/absence of each species in each non-empty community collected (81 communities) on the roofs and the communities of each sample were then clustered hierarchically according to the species composition. Colored boxes indicate the presence of a species in a specific dish. The box color represents the season at which this species was present and the boxes under the matrix show on which roof it was sampled (A, B and C are in violet, red and turquoise, respectively). Grey boxes indicate that the species was sampled in the dishes collected yearly. The number of individuals genotyped for each species in each patch is indicated in the boxes. The species richness found within each patch is indicated in the patch box, under the matrix.

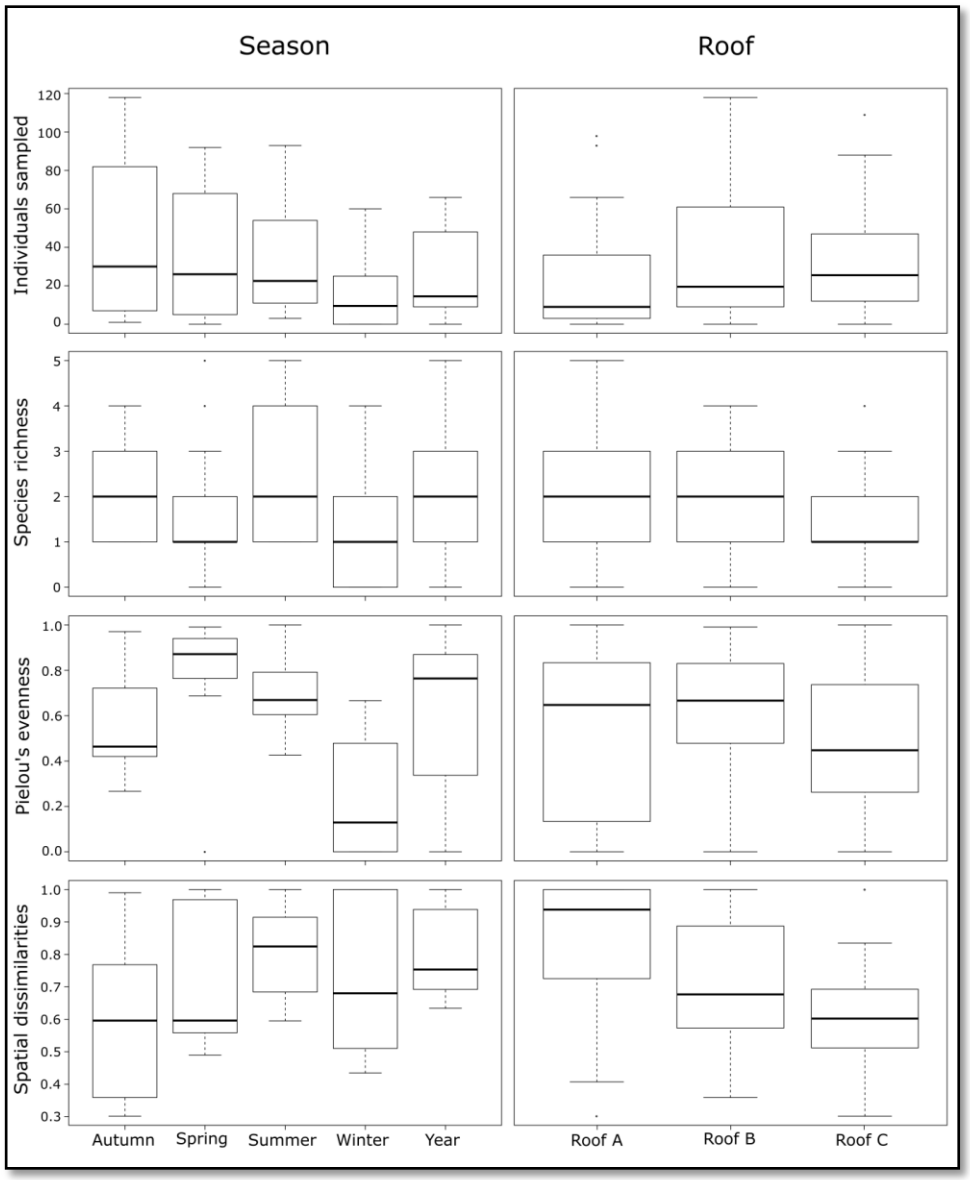

Figure 5: Summary statistics boxplots by seasons and roofs. The number of individuals sampled, the species richness, the Pielou's index of species evenness and the spatial dissimilarities are presented among communities grouped by seasons and roofs.

Modelling the spatio-temporal dynamics of Adineta sp.

Our simulations best fitted the experimental data when scenario 3 and 5 were run under permissive conditions (Supplemental figure 7a). In both cases, distinct immigration probabilities among cryptic *Adineta* species were hypothesized suggesting that this parameter could probably be the most important parameter shaping the bdelloid community dynamics. Second, the likelihood of our simulations considerably improved when exponential and normal distributions were used for the immigration, the reproduction and the survival probabilities (Supplemental figure 7a). On the one hand, the exponential distribution produced communities with one highly dominant species representing 55.1 to 73.86% of the individuals as observed experimentally (Supplemental figure 7b). On the other hand, more species (up to fourteen) were retrieved over the hundred communities simulated when the normal distribution was used (Supplemental figure 7c), better fitting the field data. As a result, the combination of those two types of distribution as used under the hybrid model produced results that slightly better fitted the experimental data (Figure 6). Third, the different values of intra and inter-specific competition strength tested did not affect the model under the combination of parameters tested.

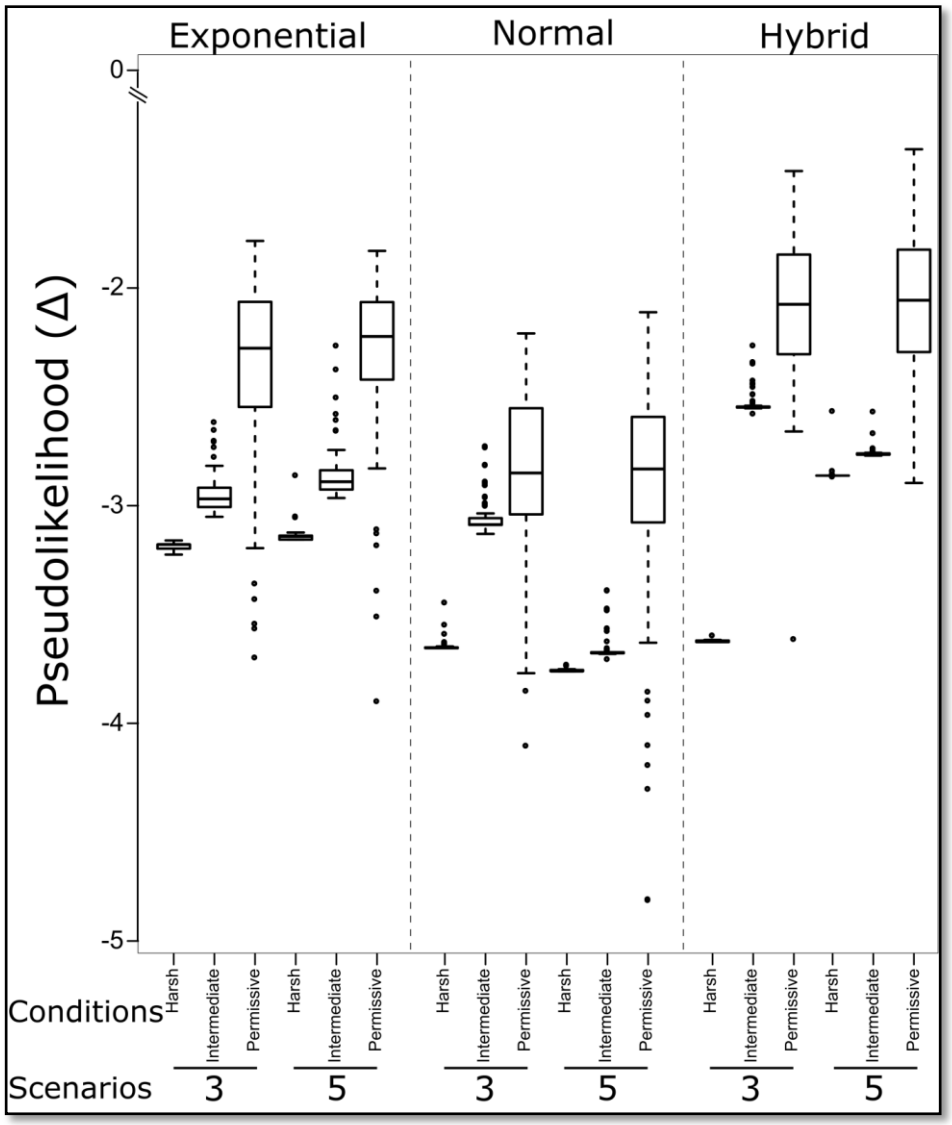

Figure 6: Fit of the simulated communities to the experimental data from the roofs. The pseudolikelihood (Δ) calculated for the simulated communities according to scenario 3 and 5 under different types of probabilities distribution for the immigration parameter (exponential, normal and a hybrid of both; see also supplemental figure 7a for other models). Pseudolikelihood values closer to 0 indicate better fit of the simulations to the data collected from the roofs experiment.

Using the scenarios that best fitted the data (3 and 5 under the hybrid distribution), we used the model to simulate the history of the communities sampled on our three roofs, as only final composition was observed empirically. These simulations suggest that most of the dishes were rapidly colonized, as 23.4 and 25.7% (scenario 3 and 5, respectively) of the communities already harbored at least one individual after the first time-step (Figure 7a). Following colonization, most communities were growing exponentially until week 12. In general, the species with the highest immigration probability dominated the community and represented 40.23 and 76.15% of all the individuals sampled according to scenario 3 and 5, respectively (Figure 7a). Yet, new species still arrived and were able to persist even though the primary colonizer had already settled (abundance exponentially increasing and evenness reaching a plateau, Figure 7b). The simulated β-diversity dynamics suggest that, at the onset, communities were quite similar, with the same species immigrating in the different Petri dishes (Figure 7b). As the rarer species immigrated over time, β-diversity slowly decreased. At the end of the simulation, only 6.4% (scenario 3) and 16.0% (scenario 5) of the dishes were empty (experimentally we observed that 10% of the plates were empty after two years). Some of those empty dishes were colonized during earlier time-steps but the immigrating individuals could not reproduce or died rapidly, while other dishes were never colonized (Figure 7c).

Our statistical analysis demonstrated a significant effect of season, with larger and more diversified communities in autumn than in winter. Therefore, we compared the simulations to the communities observed experimentally for each season separately. This suggested that harsher conditions model the communities sampled in winter best. Indeed the winter communities were better fitted by the harsh and inter mediate conditions whatever the scenario (mean = -2.20). In contrast, permissive conditions suit communities from the autumnal, spring and summer sampling best (with pseudolikelihoods mean for autumn and spring = -2.83; for summer mean = -1.62, see Supplemental figure 9).

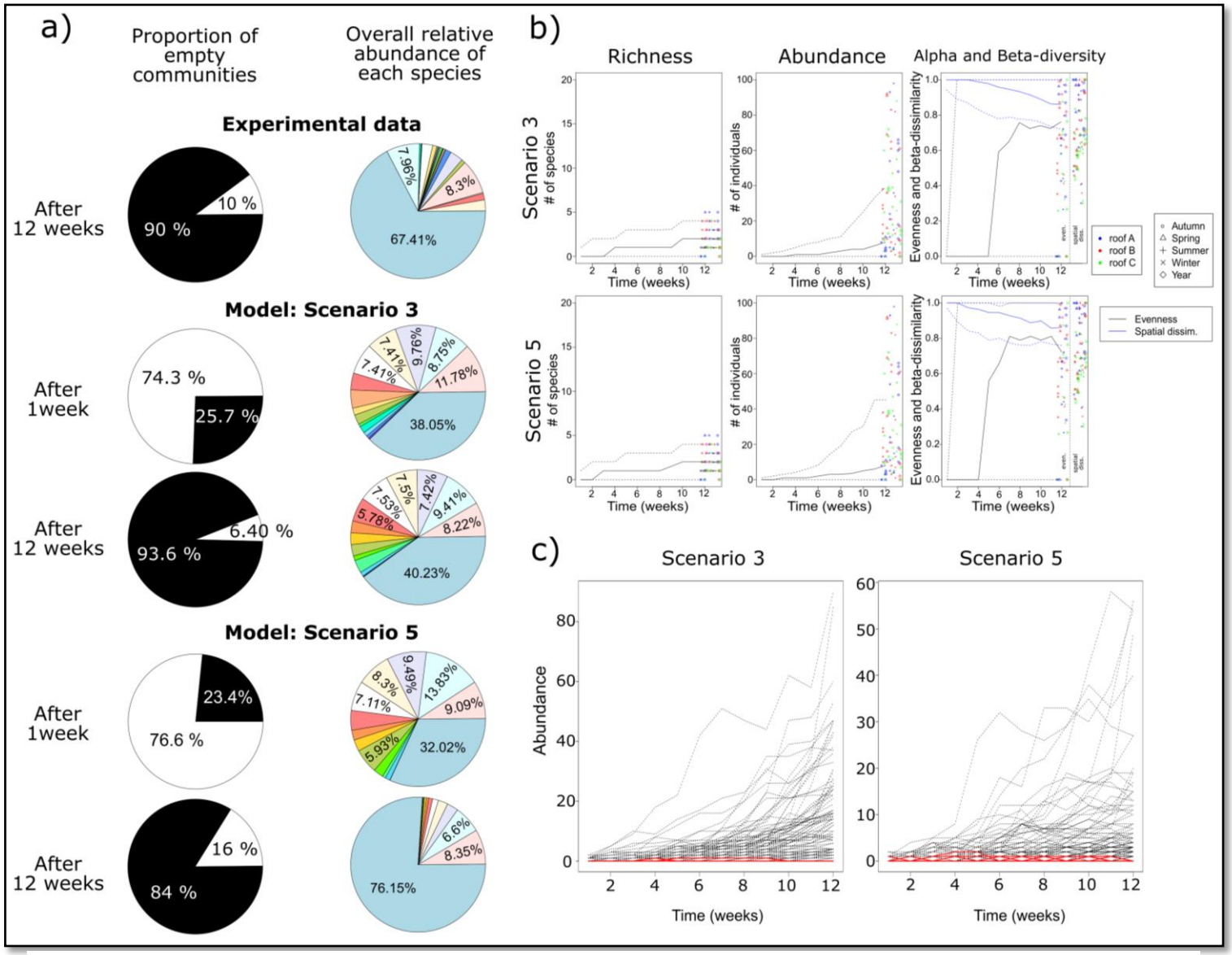

Figure 7 : Fit of the communities simulated according to the hybrid model with the experimental data. a) The overall number of colonized roof communities at sampling time (12 weeks) was 90% and 23.4-25.7% after 1 week or 84-93.6% after 12 weeks (*i.e.* time-steps) for the simulated communities. The roofs communities were colonized by 24 species overall and the relative abundance (not shown when <5%) of the dominant species represented 67.41%. Under scenario 3, simulated communities contained 20 species overall after 12 weeks, one of which accounted for 40.23% of individuals whereas 14 species dominated by one species (76.15%) were present in the simulations under scenario 5. b) The quantiles (Q50 plane line; Q05 and Q95, dotted lines) for species richness, abundance of individuals and diversity (evenness and spatial dissimilarities) were calculated at each time-step (12 weeks) for the simulated community (100 simulations) and plotted with the results of the roofs data (colored symbols) for comparison. c) The number of individual present in each of the simulated community at each time-step is plotted. The red lines correspond to the communities that were empty after time-step 12 (3 months) and the black dotted lines indicate the non-empty

Discussion

Dispersal and colonization of new habitat patches

We confronted model simulations to an extensive field experiment to examine the importance of dispersal and local processes for bdelloid rotifer community composition. Our model fitted the data best when species differed in their immigration probability, but only when this probability covaried positively with reproduction probability (scenario 5), or when all species had the same reproduction probability (scenario 3). Those results suggest that the effect of reproduction and survival (*i.e.* fitness) was low but not negligible, at least within the range of values tested. Thus, efficient immigration combined with effective reproduction (scenario 5) would explain the diversity of cryptic species observed and principally the dominance of species 9 within *A. vaga* species complex on roofs C and B. This would especially explain why the species richness observed on roof C was significantly lower. In addition, the spatial β-diversity calculated from the simulations suggests that most communities are similar at early stages of colonization, indicating that the same species tend to arrive first in most dishes (Figure 7). After this early colonization, the species with the highest survival and reproduction probabilities starts to quickly expand while some new species immigrate independently in the different communities (spatial β-diversity slowly decreases until week 12). However, those new species cannot develop much as median β-diversity stays above 0.7, indicating that the communities present in distinct Petri dishes are roughly the same, with only a few species unique to any particular dish. This could represent the case where one species is perfectly adapted to its habitat and rare species can survive in suboptimal conditions but cannot reproduce. Inversely, rarer immigration in the communities from roof A may limit or delay colonization by species 9, leaving available space for species 13-18, 22 and 24 from more distant areas. We cannot be sure of which parameter could limit dispersal on roof A, but this roof was surrounded by other buildings while C and B were more isolated and closer to the park in which species 9 is extremely abundant. In a theoretical point of view, scenario 2 where fitness varies across species but dispersal is identical may produce a similar community structure with all species able to immigrate but only a few ones surviving in the newly colonized patch. Yet, simulations under scenario 2 produced communities with pseudolikelihood (mean = -2.7 and -4.3 for the exponential and normal distribution respectively) to fit the field data lower than scenario 3 (mean pseudolikelihood = -2.3 and -2.8 for the exponential and normal distribution respectively) and scenario 5 (mean pseudolikelihood = -2.2 and -2.8 for the exponential and normal distribution respectively). One possibility is that immigration rate was too low for all species for scenario 2 under the tested values; increasing four-fold the immigration rates indeed slightly improved the model fit for scenario 2 (mean pseudolikelihood = -2.5 and -3.1 for the exponential and normal distribution, data not shown). However, this was due to a better fit of the species evenness and β-diversity generated but a lower fit of the species richness and abundance of individuals (with 20 species per patch representing a mean of 80 individuals per patch under those parameters whereas field data suggested 1-5 species representing a mean of 16.5 individuals per patch). Modifying the distribution of the survival and reproduction probabilities (*i.e.* changing the λ factor) could further increase the simulations under scenario 2 by inducing a more stringent species filter (species richness decrease) but this would be speculative since no quantitative data about the fitness differences across *Adineta* spp. are currently available.

Even though our simulations highlight the importance of dispersal as the factor having the highest impact on community structure, it is more the order of magnitude in the differences among species that fitted experimental data best. Indeed, switching from a linear distribution of immigration

probability to the hybrid between exponential and normal distribution contributed to an increase of pseudolikelihood of the model especially for scenario 3 and 5. This observation does not automatically imply that *Adineta* species have distinct dispersal capacities but rather that immigration is also a consequence of distinct habitat preferences among species. Even if all species have similar dispersal capacities, the chances to immigrate may be positively correlated with its presence in the nearby habitats (source-sink dynamic). This is corroborated by the fact that the most abundant species (species 9 representing 67.41% of all individuals genotyped) in our study was omnipresent in Belgium and especially in Namur (Figure 3). It is not clear whether geographical distance between sites have an effect on species composition of bdelloid assemblages (Fontaneto and Ricci, 2006b; Fontaneto *et al*, 2008, Fontaneto *et al*, 2011), but it is the case for other microscopic species (Soininen *et al*, 2007; Lopes *et al*, 2016).

Our results indicate that most species have low chances to colonize our local dishes, a few species have fair chances and only a restricted number of species have probabilities high enough to immigrate in multiple dishes within the time window of the study. The species with higher chances to immigrate into our dishes could correspond to the species present in the same geographical region as suggested by the presence of dominant species 9 around our experimental setting. However, species with fair chances to immigrate may be the ones from more distant areas (*e.g.* other European countries) as three species were found across the continent (species 11, 12 and 14). Finally, the two species (3 and 5) that were also sampled in the US could be indicative of low, but nonzero, immigration probabilities for geographically distant species. Although Fontaneto *et al* (2008) presented a similar conclusion, this study enabled us to more finely describe the spatial and temporal dispersal dynamics for *Adineta sp.*, if not all bdelloid rotifers. Bdelloid rotifers can passively disperse with the wind and we sampled here four species (3, 5, 12 and 14) that have already been isolated in other European countries or even in the US. Fontaneto *et al* (2008) showed that although *A. vaga* geographical distribution was negatively correlated with species delimitation resolution (*i.e.* morphospecies are cosmopolitan whereas genetic clusters are generally locally distributed), cases of large distribution were detected for the lowest taxonomic rank.

Finally, the total number of species present in the simulated community (at best fourteen species in total under scenario 3) was relatively low in comparison with the number of species sampled over the whole study (twenty-four species over the whole experimental data). However, as we pointed out that modifying the type of distribution improved the simulations, we think that testing additional distribution of probabilities or even simply testing different ranges of mean and standard deviation for the normal distribution could improve the model considerably.

Community development throughout seasons

Another important result inferred from our model is the impact of seasons on the dynamics of bdelloid rotifers communities. When parameterized for harsh and intermediate conditions, the model predictions compared well to our winter samples. Spring, summer and autumn samples were best predicted by the permissive scenarios. The main factor distinguishing winter from other seasons was lower daily minimal temperatures with frequent periods of frost. In another study on the temporal dynamics of *Macrotrachela quadricornifera* populations from mosses, Ricci *et al* (1989) concluded that the number of isolated individuals correlated with the average relative humidity prior sampling but not with temperature or rainfall. Indeed, relative humidity of the air impacts community dynamics since population growth is arrested during periods of desiccation while

reproduction is boosted immediately after rehydration (Ricci *et al*, 2007). Nevertheless, temperature is also playing a major role in reproductive rate. Several studies on Monogonont rotifers, a sister clade of Bdelloids, described an increase in generation time (due to lower reproductive rate and longer lifespan) when temperature decreased from 30°C to 15°C (Xiang *et al*, 2010; Kauler and Enesco, 2011). The same applies to the bdelloid rotifer *A. vaga* for which the number of laid eggs was lower and the egg development time was longer when individuals and eggs were incubated at 4°C instead of 25°C: less than 14% of the females laid one egg after 6 days at 4°C while at 25°C 87.5% of the females laid eggs within the first 20h, and 0 eggs hatched after 13 days at 4°C versus 40% hatching after 48h at 25°C (M. Terwagne and L. Herter, personal communication). Strikingly, all the eggs incubated at 4°C for 13 days rapidly hatched when put back at 25°C. Thus, freezing periods combined with low temperatures and low reproductive rates tend to reduce populations sizes in winter in bdelloid rotifers followed by re-expansions when temperature increases.

Indeed, six out of the nine in which no *Adineta* were found (7 trimestral dishes and 2 yearly dishes, Table 1 and Figure 7a) dishes were retrieved in winter and abundance was significantly lower during this season. We did not observe any other bdelloid families in those six dishes. The model suggests that some individuals arrived in those communities but that conditions were too harsh for any bdelloid species to persist over several time-steps (Figure 7c). It is unlikely that those dishes were empty due to a less efficient passive dispersal in winter as the corresponding two-yearly dishes were also empty. As the yearly dishes remained exposed to dispersal by wind for a full year, it is unlikely that no individuals ever colonized those dishes when all dishes sampled in summer or autumn were systematically colonized. The empty yearly dishes may reveal strong effects of winter conditions on rotifer communities. Temperatures were indeed significantly lower in winter, especially between December and January (Supplemental table 3), hindering population growth and explaining, at least partially, the significantly lower abundance retrieved during winter. Even if most bdelloids are able to tolerate anhydrobiosis, it remains unclear if all species have a similar survival rate in natural conditions, especially among cryptic species. In addition, it seems that juveniles are more affected than adults (Ricci and Caprioli, 2005). In contrast, the higher abundance observed in autumnal communities was correlated with more humidity. Constant higher humidity enables rehydration and facilitates population growth from bdelloid rotifers propagules that may be spread by wind throughout the year. No significant abundance differences were observed between spring and summer, which were similar in terms of meteorological conditions, except for the higher temperatures observed during summer that may be beneficial for population growth to a certain extent. Extremely high temperatures (T° max > 20°c for 136 days in summer and T° max > 30°c for 13 days) may result in fully desiccated communities that entered in a paused metabolism state. Those observations suggest that although *A. vaga* communities could survive throughout the year, the harsher conditions encountered in winter may result in an annual bottleneck followed by a new expansion that reaches a climax in autumn when temperatures and humidity are more stable. A second hypothesis would be that in autumn more suitable conditions for dispersal result in continuous immigration of the species from the metacommunity (source-sink dynamics). However, this is less likely as, in our model, immigration did not co-vary with harshness (Supplemental figure 3). This suggests that the main parameters influencing population growth throughout the year are survival and reproduction.

Co-existence, competition and differential adaptation among cryptic species

Interestingly, species 8 and 13 were also frequent and abundant throughout this study (93 individuals genotyped in 22 roof patches and 97 individuals genotyped in 18 roof patches, respectively; Figure 4) but have never been sampled outside Belgium. The remaining 21 species appeared sporadically (less than ten times within the roof patches with seven species only found once). A similar observation was made with *M. quadricornifera* populations where one electromorph was retrieved several times within a moss throughout their two-year study, representing 78.7% of the community, while the four other electromorphs appeared occasionally (Ricci *et al*, 1989). These results suggest that distinct cryptic species co-occur periodically but that only some clones expand significantly or immigrate and settle frequently as depicted by our simulations under scenario 5 and 3 respectively (Figure 1 and Supplemental figure 3).

We also observed that eight species (species 13 to 18, 22 and 24), also present in Belgium but not in Namur, were almost exclusively found on roof A, where the dominant species 9 was less frequent (Figure 4). This may reveal local adaptation of those eight species to environmental conditions specific to roof A and/or limited dispersal of species 9 to this roof. This suggests that species 9 probably presents a higher competitive strength than species 13 to 17, 22 and 24 which can only settle and expand when species 9 is absent, or rare. This remains speculative since we could not find any differences in the community modelled by the three degrees of intra and inter-species interaction strength. An inadequate value of K (habitat size, set to 150) could produce species densities much lower than real ones and reduce the impact of the competition factor. Although around 150 *Adineta* individuals have been observed in a single lichen patch, the habitat size may be much lower in this case, at least during the first time-steps modelled as the community had a low amount of resources (sterile agarose plates; K<150). A similar case, although rare, was observed for species 4 which was only detected two times in summer (12 and 18 individuals), but both times species 9 was absent. In this latter case, dispersal cannot explain the absence of the dominant species 9 as it was present in the other replicates from the same roof. The dominant abundance of species 4 in those two dishes could highlight adaptation to particularly dry and warm conditions or a difference in desiccation tolerance among cryptic *Adineta* species.

To our knowledge, no studies have focused on habitat specialization, neither on desiccation tolerance differences among cryptic species of bdelloid rotifers. In the first case, it seems tricky to distinguish between the presence of a species due to habitat specialization or to its high abundance in the metacommunity acting as a permanent source. In the second case, several reports show that there are several degrees of tolerance to desiccation among morpho-species (Eyres *et al*, 2015) but none have focused on variations within species complexes. However, three cryptic species morphologically identified as *Rotaria rotatoria* have been reported to have distinct temperature preferences (Xiang *et al*, 2016), often linked to desiccation tolerance. In the present study, we did not identify physico-chemical parameters that may vary among the different dishes. However, if there are differences between dishes, they are probably due to variations between roofs rather than differences among dishes as the three replicates of each roof tended to give similar results. In addition, the meteorological records that we used were not precise enough to make any distinction between roofs although we doubt there could be consequent climatic differences between dishes located < 150 meters apart. The eventual habitat differences from each roof are thus more likely resulting from each community assemblage itself. We did not determine the number of other zooplankton and phytoplankton species but we observed that roof A communities were, in general,

poor whereas C and B communities often presented several bdelloid species, tardigrades, nematodes, paramecia and were crowded with algae. It is not surprising to find those taxa in our communities as their dispersal capacity is comparable to rotifers and they are often transported together on sediments by wind (Nkem *et al*, 2006).

Conclusion

Under the light of the results provided by our model simulating the dynamics of bdelloid rotifers communities, we showed that ecological scenarios in which cryptic *Adineta* species present distinct immigration probabilities fitted the experimental data more accurately. This observation highlights the prevalent role of dispersal in the dynamics shaping bdelloid rotifers communities. Our simulations using different distribution types widely contributed to the improvement of the model emphasizing the distinctive dispersal probabilities among species. Given that the most represented species in the present study was the species dominating in the nearby area, we showed distinct preferences and fitness among cryptic bdelloid species. The most adapted species (species 9) being extremely abundant around Namur, subsequent short-range dispersal of this species is more frequent than long-range dispersal of species from other Belgian regions or other countries. However, we did not retrieve the *Adineta sp.* that was exclusively present in the soil patches around Namur (species E in Debortoli et al, 2016) showing that species dwelling in patches more exposed to wind results in higher dispersal. Those observations seem to indicate that some species have adapted to soil habitat unsuitable for dispersal and in which more parasites are present (Wilson et al, 2011), whereas other species colonize higher patches were they can reach high abundance and disperse frequently. Yet, studies focusing on the differential adaptation among cryptic species would be required to better understand species distribution in bdelloid rotifers.

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Supplemental information: Bdelloid rotifer communities shaped by immigration seasons, inter-specific variations and geographical distance

Supplemental results

Parameters settings and preliminary experiments

In a preliminary study, we used lab cultures of the AD008 clone which supplied the reference *Adineta vaga* genome (Flot et al, 2013) to gather precise data on its life cycle and used that information to calibrate our model. We isolated 36 eggs in individual (P generation) wells (12-wells plates) coated with 2ml of 2% agarose (UltraPure Agarose, Invitrogen) in sterile conditions and monitored their reproductive output and survival for 34 days. After the hatching of P, each new laid egg (generation F1 and next ones) was counted and removed from the original well. On average, the isolated egg (P) hatched after 48h of development and the juvenile reached maturity after 48 additional hours. When mature, the individual started to lay F1 eggs to reach a maximum of 6 eggs laid on average during day 6 after hatching (Supplemental figure 2f). After this peak, the fecundity slowly decreased until 0 at day 16 after hatching resulting to a mean of 20.2 eggs laid per capita (range: 8-28). The mean survival was above 95% before the peak in fecundity (day 6) after which it decreased slowly until the end of the reproductive period (day 16). Interestingly, around 40% of the P generation was still alive after 16 days and kept living until day 34 indicating that the reproductive effort is the main cause for death under lab conditions.

Meta-community structure

The GMYC method significantly delimited 117 species within *Adineta*, among which twenty-four species were retrieved in our two-year study on the roofs (Supplemental figure 4). The mean genetic distance observed within species ranged from 0 to 0.0241 whereas the inter-species distance ranged from 0.0426 to 0.2178 (estimated using Tamura-Nei model; Tamura and Nei 1993; Supplemental table 5). While the most frequent species 9 represented 67.41% of the individuals sampled (67.41%) (Figure 4), one COI haplotype (Haplotype 1) within species 9 was particularly abundant throughout Belgium (Figure 3).

In three cases, haplotypes sampled on the roofs of UNamur were regrouped within GMYC species together with haplotypes from other European countries (Figure 3): Turkey (species 11 and 12) and Slovenia (species 14). This was also observed for several other haplotypes sampled in Belgium (Supplemental figure 4 and 5): Serbia (species K), United Kingdom (species L, N, BE and CC), Ukraina (species N), France and Sweden (species BR), Italy and Poland (species CC). Six other species were built from haplotypes non-sampled in Belgium but in several other European countries (species AB, AH, BF, BP, BQ and CD). Finally, in five cases, haplotypes from European countries clustered with sequences sampled in other continents, two originating from Belgium and found on the roofs (species 3 and 5) and three from other European countries (species Y, AG and BN).

The correspondence analysis showed that several communities correlated with the presence of eight species (species 10, 13 to 18, 22 and 24; Supplemental figure 8 and that species 8 and 9 were present at lower densities or absent in those patches. This seemed highly correlated with roof A as the three patches from this roof often harbored communities characterized by the presence of those

species and only low densities of species 8 and 9 (Figure 4). On roof C, the abundance of species 8 and 9 was much higher (5.30 and 92.16%, respectively) while species 13 to 18, 20 and 24 were absent (Figure 4). A similar observation was found on roof B where species 8 and 9 represented 14.35 and 67.12% of the individuals genotyped while species 13 to 18, 20 and 24 were rare (Figure 4). We also noted that species 4 and 5 were retrieved twice during the experiment when species 9 was absent, both times in the same patch from roof B during the summer time-point.

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Supplemental figures and tables legends

Supplemental Figure 1: Rarefaction curve calculated to estimate the adequate subsampling size for genotyping. This was calculated from the dataset of Debortoli et al (2016) in which all individuals sampled were genotyped with the mtCOI marker. The number of individuals sampled in each of the 36 patches (boxes) and the corresponding number of haplotypes retrieved are plotted. Interestingly, more diversified communities often contained less than 10 (7, 8, 17 and 23) individuals while most of the bigger communities harbored a single haplotype (11, 16 and 29). Three theoretical subsampling values (10, 15 and 20 individuals) are indicated by the red, orange and green lines respectively. With a subsample of 10 individuals, there are chances to miss haplotypes, *i.e.* underestimate the diversity, in 6 out of 36 patches (16.67%) whereas only 3 or 2 (8.34% and 5.56%) communities would be underestimated by genotyping 15 or 20 individuals, respectively. Here, a subset of 24 individuals was genotyped.

Supplemental Figure 2: Parameters settings for the simulations. a) Three values of Y (low-density fecundity per individual) were tested in equation 4 and the resulting simulated number of eggs laid per capita at each time-step is presented in the boxplot. b) Boxplots showing the population growth at each time-step resulting from the different combinations of values for G_i (equation 1) and Y (equation 4) tested. c) Boxplots indicating the total number of eggs laid by each individual throughout its lifetime. d) Number of individuals in a given patch computed for distinct combinations of G_i (equation 1) and Y (equation 4). e) Fraction of individuals present at time-step 1 that survive through time-step 5 and time-step 12 under three values of survival probabilities s_i (0.9, 0.6 and 0.3, equation 2). f) Number of eggs laid each day after hatching calculated from the preliminary experiment (red line) and from the simulations (blue line).

Supplemental Figure 3: Probability distributions tested for the model under five distinct scenarios. In scenario 1, we assumed all species to have similar fitness, dispersal rate and life-cycles resembling the assumptions underlying neutral models. In scenario 2, we also assumed similar dispersal rates, but distinct fitness, depicted by distinct probabilities to survive and reproduce. In scenario 3, we assumed that only the dispersal rate varied across species. In scenario 4, we assumed that fitness and dispersal rate co-varied negatively. In scenario 5, all parameters co-varied positively. In the simulations based on b) exponential or c) normal distributions, a few or several species have much higher chances to immigrate, reproduce and survive at each time-step. d) The hybrid distribution was built from a normal distribution to which we added one species with higher probabilities. This curve models an intermediate case in which one species is outcompeting the others and several species are able to persist in sub-optimal conditions. The vectors corresponding to those five scenarios and three conditions that were used in the model are provided in Supplemental Table 4

Supplemental Figure 4: Ultrametric tree built from our local mtCOI dataset and *Adineta sp*. sequences available on GenBank. The 364 mtCOI sequences aligned using MAFFT (589 bp) and used as input for BEAST v1.6.2 are presented in supplemental table 1. The GTR+Γ4+I substitution model was selected by jModelTest 3.8 (Posada 2008). Three MCMC chain were run independently for 10^8 generations with sampling every 10,000 generations and combined with the LogCombiner package to avoid local optimum. The tree with maximal clade credibility among the last 1,000 trees sampled by BEAST was determined using TreeAnnotator v.1.6.2 as implemented in the BEAST package (Drummond and Rambaut 2007). This ultrametric tree was used as input for the GMYC analysis using the R package "splits" (http://r-forge.r-project.org/projects/splits/). Haplotypes that clustered within a same evolutionary entity are linked by red branches. Red labels indicate haplotypes sampled on the roofs during our experiment and blue labels represent haplotypes collected in Belgium during other studies. Each of the 117 cluster (*i.e.* species) is indicated by a colored bar according to the distribution of the species: white bars indicate species that were sampled in a single country but not Belgium, brown bars are species sampled only on the roofs of our experiment, green bars are species present throughout Belgium (roofs are included) pink bars are species retrieved in several European countries and yellow bars indicate species that were found on different continents. Species labeled with letters (A-CR) show species not sampled in the present study and those labeled with red numbers (1-24) indicate the 24 species sampled on the UNamur roofs. The GMYC statistics are presented in the associated table.

Supplemental Figure 5: *Adineta* species distribution. Species delimitation is given in supplemental figure 4 and the origin of the corresponding haplotype is indicated in supplemental table 1. Only the species that are found in more than one country are represented here. The species label indicates if it was sampled on the roofs of our study (red numbers) or from other studies (letters). The colored boxes next to the species label show the countries in which each species has been collected. The squared enlargement of Belgium represents the different sampling sites within the country and used within the tree of supplemental figure 4 (Roofs, Park Namur, Haugimont, LLN and Speedy).

Supplemental Figure 6: Boxplots of the climatic parameters recorded for each season. The corresponding raw data, measured daily, provided by the IRM (Institut Royal Météorologique, Belgium) is given in supplemental table 2. The maximum and minimum relative humidity in the air (%) and temperature (°C) recorded, the highest wind speed (km/h) and the amount of precipitations (mm) is presented.

Supplemental Figure 7: Model fit to the experimental data. a) Likelihoods of the simulated communities fitting the roof data under linear, exponential (only scenario 2-5) and normal distributions (only scenario 2-5) for the probabilities of immigration, survival and reproduction at each time-step. b) and c) are pie charts representing the proportion of each species in the communities simulated by the model according to exponential and normal distributions. The results presented are the simulation of scenario 3 and 5 under permissive conditions which gave the highest likelihood to fit the experimental data from the roofs.

Supplemental Figure 8: Correspondence analysis representing the structure of each community sampled on the roofs. The plotted component 1 (16.1%) and 2 (15.2%) contributed to 31.3% of the total variability. Most communities from roof B and C harbored majorly species 8 and 9 and were grouped. In contrast, species 10, 13, 14, 15, 16, 17, 18, 22 and 24 were almost exclusively found on roof A. Species 4 and 5 were retrieved twice on the roof B replicates.

Supplemental Figure 9: Fit of the simulated communities to the experimental data for each season independently. The pseudolikelihood (Δ) was calculated for the hybrid model with the parameters of scenario 3 and 5 which provided the best results on Figure 6. Pseudolikelihood values closer to 0 indicate better fit of the simulations to the data collected from the roofs experiment.

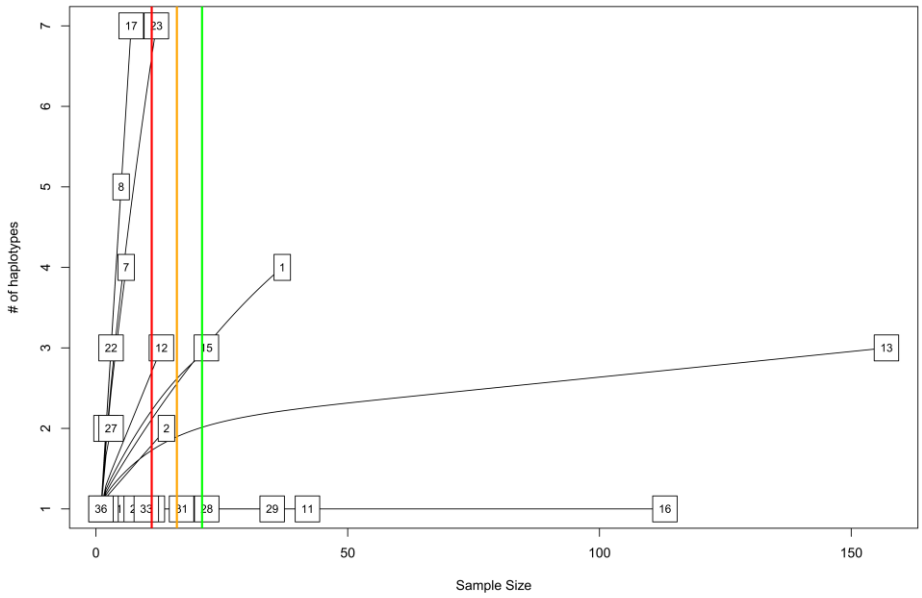
Supplemental Table 1: List of mtCOI sequences retrieved from GenBank and used for the phylogenetic analyses. The sequence names are the same used on figure 4 and supplemental figure 2. The location indicates in which country the corresponding individual was sampled when this information was available.

Supplemental Table 2: Daily meteorological records provided by the IRM institute for the period covering the two-year roof experiment. The seasons are indicated by colors: blue for winter, green for spring, yellow for summer and orange for autumn.

Supplemental Table 3: Results of the ANOVA giving the climatic variation through seasons. The R^2 indicates the fit of the data to a linear model.

Supplemental Table 4: List of values used to create the different vectors used as probability distributions for each parameter in the model. The vector codes are in R language.

Supplemental Table 5: Intra and interspecific genetic distance for the 24 *Adineta* species sampled on the roofs. The distances were calculated using the Tamura-Nei model (Tamura and Nei 1993). The smallest and highest distances between species are highlighted in grey. The intraspecific distances (n/c when not applicable) are presented on the diagonal and the highest value is highlighted in black.


# of haplotypes
Sample Size
0
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100
150
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16

Figure S1

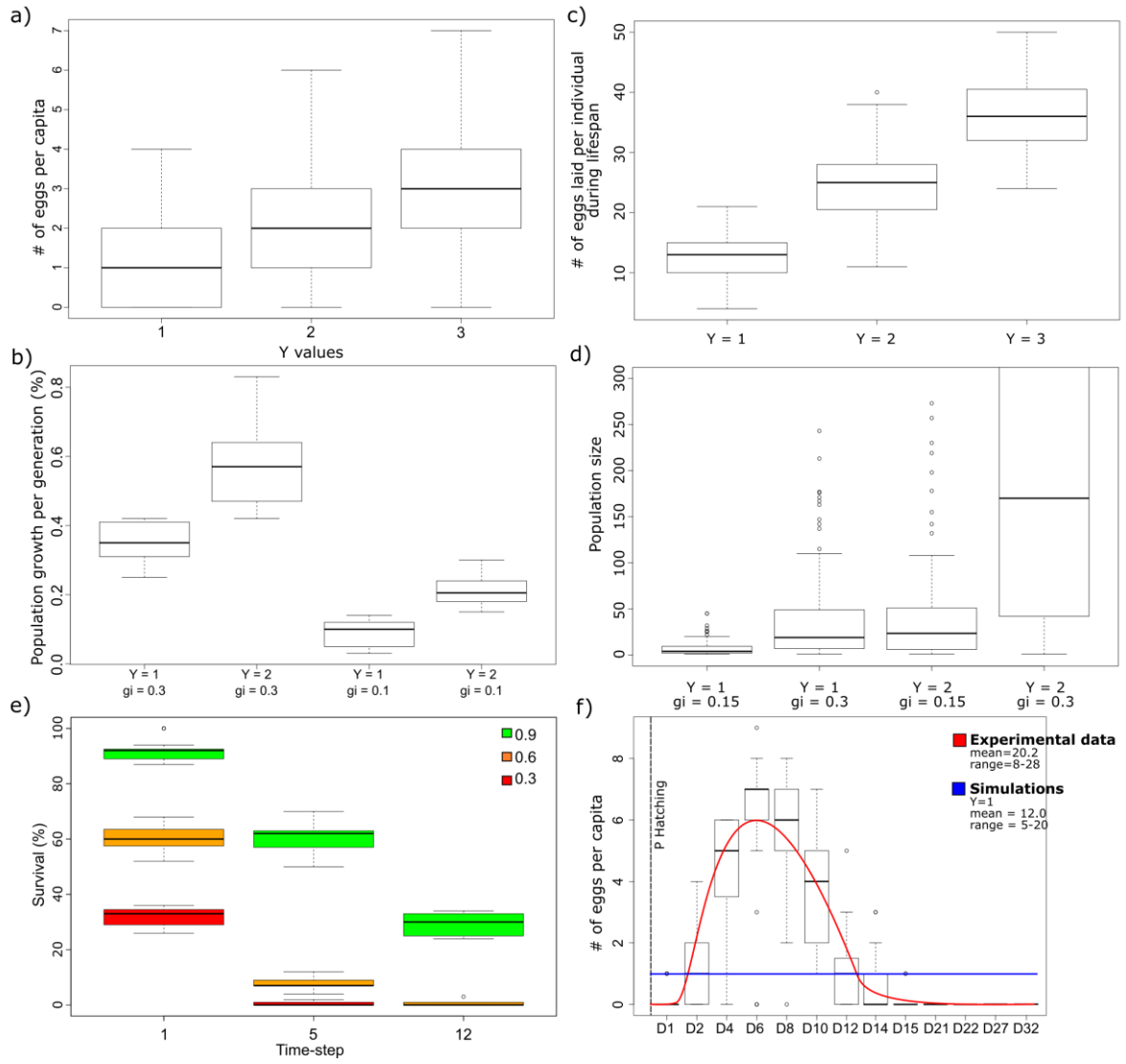

Figure S2

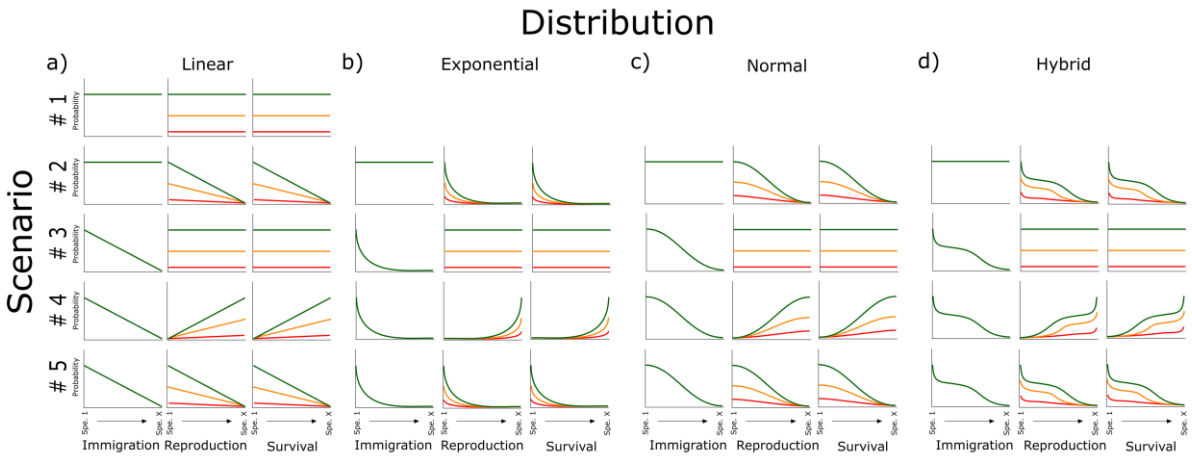

Figure S3

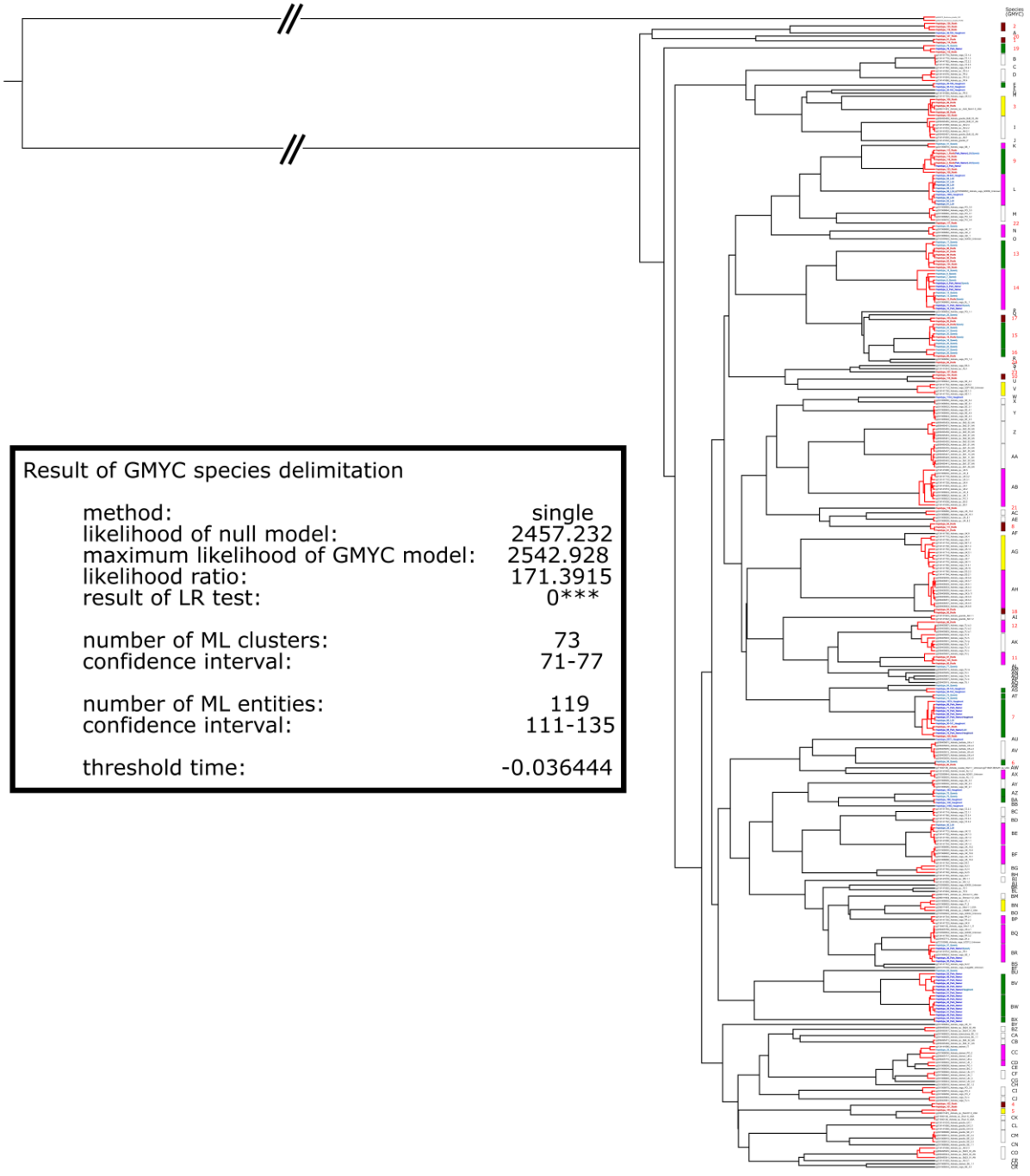
Result of GMYC species delimitation
method: single
likelihood of null model: 2457.232
maximum likelihood of GMYC model: 2542.928
likelihood ratio: 171.3915
result of LR test: 0***
number of ML clusters: 73
confidence interval: 71-77
number of ML entities: 119
confidence interval: 111-135
threshold time: -0.036444

Figure S4

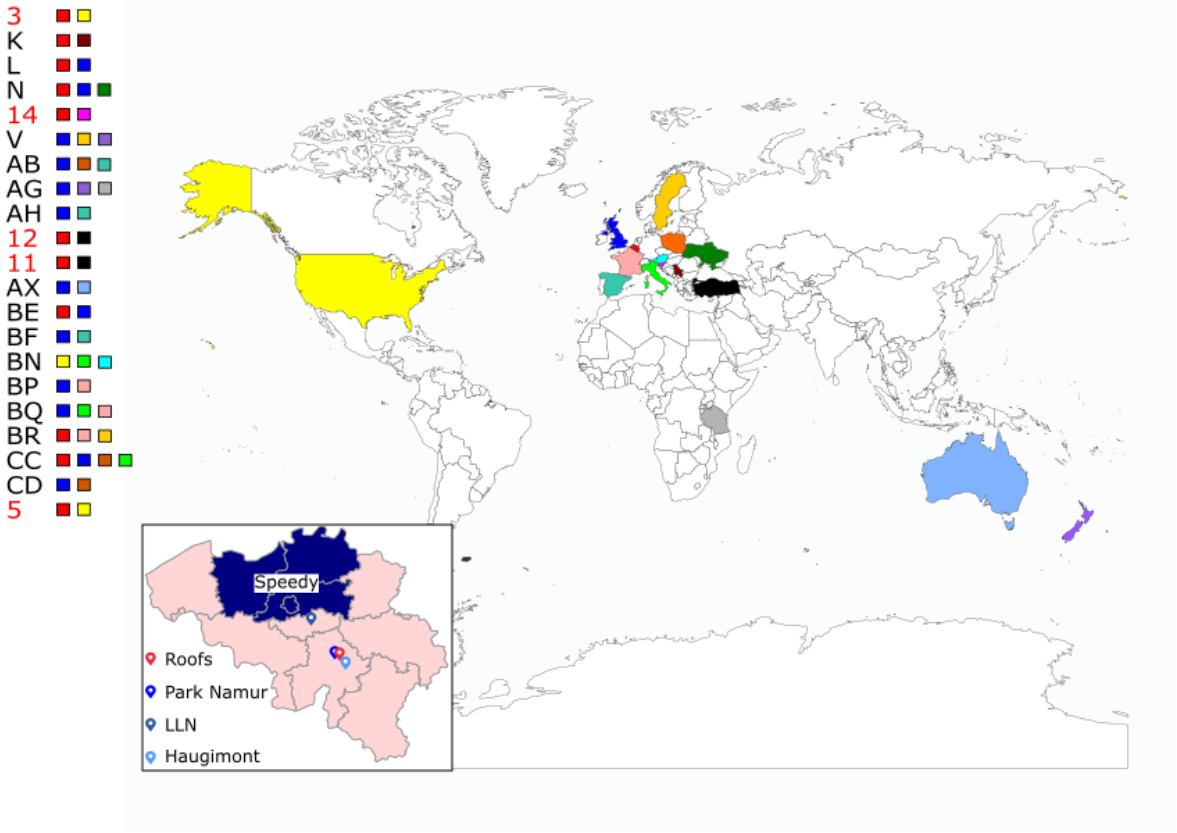

Figure S5

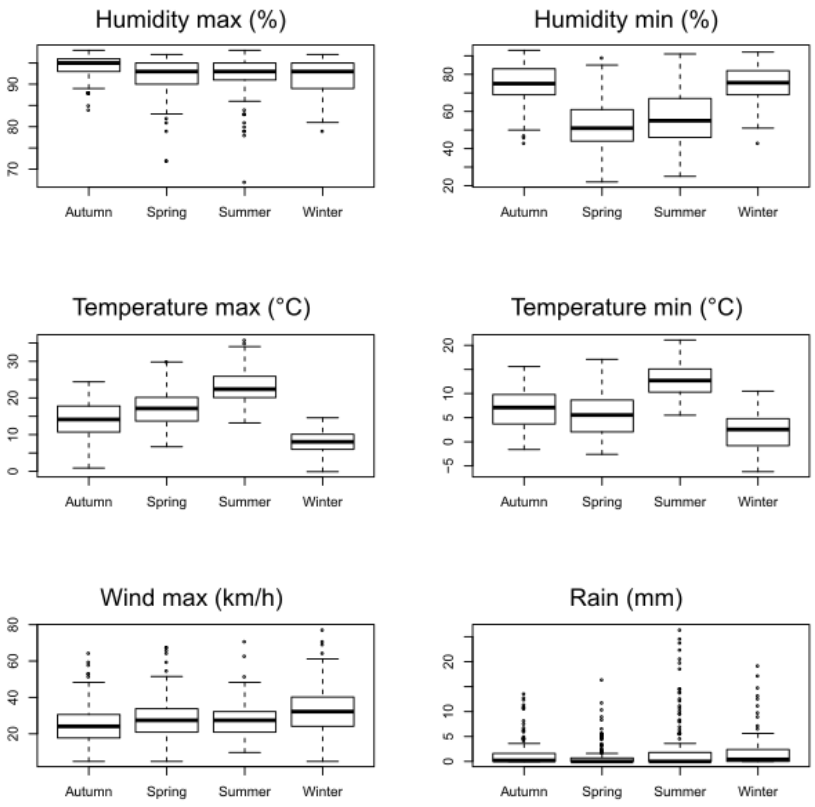

Figure S6

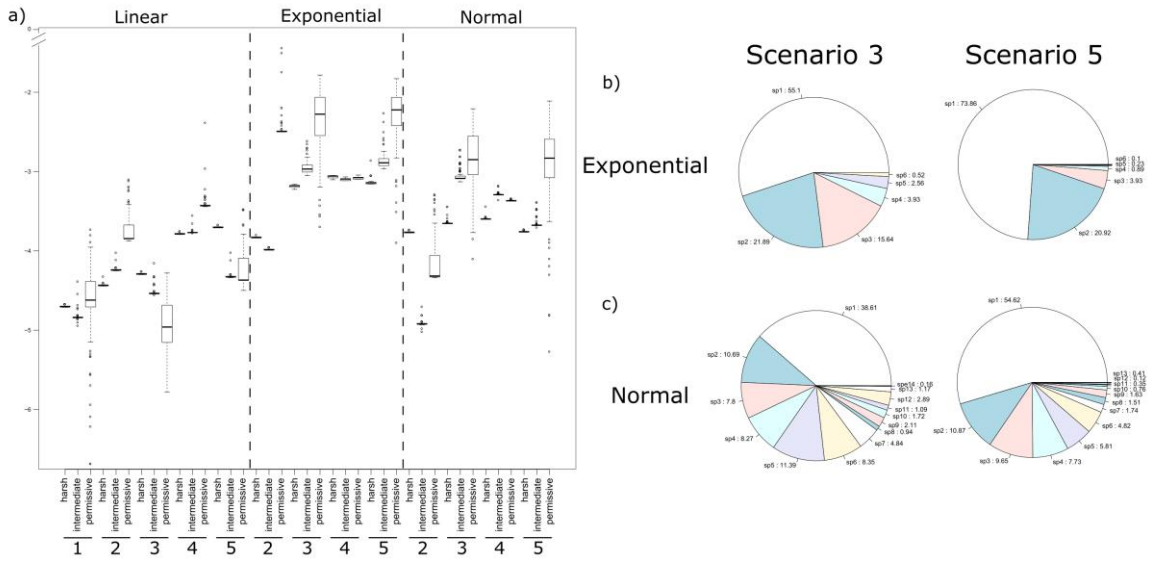

Figure S7

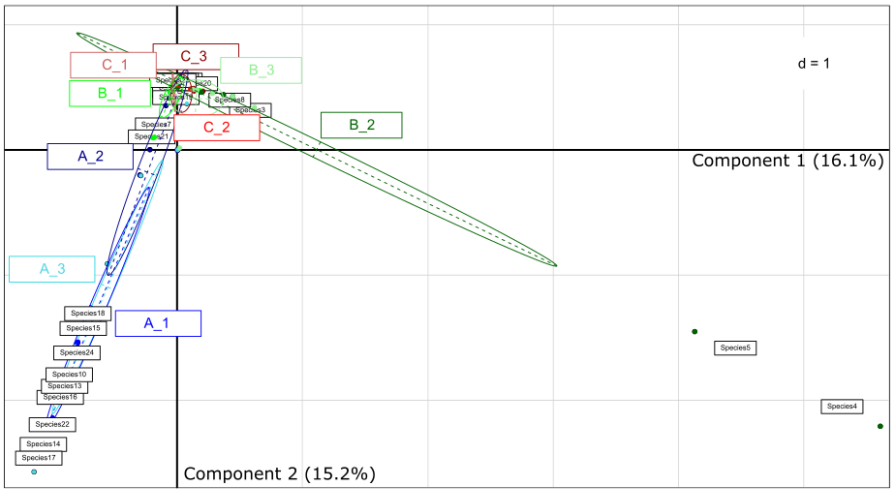

Figure S8

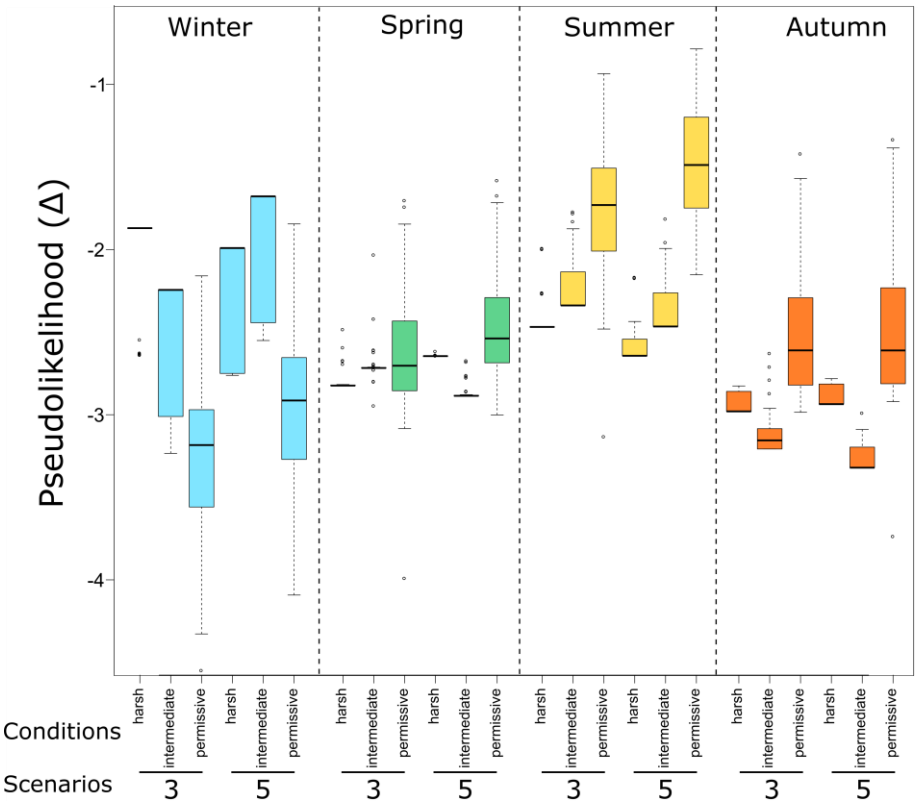
Winter
Spring
Summer
Autumn
Pseudolikelihood (Δ)
-1
-2
-3
-4
Conditions
harsh
intermediate
permissive
Scenarios
3
5

Figure S9

Table 1

Sequence name	Accession	GI	Location
Adineta barbata isolate UK.a	EU751029.1	209405613	United Kingdom
Adineta barbata isolate UK.a.1	EU751023.1	209405601	United Kingdom
Adineta barbata isolate UK.a.2	EU751024.1	209405603	United Kingdom
Adineta barbata isolate UK.a.3	EU751025.1	209405605	United Kingdom
Adineta barbata isolate UK.a.4	EU751026.1	209405607	United Kingdom
Adineta barbata isolate UK.a.5	EU751027.1	209405609	United Kingdom
Adineta barbata isolate UK.a.6	EU751028.1	209405611	United Kingdom
Adineta gracilis isolate Bd8_01	KJ543598.1	659495485	Antarctica
Adineta gracilis isolate Bd8_02	KJ543599.1	659495487	Antarctica
Adineta gracilis isolate Bd8_03	KJ543600.1	659495489	Antarctica
Adineta gracilis isolate IT	EF173180.1	134141632	Italy
Adineta gracilis isolate SE 1.1	GQ465634.1	301068906	Sweden
Adineta gracilis isolate SE 2.1	GQ465635.1	301068908	Sweden
Adineta gracilis isolate SE 2.2	GQ465636.1	301068910	Sweden
Adineta gracilis isolate SE 2.3	GQ465637.1	301068912	Sweden
Adineta gracilis isolate SE 2.4	GQ465638.1	301068914	Sweden
Adineta gracilis isolate UK.1	EF173181.1	134141634	United Kingdom
Adineta gracilis isolate UK.2.1	EF173197.1	134141666	United Kingdom
Adineta gracilis isolate UK.2.2	EF173198.1	134141668	United Kingdom
Adineta gracilis isolate UK.2.3	EF173199.1	134141670	United Kingdom
Adineta grandis isolate AN.1.1	EF173184.1	134141640	Antarctica
Adineta grandis isolate AN.1.2	EF173185.1	134141642	Antarctica
Adineta oculata clone War1.1	DQ078515.1	71840136	Unknown
Adineta ricciae isolate Adir1.1	HM032930.1	296011503	Unknown
Adineta ricciae isolate AU 1.3	GQ465591.1	301068820	Australia
Adineta ricciae isolate AU.1.1	EF173187.1	134141646	Australia
Adineta ricciae isolate AU.1.2	EF173188.1	134141648	Australia
Adineta ricciae voucher AD001	KM043179.1	703599944	Unknown
Adineta sp. Adi2 isolate RamO1.8	HM032919.1	296011481	USA
Adineta sp. Adi2 isolate Shmoo1.12	HM032918.1	296011693	USA
Adineta sp. Adi2 isolate Shmoo1.3	HM032917.1	296011691	USA
Adineta sp. Adi3 isolate RamO1.1	HM032921.1	296011485	USA
Adineta sp. Adi3 isolate RamO1.10	HM032922.1	296011487	USA
Adineta sp. Adi3 isolate RamO1.11	HM032920.1	296011483	USA
Adineta sp. Adi3 isolate RamO1.12	HM032923.1	296011489	USA
Adineta sp. Adi4 isolate Mem1.10	HM032926.1	296011495	USA
Adineta sp. Adi4 isolate Mem1.8	HM032924.1	296011491	USA
Adineta sp. Adi4 isolate Mem1.9	HM032925.1	296011493	USA
Adineta sp. Adi5 isolate LRat1.2	HM032929.1	296011501	USA
Adineta sp. Adi5 isolate LRatM1.2	HM032928.1	296011499	USA
Adineta sp. Adi5 isolate Mem1.1	HM032927.1	296011497	USA
Adineta sp. AN.1	EF173189.1	134141650	Antarctica
Adineta sp. AN.2.1	EF173190.1	134141652	Antarctica
Adineta sp. AN.2.2	EF173191.1	134141654	Antarctica
Adineta sp. AN.2.3	EF173192.1	134141656	Antarctica
Adineta sp. AN.2.4	EF173193.1	134141658	Antarctica
Adineta sp. AN.3.1	EF173194.1	134141660	Antarctica
Adineta sp. AN.3.2	EF173195.1	134141662	Antarctica
Adineta sp. AU.1	EF173186.1	134141644	Australia

Adineta sp. Bd1_01	KJ543570.1	659495429	Antarctica
Adineta sp. Bd1_02	KJ543571.1	659495431	Antarctica
Adineta sp. Bd1_03	KJ543572.1	659495433	Antarctica
Adineta sp. Bd1_04	KJ543573.1	659495435	Antarctica
Adineta sp. Bd1_05	KJ543574.1	659495437	Antarctica
Adineta sp. Bd1_06	KJ543575.1	659495439	Antarctica
Adineta sp. Bd1_07	KJ543576.1	659495441	Antarctica
Adineta sp. Bd1_08	KJ543577.1	659495443	Antarctica
Adineta sp. Bd1_09	KJ543578.1	659495445	Antarctica
Adineta sp. Bd1_10	KJ543579.1	659495447	Antarctica
Adineta sp. Bd1_11	KJ543580.1	659495449	Antarctica
Adineta sp. Bd2_01	KJ543581.1	659495451	Antarctica
Adineta sp. Bd2_02	KJ543582.1	659495453	Antarctica
Adineta sp. Bd2_03	KJ543583.1	659495455	Antarctica
Adineta sp. Bd2_04	KJ543584.1	659495457	Antarctica
Adineta sp. Bd2_05	KJ543585.1	659495459	Antarctica
Adineta sp. Bd2_06	KJ543586.1	659495461	Antarctica
Adineta sp. Bd2_07	KJ543587.1	659495463	Antarctica
Adineta sp. Bd2_08	KJ543588.1	659495465	Antarctica
Adineta sp. Bd23_01	KJ543626.1	659495541	Antarctica
Adineta sp. Bd23_02	KJ543627.1	659495543	Antarctica
Adineta sp. Bd23_03	KJ543628.1	659495545	Antarctica
Adineta sp. Bd24_01	KJ543629.1	659495547	Antarctica
Adineta sp. Bd24_02	KJ543630.1	659495549	Antarctica
Adineta sp. Bd4_01	KJ543590.1	659495469	Antarctica
Adineta sp. Bd4_02	KJ543591.1	659495471	Antarctica
Adineta sp. CWB-2005 clone HuH1.4a	DQ078516.1	71840138	Unknown
Adineta sp. CWB-2005 clone Rou1.6	DQ078513.1	71840132	USA
Adineta sp. CWB-2005 clone Rou1.8	DQ078514.1	71840134	USA
Adineta sp. ES.1	EF173182.1	134141636	Spain
Adineta sp. ES.2	EF173183.1	134141638	Spain
Adineta sp. FR.1	EF173200.1	134141672	France
Adineta sp. FR.2	EF173202.1	134141676	France
Adineta sp. FR.3.1	EF173205.1	134141682	France
Adineta sp. FR.3.2	EF173206.1	134141684	France
Adineta sp. FR.4	EF173207.1	134141686	France
Adineta sp. FR.5	EF173208.1	134141688	France
Adineta sp. MX.1.1	EF173203.1	134141678	Mexico
Adineta sp. MX.1.2	EF173204.1	134141680	Mexico
Adineta sp. PO 1	GQ465592.1	301068822	Poland
Adineta sp. TZ.1	EF173210.1	134141692	Tanzania
Adineta sp. TZ.2	EF173211.1	134141694	Tanzania
Adineta sp. UK 6	GQ465593.1	301068824	United Kingdom
Adineta sp. UK 7	GQ465597.1	301068832	United Kingdom
Adineta sp. UK 8.1	GQ465594.1	301068826	United Kingdom
Adineta sp. UK 8.2	GQ465595.1	301068828	United Kingdom
Adineta sp. UK 9	GQ465596.1	301068830	United Kingdom
Adineta sp. UK.1	EF173196.1	134141664	United Kingdom
Adineta sp. UK.2	EF173201.1	134141674	United Kingdom
Adineta sp. UK.3.1	EF173222.1	134141716	United Kingdom

Adineta sp. UK.3.2	EF173223.1	134141718	United Kingdom
Adineta sp. UK.4	EF173228.1	134141728	United Kingdom
Adineta sp. UK.5	EF173209.1	134141690	United Kingdom
Adineta steineri isolate BG 1	GQ465598.1	301068834	Bulgaria
Adineta steineri isolate IT	EF173212.1	134141696	Italy
Adineta steineri isolate PO 1	GQ465600.1	301068838	Poland
Adineta steineri isolate PO 2	GQ465599.1	301068836	Poland
Adineta steineri isolate SE 1.1	GQ465639.1	301068916	Sweden
Adineta steineri isolate SE 1.2	GQ465640.1	301068918	Sweden
Adineta steineri isolate UK 1	GQ465601.1	301068840	United Kingdom
Adineta steineri isolate UK.a	EU751080.1	209405715	United Kingdom
Adineta steineri isolate UK.b	EU751081.1	209405717	United Kingdom
Adineta steineri isolate Ukr 1	GQ465602.1	301068842	Ukraina
Adineta steineri isolate Ukr 2.1	GQ465604.1	301068846	Ukraina
Adineta steineri isolate Ukr 2.2	GQ465603.1	301068844	Ukraina
Adineta steineri isolate Ukr 3	GQ465605.1	301068848	Ukraina
Adineta tuberculosa isolate SE 1.1	GQ465641.1	301068920	Sweden
Adineta tuberculosa isolate SE 1.2	GQ465642.1	301068922	Sweden
Adineta vaga	JX184001.1	402761739	Unknown
Adineta vaga	DQ079961.1	83655499	Unknown
Adineta vaga	AY218092.1	37788254	Unknown
Adineta vaga clone WAv1.1	DQ078512.1	71840130	Italy
Adineta vaga isolate A.vaga.ES.3	EF650483.1	157365246	Spain
Adineta vaga isolate AT 1	GQ465606.1	301068850	Austria
Adineta vaga isolate AU.1	EF173234.1	134141740	Australia
Adineta vaga isolate AU.2	EF173235.1	134141742	Australia
Adineta vaga isolate AU.3	EF173236.1	134141744	Australia
Adineta vaga isolate AU.4	EF173237.1	134141746	Australia
Adineta vaga isolate AU.5	EF173238.1	134141748	Australia
Adineta vaga isolate ES.1	EF173245.1	134141762	Spain
Adineta vaga isolate ES.2.1	EF173246.1	134141764	Spain
Adineta vaga isolate ES.2.2	EF173247.1	134141766	Spain
Adineta vaga isolate FR.2.1	EF173226.1	134141724	France
Adineta vaga isolate FR.2.2	EF173227.1	134141726	France
Adineta vaga isolate FR.3.1	EF173243.1	134141758	France
Adineta vaga isolate FR.3.2	EF173244.1	134141760	France
Adineta vaga isolate G3P11B5	EF173220.1	134141712	Unknown
Adineta vaga isolate G3P11B5	EF173220.1	134141712	Unknown
Adineta vaga isolate IT 2	GQ465607.1	301068852	Italy
Adineta vaga isolate NZ.1.1	EF173230.1	134141732	New-Zealand
Adineta vaga isolate NZ.1.2	EF173231.1	134141734	New-Zealand
Adineta vaga isolate NZ.1.3	EF173232.1	134141736	New-Zealand
Adineta vaga isolate NZ.1.4	EF173233.1	134141738	New-Zealand
Adineta vaga isolate PO 1.1	GQ465608.1	301068854	Poland
Adineta vaga isolate PO 1.2	GQ465609.1	301068856	Poland
Adineta vaga isolate PO 2	GQ465610.1	301068858	Poland
Adineta vaga isolate PO 3.1	GQ465611.1	301068860	Poland
Adineta vaga isolate PO 3.2	GQ465612.1	301068862	Poland
Adineta vaga isolate PO 3.3	GQ465613.1	301068864	Poland
Adineta vaga isolate PO 3.4	GQ465614.1	301068866	Poland

Adineta vaga isolate PO 3.5	GQ465615.1	301068868	Poland
Adineta vaga isolate PO 3.6	GQ465616.1	301068870	Poland
Adineta vaga isolate PO 3.7	GQ465617.1	301068872	Poland
Adineta vaga isolate PO 3.8	GQ465619.1	301068876	Poland
Adineta vaga isolate PO 4	GQ465618.1	301068874	Poland
Adineta vaga isolate SB 1	GQ465620.1	301068878	Serbia
Adineta vaga isolate SE 1	GQ465643.1	301068924	Sweden
Adineta vaga isolate SE 2.1	GQ465644.1	301068926	Sweden
Adineta vaga isolate SE 2.2	GQ465645.1	301068928	Sweden
Adineta vaga isolate SE 2.3	GQ465646.1	301068930	Sweden
Adineta vaga isolate SE 3.1	GQ465647.1	301068932	Sweden
Adineta vaga isolate SE 3.2	GQ465648.1	301068934	Sweden
Adineta vaga isolate SE 3.3	GQ465649.1	301068936	Sweden
Adineta vaga isolate SE 3.4	GQ465650.1	301068938	Sweden
Adineta vaga isolate SE 4.1	GQ465651.1	301068940	Sweden
Adineta vaga isolate SE 4.2	GQ465652.1	301068942	Sweden
Adineta vaga isolate SE 4.3	GQ465653.1	301068944	Sweden
Adineta vaga isolate SE 4.4	GQ465654.1	301068946	Sweden
Adineta vaga isolate SE 4.5	GQ465655.1	301068948	Sweden
Adineta vaga isolate SE 4.6	GQ465656.1	301068950	Sweden
Adineta vaga isolate SE 4.7	GQ465657.1	301068952	Sweden
Adineta vaga isolate SE 5.1	GQ465658.1	301068954	Sweden
Adineta vaga isolate SE 5.2	GQ465659.1	301068956	Sweden
Adineta vaga isolate SL 1	GQ465621.1	301068880	Slovenia
Adineta vaga isolate TU.a	EU751167.1	209405889	Turkey
Adineta vaga isolate TU.a.1	EU751164.1	209405883	Turkey
Adineta vaga isolate TU.a.2	EU751165.1	209405885	Turkey
Adineta vaga isolate TU.a.3	EU751166.1	209405887	Turkey
Adineta vaga isolate TU.b	EU751168.1	209405891	Turkey
Adineta vaga isolate TU.c	EU751169.1	209405893	Turkey
Adineta vaga isolate TU.d	EU751170.1	209405895	Turkey
Adineta vaga isolate TU.e	EU751171.1	209405897	Turkey
Adineta vaga isolate TU.f	EU751172.1	209405899	Turkey
Adineta vaga isolate TU.g	EU751173.1	209405901	Turkey
Adineta vaga isolate TU.h	EU751174.1	209405903	Turkey
Adineta vaga isolate TU.i	EU751175.1	209405905	Turkey
Adineta vaga isolate TU.j	EU751176.1	209405907	Turkey
Adineta vaga isolate TU.k	EU751177.1	209405909	Turkey
Adineta vaga isolate TU.l	EU751178.1	209405911	Turkey
Adineta vaga isolate TU.m	EU751179.1	209405913	Turkey
Adineta vaga isolate TU.n	EU751213.1	209405981	Turkey
Adineta vaga isolate TU.o	EU751214.1	209405983	Turkey
Adineta vaga isolate TZ.1.1	EF173251.1	134141774	Tanzania
Adineta vaga isolate TZ.1.2	EF173252.1	134141776	Tanzania
Adineta vaga isolate TZ.1.3	EF173253.1	134141778	Tanzania
Adineta vaga isolate TZ.2.1	EF173254.1	134141780	Tanzania
Adineta vaga isolate TZ.2.2	EF173255.1	134141782	Tanzania
Adineta vaga isolate TZ.2.3	EF173256.1	134141784	Tanzania
Adineta vaga isolate TZ.2.4	EF173257.1	134141786	Tanzania
Adineta vaga isolate TZ.2.5	EF173258.1	134141788	Tanzania

Adineta vaga isolate TZ.3.1	EF173259.1	134141790	Tanzania
Adineta vaga isolate TZ.3.2	EF173260.1	134141792	Tanzania
Adineta vaga isolate TZ.3.3	EF173261.1	134141794	Tanzania
Adineta vaga isolate UK 15	GQ465623.1	301068884	United Kingdom
Adineta vaga isolate UK 16.1	GQ465624.1	301068886	United Kingdom
Adineta vaga isolate UK 16.2	GQ465625.1	301068888	United Kingdom
Adineta vaga isolate UK 17	GQ465626.1	301068890	United Kingdom
Adineta vaga isolate UK 18	GQ465627.1	301068892	United Kingdom
Adineta vaga isolate UK 19.1	GQ465628.1	301068894	United Kingdom
Adineta vaga isolate UK 19.2	GQ465629.1	301068896	United Kingdom
Adineta vaga isolate UK 19.3	GQ465630.1	301068898	United Kingdom
Adineta vaga isolate UK 19.4	GQ465631.1	301068900	United Kingdom
Adineta vaga isolate UK 19.5	GQ465632.1	301068902	United Kingdom
Adineta vaga isolate UK.1.1	EF173213.1	134141698	United Kingdom
Adineta vaga isolate UK.1.2	EF173214.1	134141700	United Kingdom
Adineta vaga isolate UK.1.3	EF173215.1	134141702	United Kingdom
Adineta vaga isolate UK.1.4	EF173216.1	134141704	United Kingdom
Adineta vaga isolate UK.10	EF173248.1	134141768	United Kingdom
Adineta vaga isolate UK.11	EF173249.1	134141770	United Kingdom
Adineta vaga isolate UK.12	EF173250.1	134141772	United Kingdom
Adineta vaga isolate UK.13	EF173239.1	134141750	United Kingdom
Adineta vaga isolate UK.2	EF173217.1	134141706	United Kingdom
Adineta vaga isolate UK.3	EF173218.1	134141708	United Kingdom
Adineta vaga isolate UK.4	EF173219.1	134141710	United Kingdom
Adineta vaga isolate UK.5.1	EF173221.1	134141714	United Kingdom
Adineta vaga isolate UK.5.2	EF173224.1	134141720	United Kingdom
Adineta vaga isolate UK.6	EF173225.1	134141722	United Kingdom
Adineta vaga isolate UK.7	EF173229.1	134141730	United Kingdom
Adineta vaga isolate UK.8.1	EF173240.1	134141752	United Kingdom
Adineta vaga isolate UK.8.2	EF173241.1	134141754	United Kingdom
Adineta vaga isolate UK.9	EF173242.1	134141756	United Kingdom
Adineta vaga isolate UK.a	EU751078.1	209405711	United Kingdom
Adineta vaga isolate UK.a.1	EU751077.1	209405709	United Kingdom
Adineta vaga isolate UK.b	EU751271.1	209406097	United Kingdom
Adineta vaga isolate UK.b.1	EU751247.1	209406049	United Kingdom
Adineta vaga isolate UK.b.10	EU751256.1	209406067	United Kingdom
Adineta vaga isolate UK.b.11	EU751257.1	209406069	United Kingdom
Adineta vaga isolate UK.b.12	EU751258.1	209406071	United Kingdom
Adineta vaga isolate UK.b.13	EU751259.1	209406073	United Kingdom
Adineta vaga isolate UK.b.14	EU751260.1	209406075	United Kingdom
Adineta vaga isolate UK.b.15	EU751261.1	209406077	United Kingdom
Adineta vaga isolate UK.b.16	EU751262.1	209406079	United Kingdom
Adineta vaga isolate UK.b.17	EU751263.1	209406081	United Kingdom
Adineta vaga isolate UK.b.18	EU751264.1	209406083	United Kingdom
Adineta vaga isolate UK.b.19	EU751265.1	209406085	United Kingdom
Adineta vaga isolate UK.b.2	EU751248.1	209406051	United Kingdom
Adineta vaga isolate UK.b.20	EU751266.1	209406087	United Kingdom
Adineta vaga isolate UK.b.21	EU751267.1	209406089	United Kingdom
Adineta vaga isolate UK.b.22	EU751268.1	209406091	United Kingdom
Adineta vaga isolate UK.b.23	EU751269.1	209406093	United Kingdom

Adineta vaga isolate UK.b.24	EU751270.1	209406095	United Kingdom
Adineta vaga isolate UK.b.3	EU751249.1	209406053	United Kingdom
Adineta vaga isolate UK.b.4	EU751250.1	209406055	United Kingdom
Adineta vaga isolate UK.b.5	EU751251.1	209406057	United Kingdom
Adineta vaga isolate UK.b.6	EU751252.1	209406059	United Kingdom
Adineta vaga isolate UK.b.7	EU751253.1	209406061	United Kingdom
Adineta vaga isolate UK.b.8	EU751254.1	209406063	United Kingdom
Adineta vaga isolate UK.b.9	EU751255.1	209406065	United Kingdom
Adineta vaga isolate Ukr 1	GQ465633.1	301068904	Ukraina
Adineta vaga isolate Ukr 2	GQ465622.1	301068882	Ukraina
Adineta vaga UCD12	DQ089725.1	72153586	Unknown
Adineta vaga voucher AD003	KM043180.1	703599946	Unknown
Adineta vaga voucher AD004	KM043181.1	703599948	Unknown
Adineta vaga voucher AD005	KM043182.1	703599950	Unknown
Adineta vaga voucher AD006	KM043183.1	703599952	Unknown
Adineta vaga voucher AD008	KM043184.1	703599954	Unknown
Adineta vaga voucher AvagaB2	KF582480.1	557477039	Unknown
Brachionus plicatilis isolate 1ERA1	AF266895.1	9828277	Unknown
Brachionus plicatilis isolate 6TUR2	AF266853.1	9828193	Unknown

Table 2

			Temperature (°C)		Humidity (%)		Wind (km/h)	Rain (mm)
TP	Year	Date	Min	Max	Min	Max	Max	Total
3	First	11/12/2013	-3.8	7.7	85	97	4.8	0.2
3	First	12/12/2013	-4.1	5.1	82	96	19.3	0
3	First	13/12/2013	-4.6	7	77	96	25.7	0.4
3	First	14/12/2013	3.4	7.4	83	94	29	2.8
3	First	15/12/2013	5.8	9.8	65	87	38.6	0
3	First	16/12/2013	3.3	13.4	43	82	32.2	0
3	First	17/12/2013	2.8	11.3	62	88	30.6	0
3	First	18/12/2013	6.1	10.1	73	85	30.6	0
3	First	19/12/2013	0.6	10.9	76	95	38.6	9
3	First	20/12/2013	2.3	8.2	77	96	27.4	0.2
3	First	21/12/2013	4.4	7	64	87	40.2	0.8
3	First	22/12/2013	4.7	9.5	84	92	38.6	5.6
3	First	23/12/2013	5.9	12.1	66	88	54.7	0
3	First	24/12/2013	8	12	64	92	69.2	19.2
3	First	25/12/2013	3.6	10.9	80	94	30.6	5.6
3	First	26/12/2013	2.9	7.4	82	96	20.9	0
3	First	27/12/2013	4.7	12	79	92	53.1	0.6
3	First	28/12/2013	5.8	12.3	82	95	35.4	13.2
3	First	29/12/2013	2.9	7.2	81	92	33.8	2
3	First	30/12/2013	1.7	7	76	94	33.8	0.2
3	First	31/12/2013	5.3	9.4	53	84	35.4	0.6
3	First	01/01/2014	5.4	9.7	77	89	35.4	1.2
3	First	02/01/2014	7.2	10.4	81	89	35.4	3.4
3	First	03/01/2014	7.6	12.6	64	89	77.2	2
3	First	04/01/2014	7.6	10.7	69	86	32.2	0.8
3	First	05/01/2014	4	8.5	82	90	32.2	1.2
3	First	06/01/2014	7.5	13.5	77	89	45.1	0.4
3	First	07/01/2014	9.9	13.9	69	87	43.5	0.2
3	First	08/01/2014	6.8	11.9	75	91	56.3	0.8
3	First	09/01/2014	6.5	12.1	76	90	46.7	2.6
3	First	10/01/2014	3.9	8.5	79	90	27.4	0
3	First	11/01/2014	-0.9	10	80	94	32.2	0.2
3	First	12/01/2014	-1.9	6.6	69	97	24.1	0.2
3	First	13/01/2014	3.8	9.9	74	90	30.6	0
3	First	14/01/2014	3.6	7.3	84	93	24.1	0.6
3	First	15/01/2014	2.6	6.8	85	91	32.2	2.6
3	First	16/01/2014	6.8	9.6	85	92	35.4	2.6
3	First	17/01/2014	7.4	10.4	71	87	30.6	0
3	First	18/01/2014	1.9	11.8	64	93	25.7	0
3	First	19/01/2014	6.7	9.7	69	91	27.4	0
3	First	20/01/2014	5.1	6.9	92	95	22.5	9.8
3	First	21/01/2014	2.2	6.8	83	95	22.5	0.8
3	First	22/01/2014	-0.2	5.6	74	91	22.5	0
3	First	23/01/2014	-0.1	5.3	88	93	33.8	3.2
3	First	24/01/2014	-2.7	6.2	75	95	20.9	0
3	First	25/01/2014	-3.3	6.4	70	96	59.5	2
3	First	26/01/2014	4.3	7.4	75	92	37	7.2
3	First	27/01/2014	2.7	7.2	81	90	33.8	2.4

Table 3

Variable	df	F value	p-value	R^2
T° Min	3	222	$<2e^{-16}$	0.500
T° Max	3	338.8	$<2e^{-16}$	0.604
Humidity Min	3	163.8	$<2e^{-16}$	0.424
Humidity Max	3	17.03	$1.16e^{-10}$	0.068
Rain	3	4.318	0.005	0.015
Wind Max	3	14.23	$5.35e^{-09}$	0.056

Table 4

Linear_distribution							
Model_ID	ReproProbMin	ReproProbMax	MetaCommMin	MetaCommMax	SurvProbMin	SurvProbMax	n
1_linear_permissive	0.344	0.344	0.003	0.003	0.9	0.9	117
1_linear_intermediate	0.177	0.177	0.003	0.003	0.45	0.45	117
1_linear_harsh	0.0344	0.0344	0.003	0.003	0.09	0.09	117

```
seq(["SurvProbMin"], ["SurvProbMax"], length.out = ["n"]))
seq(["ReproProbMin"],["ReproProbMax"], length.out = ["n"]))
seq(["MetaCommMin"], ["MetaCommMax"], length.out = ["n"]))
```

Triangular_distribution							
Model_ID	ReproProbMin	ReproProbMax	MetaCommMin	MetaCommMax	SurvProbMin	SurvProbMax	n
2_triangular_permissive	0.01	0.344	0.003	0.003	0.01	0.9	117
2_triangular_intermediate	0.005	0.177	0.003	0.003	0.005	0.45	117
2_triangular_harsh	0.001	0.0344	0.003	0.003	0.001	0.09	117
3_triangular_permissive	0.344	0.344	10E-10	0.005	0.9	0.9	117
3_triangular_intermediate	0.177	0.177	10E-10	0.005	0.45	0.45	117
3_triangular_harsh	0.0344	0.0344	10E-10	0.005	0.09	0.09	117
4_triangular_permissive	0.344	0.01	10E-10	0.005	0.9	0.01	117
4_triangular_intermediate	0.177	0.005	10E-10	0.005	0.45	0.005	117
4_triangular_harsh	0.0344	0.001	10E-10	0.005	0.09	0.001	117
5_triangular_permissive	0.01	0.344	10E-10	0.005	0.01	0.9	117
5_triangular_intermediate	0.005	0.177	10E-10	0.005	0.005	0.45	117
5_triangular_harsh	0.001	0.0344	10E-10	0.005	0.001	0.09	117

```
seq(["SurvProbMin"], ["SurvProbMax"], length.out = ["n"]))
seq(["ReproProbMin"],["ReproProbMax"], length.out = ["n"]))
seq(["MetaCommMin"], ["MetaCommMax"], length.out = ["n"]))
```

Exponential_distribution							
Model_ID	ReproProbMin	ReproProbMax	MetaCommMin	MetaCommMax	SurvProbMin	SurvProbMax	n
2_Exp_permissive	1	10	5.9	5.9	0.1	10	117
2_Exp_intermediate	1.5	10	5.9	5.9	1	10	117
2_Exp_harsh	3	10	5.9	5.9	2	10	117
3_Exp_permissive	1	1	1	100	0.1	0.1	117
3_Exp_intermediate	2	2	1	100	0.8	0.9	117
3_Exp_harsh	3	3	1	100	2.5	2.5	117
4_Exp_permissive	1	10	100	1	0.1	10	117
4_Exp_intermediate	1.5	10	100	1	1	10	117
4_Exp_harsh	3	10	100	1	2	10	117
5_Exp_permissive	1	10	1	100	0.1	10	117
5_Exp_intermediate	1.5	10	1	100	1	10	117
5_Exp_harsh	3	10	1	100	2	10	117

```
dexp(seq(["SurvProbMin"], ["SurvProbMax"], length.out = ["n"]))
dexp(seq(["ReproProbMin"],["ReproProbMax"], length.out = ["n"]))
dexp(seq(["MetaCommMin"], ["MetaCommMax"], length.out = ["n"]))
```

Normal_distribution							
Model_ID	ReproProbMin	ReproProbMax	MetaCommMin	MetaCommMax	SurvProbMin	SurvProbMax	n
2_Normal_permissive	0.1	2	0.025	0.025	0.1	2	117
2_Normal_intermediate	1.2	2	0.025	0.025	1.2	2	117
2_Normal_harsh	2	2	0.025	0.025	2	2	117
3_Normal_permissive	0.5	0.5	0.25	0	0.1	0.1	117
3_Normal_intermediate	1.3	1.3	0.25	0	0.8	0.9	117
3_Normal_harsh	2	2	0.25	0	2.5	2.5	117
4_Normal_permissive	0.1	2	0.25	0	0.1	2	117
4_Normal_intermediate	1.2	2	0.25	0	1.2	2	117
4_Normal_harsh	2	2	0.25	0	2	2	117
5_Normal_permissive	0.1	2	0	0.25	0.1	2	117
5_Normal_intermediate	1.2	2	0	0.25	1.2	2	117
5_Normal_harsh	2	2	0	0.25	2	2	117

```
dnorm(seq(["SurvProbMin"], ["SurvProbMax"], length.out = ["n"]))*2.25
dnorm(seq(["ReproProbMin"],["ReproProbMax"], length.out= ["n"]))
dnorm(seq(["MetaCommMin"], ["MetaCommMax"], length.out = ["n"]), mean=0.0005, sd=0.01)/600
```

Hybrid_distribution							

```
MetaComm    <- c(0.1,
                 dnorm(seq(0.0, 0.5, length.out = 23), mean = 0.005, sd = 0.1)/150,
                 seq(10E-5,10E-10, length.out = 93))
```

Table 5

	Spe_2	Spe_20	Spe_1	Spe_19	Spe_3	Spe_9	Spe_22	Spe_13	Spe_14	Spe_17	Spe_15	Spe_16	Spe_24	Spe_23	Spe_10	Spe_21	Spe_8	Spe_18	Spe_12	Spe_11	Spe_7	Spe_6	Spe_4	Spe_5
Spe_2	**0,0083 (0,0033)**	0,0202	0,0237	0,0229	0,0242	0,0236	0,0244	0,0227	0,0207	0,0213	0,0192	0,0218	0,0212	0,0228	0,0225	0,0254	0,0196	0,0222	0,0246	0,0226	0,0246	0,0238	0,0308	0,0261
Spe_20	0,1719	**n/c**	0,0204	0,0248	0,0175	0,0216	0,021	0,0268	0,0209	0,0206	0,0207	0,0222	0,0231	0,0225	0,0227	0,0265	0,0252	0,0206	0,0274	0,0208	0,0241	0,0244	0,0253	0,0246
Spe_1	0,1802	0,1625	**0,0071 (0,0032)**	0,0246	0,0192	0,0243	0,024	0,0234	0,0195	0,0232	0,022	0,0228	0,0256	0,0203	0,0234	0,0253	0,0237	0,0218	0,0233	0,0223	0,0264	0,0208	0,0255	0,0266
Spe_19	0,1621	0,1892	0,1654	**0,0096 (0,0039)**	0,0218	0,021	0,0227	0,0221	0,0215	0,0227	0,0226	0,0244	0,0235	0,0211	0,0246	0,0272	0,0207	0,0205	0,0278	0,0206	0,0236	0,0234	0,0259	0,0205
Spe_3	0,1833	0,1582	0,1454	0,1637	**0,0068 (0,0023)**	0,024	0,0221	0,0263	0,0186	0,0199	0,0185	0,0188	0,0197	0,0191	0,0226	0,0202	0,0204	0,0165	0,0248	0,0212	0,0267	0,0205	0,0241	0,0216
Spe_9	0,1862	0,1825	0,1742	0,1428	0,1551	**0,0166 (0,0033)**	0,0187	0,0216	0,0159	0,0181	0,0177	0,0203	0,0189	0,0186	0,0209	0,0239	0,0205	0,0185	0,0241	0,0177	0,021	0,0196	0,0226	0,0249
Spe_22	0,1848	0,1785	0,1748	0,1713	0,1555	0,1205	**n/c**	0,022	0,0162	0,019	0,0158	0,0193	0,0171	0,019	0,021	0,0222	0,0181	0,0215	0,0208	0,0209	0,0238	0,0209	0,023	0,0199
Spe_13	0,1678	0,1888	0,1656	0,1365	0,1694	0,1295	0,1597	**0,0055 (0,0028)**	0,0164	0,0194	0,0191	0,0209	0,0206	0,0177	0,0198	0,0227	0,0194	0,0221	0,0197	0,0185	0,0225	0,0175	0,0218	0,0235
Spe_14	0,1601	0,1654	0,1372	0,1521	0,1265	0,1004	0,1019	0,1069	**0,0153 (0,003)**	0,0117	0,0118	0,011	0,0133	0,0147	0,0157	0,0159	0,0166	0,0159	0,0157	0,0151	0,0184	0,0165	0,0206	0,0231
Spe_17	0,1736	0,169	0,1697	0,1587	0,1422	0,1279	0,1287	0,1185	0,0707	**0,0122 (0,0048)**	0,0106	0,0146	0,0133	0,0158	0,0166	0,0205	0,0179	0,0184	0,0196	0,018	0,018	0,0211	0,0184	0,0238
Spe_15	0,148	0,1675	0,1621	0,1524	0,1264	0,1182	0,1143	0,1206	0,0625	0,0565	**0,0131 (0,0033)**	0,0097	0,0121	0,0145	0,0171	0,017	0,0139	0,0154	0,0188	0,0159	0,0182	0,0198	0,0216	0,0227
Spe_16	0,1637	0,1785	0,1606	0,1619	0,1294	0,1296	0,1305	0,122	0,0616	0,0738	0,0426	**0,0129 (0,0039)**	0,0125	0,0156	0,0177	0,0201	0,0164	0,0153	0,0173	0,0172	0,0196	0,022	0,0198	0,023
Spe_24	0,1655	0,1906	0,1911	0,1598	0,141	0,1281	0,1277	0,1306	0,0748	0,0704	0,0663	0,0683	**n/c**	0,0178	0,0179	0,0208	0,0158	0,0178	0,0195	0,0183	0,0187	0,0201	0,023	0,0218
Spe_23	0,1686	0,1712	0,1503	0,1527	0,1349	0,1182	0,1244	0,131	0,1029	0,0961	0,1022	0,1083	0,1206	**n/c**	0,016	0,021	0,0162	0,0167	0,0175	0,0165	0,0192	0,0175	0,0216	0,0218
Spe_10	0,1715	0,1933	0,1744	0,1662	0,1611	0,1354	0,1259	0,1301	0,1047	0,1118	0,1172	0,1236	0,1281	0,1162	**0,007 (0,0039)**	0,0183	0,0195	0,0226	0,0209	0,0198	0,0229	0,021	0,0256	0,0242
Spe_21	0,1836	0,2178	0,1918	0,2079	0,1533	0,1688	0,174	0,1574	0,1267	0,1446	0,1304	0,1506	0,1609	0,1615	0,1466	**n/c**	0,0221	0,0207	0,0214	0,018	0,0257	0,0203	0,0273	0,0273
Spe_8	0,1543	0,1857	0,1877	0,1633	0,1608	0,165	0,1454	0,1598	0,1514	0,1496	0,1332	0,1423	0,1322	0,1377	0,1563	0,1467	**0,0046 (0,0022)**	0,0195	0,0212	0,0174	0,0208	0,0176	0,0249	0,0214
Spe_18	0,1632	0,1575	0,1466	0,1476	0,1147	0,1218	0,1375	0,1404	0,0968	0,114	0,1025	0,0984	0,1237	0,1132	0,1471	0,1402	0,1579	**0,0017 (0,0017)**	0,0183	0,0197	0,0195	0,0212	0,021	0,0223
Spe_12	0,1899	0,2016	0,1769	0,1856	0,1508	0,1625	0,1273	0,1343	0,1241	0,1375	0,1274	0,1241	0,1391	0,1215	0,1334	0,1566	0,1451	0,1369	**0,0079 (0,0027)**	0,0154	0,0289	0,0216	0,023	0,0272
Spe_11	0,1734	0,1625	0,1679	0,1349	0,1353	0,119	0,1468	0,1272	0,1131	0,1266	0,1027	0,1303	0,1281	0,1047	0,1452	0,1519	0,1449	0,1417	0,0836	**0,0131 (0,0033)**	0,0212	0,0188	0,023	0,0213
Spe_7	0,194	0,1848	0,2124	0,1723	0,2015	0,1668	0,1717	0,1611	0,1383	0,1242	0,1221	0,1332	0,134	0,1427	0,1698	0,1878	0,1691	0,1517	0,192	0,1558	**0,0158 (0,0029)**	0,0209	0,022	0,0289
Spe_6	0,1773	0,1881	0,1524	0,1442	0,1452	0,1342	0,1451	0,1353	0,1252	0,1573	0,145	0,1619	0,1538	0,1189	0,1527	0,1681	0,1448	0,1502	0,1592	0,1409	0,1659	**0,0017 (0,0014)**	0,0233	0,0192
Spe_4	0,2071	0,1953	0,1787	0,1683	0,1539	0,1539	0,1734	0,1507	0,1275	0,1288	0,1271	0,1242	0,1419	0,1535	0,1938	0,1912	0,1813	0,1311	0,1633	0,1525	0,1601	0,1633	**0,0051 (0,0031)**	0,0169
Spe_5	0,1782	0,1936	0,1701	0,1416	0,1476	0,153	0,1502	0,1458	0,1428	0,1426	0,1415	0,1507	0,1423	0,1476	0,1818	0,197	0,1765	0,1417	0,196	0,1418	0,1982	0,1466	0,0925	**0,0241 (0,007)**

General discussion & Perspectives

In the precedent chapters, the different experiments conducted to understand the long-term survival of bdelloid rotifers in absence of conventional sex and their direct interpretations have been presented. Throughout this thesis we tackled several aspects of the evolution of those intriguing animals and it clearly appears that, in spite of being asexuals, their evolution is much more complex than simple lineages of females reproducing clonally. Structures turn out to be extremely dynamics from genomes to communities. In this final part, I will discuss the dynamics possibly ruling the evolution of *Adineta* species by affecting genomic the diversification/homogenization balance and the community structure over time.

Gene conversion maintain genome homogeneity

As presented throughout this thesis, it is now well established that bdelloid rotifers reproduce by apomictic parthenogenesis through which oocytes are produced by mitotic divisions of the germ line. As a result, each egg develops into a daughter that is theoretically a clone of the mother except for point mutations and mitotic crossovers (Symington et al, 2014). Indeed twelve individuals collected from the same lichen patch harbored identical genotypes for the five sequenced loci (see Chapter 1). Even if, over generations, point mutations should theoretically accumulate in the genome of asexual species until being detrimental, this was not observed in bdelloid rotifers as abundant gene conversion seemed to limit this effect (Flot et al, 2013). The exact mechanisms responsible for gene conversion in bdelloids are not yet known but have been suggested to be promoted by cycles of DNA breakage/repair occurring during desiccation/rehydration events. This hypothesis could partly explain how mutations accumulation (diversification) is balanced by gene conversion (homogenization) in bdelloid species dwelling in terrestrial habitats that frequently dry (mosses, lichens, ponds…). Alternatively, DNA breaks induced by molecular actors (e.g. involving spo11) could trigger gene conversion in permanently hydrated species. Yet, it seems interesting here to highlight that gene conversion does not automatically have a positive impact on genomes. Conversions can purge the genome from deleterious mutations that accumulated over generations but that is highly dependent to the genomic environment in which conversion occur. In example, a recessive allele can accumulate detrimental mutations if a healthy allelic gene maintains the function. The healthy allele having a 50:50 chance to be used as

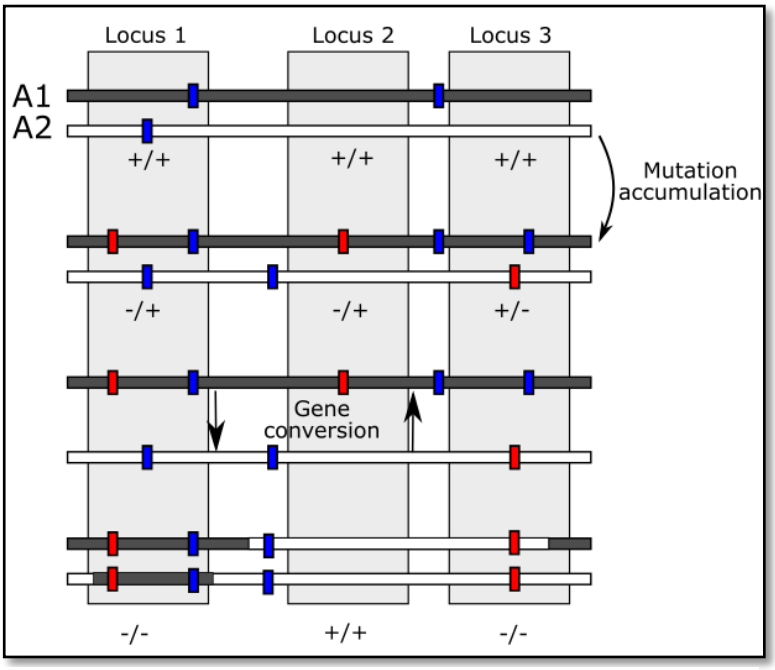

Figure 1 : Schematic representation of the impact of gene conversion in relation to the genomic environment. Two allelic regions (grey and white) separated by a few neutral mutations (blue bars) are represented. Over generations mutations accumulate, some of which can be detrimental (red bars, minus symbol) but masked by the dominant healthy allele (plus symbol). As each allele has 50:50 chances to be used as template during gene conversion, detrimental mutation can be either revealed or suppressed. The effect of gene conversion on the global genomic environment will then determine the fate of the affected lineage.

template; gene conversion could reveal the masked recessive deleterious alleles. The subsistence of the lineage in which gene conversion occurs will depend on the average resulting fitness, all loci being taken into account. It appears thus essential that gene conversion occurs frequently enough to limit the accumulation of "masked" mutation in recessive alleles (Figure 1). Comparing the genomes of two populations originating from a same strain, one being maintained hydrated and the other frequently going through desiccation cycles, could enable to quantify the impact of desiccation in balancing mutations accumulation. Similarly, transcriptomics studies to compare the expression of genes involved in DNA breaks could demonstrate if such genes could trigger mechanisms that replace desiccation in aquatic bdelloid species to mediate gene conversion.

Tetraploidy and genetic transfers enable genomic diversification

Even though point mutations can lead to genetic diversification and adaptation over the long term, this process is slow compared with the potential rate of adaptation conferred by sex. As explained in the introduction, in the one hand an asexual genome has to accumulate each beneficial mutation one by one to reach optimal fitness; in the other hand, beneficial mutations may not be maintained because they appear in a bad genomic background (e.g. a genome that already accumulated several detrimental mutations). In contrast, mutations can occur independently in distinct genomes and then be reunited by recombination in sexual populations which theoretically maintain lower levels of detrimental mutation accumulation. This is nicely depicted in monogonont rotifers in which clonal lineages can reproduce sexually to rapidly respond to environmental stresses. In bdelloid rotifers, the theoretical disadvantage of asexuality in terms of adaptive efficiency can be balanced by the degenerate tetraploid nature of their genome and the acquisition of new genes through horizontal genetic transfers.

First, some genes being present in four copies (grouped in two pairs, see introduction), two copies can accumulate mutations which can eventually lead to new functions (neo-functionalization) while the two other copies vary much less and ensure the maintenance of the initial function. This is possible if gene conversion is taken into account, conversion within allelic pairs being more frequent than conversion between allelic pairs. As a result, the genetic distance within an allelic pair is maintained around 3% on average while the distance between pairs can be higher than 20%. Because this average distance of 3% is the result of a balance between gene conversion and point mutations, it could be possible to estimate the frequency of gene conversion just by knowing the mutation rate, one conversion occurring roughly every three mutations on the 100 bp DNA fragment. As, in several cases, one allelic pair is not expressed in bdelloid rotifers (Boris Hespeels, pers. comm.), it could indeed reveal the loss of function for this pair. Comparing the expression profiles of allelic pairs could thus reveal examples of gene neo-functionalization and the impact of this mechanism in the evolution of bdelloid rotifers.

Second, bdelloid rotifers have been shown to harbor 8-10% of genes from non-metazoan origins (Gladyshev et al, 2008; Flot et al, 2013; Eyres et al, 2015). Acquiring foreign genes that may play a role in the response to environmental changes is an alternative way to diversify in absence of sex (Keeling and Palmer, 2008). Most genes acquired horizontally are predicted to originate from bacteria, fungi, protists and plants that are frequent within the same habitats than bdelloid rotifers (Gladyshev et al, 2008; Flot et al, 2013; Eyres et al, 2015). Thus, one appealing hypothesis is that bdelloid integrate the DNA released by organisms less tolerant to desiccation found within the same habitat patches. Similarly, *Rotaria socialis* seems to have taken up more genes from protists of class

Oligohymenophorea, both being epibiont of *Asellus aquaticus* (Eyres et al, 2015). Yet, different physical barriers have to be overpassed; environmental DNA has to enter the germ line nuclei and has to be integrated in the genome in order to be transmitted vertically; it has to be domesticated by the recipient genome and have a selective advantage in order to be maintained. Environmental DNA is known to be unstable, degrading in a few days once cell integrity is compromised (Lindahl, 1993); the donor and the receiver should thus be geographically close to each other when entering desiccation to enable HGTs. In addition, the germovitellarium in which oocytes matures and develop into eggs are in the close vicinity with the gut, especially when desiccated (Marotta et al, 2010). Nor connections between those organs neither cell pathways for the processing of environmental DNA have been shown yet. However, the fact that those organs are organized in syncytium could facilitate the delivery of foreign DNA into the oocytes once into the germovitellarium.

The integration of the environmental DNA into the genome could be mediated by several molecular mechanisms such as non-homologous end-joining (NHEJ), homologous recombination (HR) or other mechanisms involving viruses or transposons for example. The transfers and integration of long fragments of DNA (>40kb) encoding multiple genes was shown to be mediated by a previously unknown transposable element (TE), called *terminons*, in *Adineta vaga* (Arkhipova et al, 2017). Those giant TEs can extend telomeres by tens of kb, eventually counteracting terminal erosion, and could possibly explain the frequency of gene transfers in bdelloid rotifers. Indeed, a significant number of foreign genes are shown to colocalize with transposable elements (TEs) in telomeric regions, suggesting their concerted acquisition (Gladyshev et al, 2008; Flot et al, 2013). Several cases of TE-mediated horizontal gene transfers have been described in eukaryotes (Kidwell, 1993; Syvanen and Kado, 2001; Zaneveld et al, 2008), often transmitted by retroviruses that act as vectors (Yohn et al, 2005). However, the fact that a large repertoire of TE families, but a very low copy number of TEs indicates the existence of efficient mechanisms limiting their proliferation within the genome (around 3% of the genome size only; Flot et al, 2013). The proximity of TEs with foreign genes could trigger the surveillance response by the host genome through the expression of RNA silencing pathways used to preserve genome integrity (Rodriguez and Arkhipova, 2016). RNAs libraries are indeed highly enriched in known TEs present in *A. vaga* genome. This process may result in the adaptation of foreign genes to the receiver genome by adjusting the transcriptional activity and the incorporation into metabolic pathways (Rodriguez and Arkhipova, 2016).

Transcriptomics studies show that most genes from non-metazoan origin observed in bdelloid rotifers are expressed and have predicted functions for the catabolism of complex polysaccharide or response to stress probably playing an adaptive role in bdelloid rotifers (Eyres et al, 2015). Even though the exact role of the acquired genes have to be confirmed, this observation highlights the role of foreign gene acquisition in the evolution of bdelloid rotifers, especially for the adaptation to new habitats presenting different stressors and food. Furthermore, it appeared that non-desiccating species also independently accumulated foreign genes confirming the importance of HGTs in the adaptation of all bdelloid rotifers. Another observation from comparative transcriptomics and genomics studies is the domestication of the transferred genes through time (Pàl et al, 2005). Indeed, ancient HGTs have GC content similar to the receiver genomes and are present in two to four copies while genes recently acquired harbor distinct GC content and are often in a single copy (Flot et al, 2013; Eyres et al, 2015). Furthermore, ancient HGTs harbor short introns indicating a homogenization with the core genome of bdelloid rotifers while recent HGTs are often intron-less (Flot et al, 2013; Eyres et al, 2015). Here, if desiccation favors the transfer of foreign DNA

by fragmenting the genomes (Hespeels et al, 2014), it can also impact the maintenance of the acquired genes. Gene conversion resulting from the repair of DNA DSBs can either cause the loss of the horizontally acquired gene if the original sequence without HGT found on the allelic pair is used as template or promote its stabilization if the copy harboring the HGT is used as template. In the first case, both allelic regions will retrieve the sequence preceding the transfer whereas both copies will harbor the HGT, stabilizing it in the second case. Interestingly, the ancient horizontally acquired genes (common to *Rotaria* and *Adineta* species) associated with desiccation tolerance are being differentially lost in aquatic species showing that conferring a selective advantage is necessary for the maintenance of HGTs (Eyres et al, 2015). Under those considerations, the frequency of HGTs (5.1 and 21.8 events per million years in non-desiccating and desiccating species, respectively) from non-metazoan origin in bdelloids estimated by Eyres et al (2015) is probably an underestimation of the actual rate of transfers.

Another important factor impacting the rate at which bdelloid could acquire foreign genes is the genetic distance separating the donor and receiver genomes (Wilson et al, in review). In the cases discussed above, we mostly talked about inter-kingdom events because inter-metazoan and especially inter-bdelloidea transfers are much more complex to detect given the lack of genomic data on the communities dwelling in moss and lichen patches. Even though the patterns of DNA transfer between *Adineta* species (Debortoli et al, 2016) are likely to be due to contaminations (discussed in chapter 2) our different observations should motivate additional experiments focusing on this topic. A particularly puzzling case (see introduction of chapter 3) is the observation of one individual morphologically identified as *Habrotrocha* which harbored two alleles corresponding to the expected *Habrotrocha* sequences and one allele identical to the ones identified in *Philodina* species for the Nu1054 marker. Even more interesting is the fact that we could retrieve those three alleles in multiple daughters, but not all, of the first isolate (2A5) used to start clonal cultures. Such case may indicate that the acquired Nu1054 copy is unstable and rapidly eliminated in the progeny, that our PCR-based approach is unsuitable for detecting all cases of HGTs, or that environmental DNA persist in the cultures for weeks despite several steps of cleaning and re-isolation. Here, contaminations seem very unlikely to explain the presence of one additional allele in individual 2A5 as it would require that the contaminant DNA was extremely abundant in the first isolate (isolated from the moss patch in <5µL of water) that was then cultured in big Petri dishes (30mL). Similarly, it could be that bacteria containing a homolog of Nu1054 are present in the cultures explaining why we retrieve one additional Nu1054 copy in some daughters. However, we would expect to retrieve this additional contaminant copy more often as bacteria would be homogeneously distributed in the whole Petri dish culture and not restricted to a few bdelloid clones. Alternatively, it could be that our Nu1054 marker is working differentially on distinct copies, preferentially amplifying the *Habrotrocha* alleles and only rarely the *Philodina* copy. In example, this marker could not amplify the alleles of one species (species F) in Debortoli et al (2016). It could also be that the acquired *Philodina* copy is transmitted over a few generations as non-chromosomal DNA, *e.g.* like plasmids in prokaryotes, rapidly disappearing if not integrated in the core genome. Finally, even if our observations correspond to an actual inter-bdelloid species DNA transfer, it may be that the acquired copy is eliminated by gene conversion, the allelic region without the HGT used as template to convert the newly acquired sequence. Although the case of DNA transfers between bdelloid species is obscure, we cannot exclude this possibility as they would probably be subjected to fewer constraints than inter-kingdom events.

First, *A. vaga* seem to group up in patches of dozens of individuals to better survive desiccation (Hespeels et al, 2014) eventually favoring the delivery of intact DNA among individuals. However, additional tests should be devoted to this simple observation to confirm that patches of individuals are the results of a real ecological process and not an experimental artifact. In Hespeels et al (2014), thousands of individuals were desiccated together in a water drop and even if the authors could demonstrate that the groups were not only formed by water tension, it remains unclear if living individuals clustered together because the slowly evaporating drop left less and less space to wander around. Furthermore, it is still unknown if distinct species would group together or separately. Second, it has been shown that *Philodina roseola* individuals tend to retain closely related DNA fragments longer than genetically distant DNA in their gut (Bininda-Emonds et al, 2016). Even if no evidence that this foreign DNA was then integrated in the receiver oocytes, this suggests that the time window during which related DNA can enter nuclei of the germ line is longer. Third, although the genetic distance among individuals from a same family (up to 36.1% divergence among *Adineta* spp. calculated using the COI marker) can be as high as the distance between individuals from distinct families (up to 36.1%), homologies can be maintained. Transfers of long DNA fragments (>20 kb) have indeed been observed among yeasts of different families (Liti et al, 2006; Novo et al, 2009). Fourth, genes acquired from other bdelloid rotifers should be more easily maintained in the receiver genome because both donors and receivers present similar genomic features (introns, GC content…). The GC content is relatively stable across bdelloid rotifers (exons GC content = 33%, 30.7% and 33% in *R. socialis, R. sordida* and *A. vaga*, respectively) and the transferred genes should already present short introns (Eyres et al, 2015). Finally, even though only 44.4% of the transcripts from *A. ricciae* were found in *Rotaria* species indicating a wide variation in gene content among families, or at least differential gene expression, almost half of the expressed genes were common showing their importance in bdelloids life cycle (Eyres et al, 2015). There are thus higher chances that transfers among bdelloids will be adaptive and maintained, especially transfers within genus (85.6 to 91.6% of transcripts in common among *Rotaria* species).

To conclude this part, even though the genomic signatures (genomic regions evolving asexually) observed in this thesis seem to exclude sex-related mechanism as explanation for the transfers of DNA among bdelloid rotifers, particularly transfers among individuals belonging to the same species, we cannot totally dismiss this possibility. The genomic structure (collinearity breakpoints and palindromes) found in *A. vaga* is not compatible with meiosis *sensu stricto* because it impede proper chromosome pairing, yet alternative mechanisms allow meiosis without pairing of homologous chromosomes (Flot et al, 2013; Signorovitch et al, 2015).

Are sex-related mechanisms plausible?

As discussed in Signorovitch *et al* (2015), meiosis requiring only pairing of telomeric regions, as found in *Oenothera* primroses, would be compatible with *A. vaga* genome and could simply explain the allele sharing patterns observed among conspecific individuals (see chapters 1 and 3). In *Oenothera*, the maternal and paternal chromosomes segregate as distinct entities during telophase 1, there is no random re-assortment but rare crossing-overs are possible between telomeric regions of homologous chromosomes (Cleland, 1972; Golczyk et al, 2008). These ensembles of chromosomes (named Renner complexes α and β) are always transmitted together through generations and two Renner complexes from the same type cannot intercross (α/α impossible). Several variants of this peculiar meiosis are described; Renner complexes can form regular tetrads or rings of a few to all

chromosomes during prophase 1 (Golczyk et al, 2014). Similarly, parasexual reproduction as found in the fungi of the genus *Candida* or in some *Paramecium* species do not require chromosome pairing as the entire DNA content of both parents are transmitted, without reduction division. In *C. albicans*, cell fusion between the two mating types a and α followed by nuclei fusion (karyogamy) is triggered by pheromones (Hull and Johnson, 1999). The resulting egg has a transiently doubled ploidy level comprising both the entire parental genomes, followed by several aneuploidy stages during which chromosome copies are randomly lost over generations until parental ploidy is re-established (Bennett and Johnson, 2003; Hickman *et al*, 2015). When diploidy is restored, the resulting daughter cell can either be a, α or a mix of both. Parasexual reproduction enables chromosome re-assortment and crossing-overs between a and α types, without ploidy reduction.

Oenothera-like mechanisms or parasexual cycles would more parsimoniously explain the patterns of allele sharing observed across several loci as described in Signorovitch et al (2015) than massive HGTs among bdelloid rotifers. But nor evidence for distinct Renner complexes neither distinct mating-types have ever been observed in bdelloid rotifers. Yet, several observations on bdelloid rotifers may question the possibility for such sex-related mechanisms.

First, in laboratory, *A. vaga* individuals appear to group and spend a few time tightly interlaced when they are about to lay eggs (personal observation), sometimes forming patches of hundreds of individuals. Even though this is frequent in dense lab cultures, it is hard to tell if this is driven by a sexual behavior or if laying eggs linked together favor the egg development and if a similar behavior occurs in nature. It is also unknown if distinct species would group together or if this is species-specific. Furthermore, no sexual organs, motile gametes or other reproductive apparatus potentially involved in cell/cell fusion, as in *Oenothera* meiosis or in parasex, have ever been observed in bdelloid rotifers (Fontaneto and Barraclough, 2015).

Second, Hsu described the formation of chromosome rings along the nucleus envelope in sexually immature *Philodina roseola* individuals and that this conformation in maintain until after the first maturation division of the oocyte (see Figure 1-4 in Hsu, 1956a). Hsu did not investigate such early maturation stages in *Habrotrocha tridens* (Hsu, 1956b). This observation has never been reported again since then, but more recent investigations mostly targeted oocytes blocked in metaphase or anaphase 1 by drugs derived from colchicine. It is possible that the chromosome ring observed in prophase 1 in *Oenothera* is disrupted at later stages.

Finally, we did not observe more than two copies of the markers sequenced in the recombining individuals, neither cases of higher ploidy in the lab cultures used for karyotyping (344 karyotypes in total, including 72 *A. vaga* karyotypes; Julie Virgo, personal communication) as expected in parasexual reproduction. Yet, it could be possible that such cases have never been observed if a first oocyte division takes place within the mother, prior egg formation. Then, within the laid egg the second division separate sister chromatids which results in the formation of an embryo with the parental ploidy level and one polar body as observed.

All those mechanisms are very speculative as several elements have never been observed in bdelloid rotifers, yet we cannot totally exclude them as they would more parsimoniously explain the intraspecific exchanges observed in this thesis and the patterns observed in Signorovitch et al (2015) than multiple independent HGTs. In addition, one may question the possibility for those sex-related mechanisms to explain interspecific exchanges. Even though, telomeric regions seem widely

conserved across taxa and thus *Oenothera*-like meiosis mechanistically plausible, such exchanges would result in a genomic signature incompatible with the degenerate tetraploidy observed as the genetic distance within allelic pairs would drastically exceed ≈3%, *i.e*. the distance between *Adineta* species is higher than 20% for EPIC25 homologs.

Frequent dispersal and habitat sorting, the dynamics of bdelloid communities

In the previous parts, we discussed the dynamics of bdelloids genome and even though the mechanisms responsible for genomic diversification remain unclear, the acquisition of genes horizontally cannot be questioned (whatever their origin). However, those events are probably rare enough (especially genes from non-metazoan origin) to be considered as a "replacement for sex". As a result, the dynamics ruling the colonization of new habitats and the response to environmental changes must be different in bdelloid rotifers than in other metazoans reproducing sexually. A given species adapted to a given habitat or conditions can hardly adapt rapidly to changes if it do not benefit from the advantages of sexual reproduction. In bdelloid rotifers, desiccated propagules are frequently dispersed passively by wind. Thus, individuals immigrate in new habitats randomly, adapted or not. For organisms less tolerant to environmental stresses and with a lower dispersal rate, colonizing an unsuitable habitat is theoretically lethal. However, for bdelloid rotifers, a few individuals can persist as 'tuns' for years until environmental conditions become more appropriate. Alternatively, 'tuns' can be dispersed again into another habitat patch, eventually more suitable. Several cases observed throughout this thesis where only a single individual of a species was found around Namur could indicate such scenario (24.13% of the GMYC species observed in the thesis were represented by a single individual; see species B in chapter 1; species B, D, E, H and J in chapter 3; species 5, 6, 19, 20 and 23 in chapter 4; species K, AL and 19 in annex 5). Even though bdelloid rotifers are renowned for their tolerance to extreme conditions data concerning their survival as 'tuns' in natural patches are still missing. Bdelloid adopt this 'tun' conformation under various kinds of stresses (food depletion, unsuitable temperatures, crowding, drugs…) and none solely desiccation-related stress. However, it is unknown if 'tuns' persisting in humid conditions are more persistent than desiccated ones or if their metabolism is totally paused or just slowed down as the mastax is still active sometimes. A bdelloid propagule persisting for years despite adverse conditions have better chances to be transported in a suitable habitat at some point than individuals surviving as tuns for only a few days. As a result, dwelling in habitats well exposed to wind would be a predominant factor in the life cycle of bdelloid species. Interestingly, soil patches (see chapter 1) harbored a widely different *Adineta* spp. community. Much less individuals were sampled on soil patches (mean = 2.09 versus 22.13 for trunk patches) and species were different than the ones sampled lichen patches collected on the trunks (species E was only found in soil patches whereas species D and F were retrieved only on trunks). Those observations may indicate that *Adineta vaga* diversified into several cryptic species with distinct habitat preferences, some species dwelling in patches often exposed to moisture (soil) and some species frequently going through cycles of desiccation/rehydration (lichen patches on trunks). Another hypothesis is that bdelloid communities in soil patches or other patches located near the ground are more exposed to parasites, such as Rotiferophthora fungi, due to increased moisture and direct connections between patches (Wilson and Sherman, 2013). In contrast, lichen patches submitted to frequent short desiccation periods and connected majorly by windblown dispersion present are parasites-free (Wilson and Sherman, 2013). However, our results suggest that bdelloid this spatio-temporal escape to parasites is not the only strategy explaining the evolutionary success of bdelloid rotifers. Indeed, some species seem to tolerate the presence of

parasites more than other species, eventually indicating cases of coevolution depicted by the Red Queen hypothesis. Yet, this has to be studied into more details. Furthermore, the spatio-temporal escape may be applicable to other cases than parasites. In this thesis, escape from other bdelloid species appeared important too, eventually due to inter-bdelloid interactions whatever those may represent (competition, predation …). In chapter 1, the two mostly represented species (species A and C) were found across all types of patches (soil or lichens) but rarely together or only at low densities. This was also the case in chapter 4 where a few species could only expand when the dominant species 9 (corresponding to C in chapter 1) was absent or rare. Interestingly, this was not linked to seasons suggesting that it was not environmental conditions that ruled community composition, but rather the roof location that limited immigration of species 9. Thus, one important conclusion about the dynamics of bdelloid communities is that dispersal reduces interactions between species that would otherwise exclude each other.

Another key point is the observation that bdelloid communities are submitted to important bottlenecks during harsher periods, as in winter (chapter 4). Although our experimental setups could not enable us to follow precisely the dynamics for periods longer than a year, the fact that some individuals survived harsh conditions allows for rapid re-expansion when conditions are more suitable (during other seasons). Here, it would be interesting to test if the most represented species in a given community will have higher chances to have a few survivors than rare species, or if stress-resistance is so different across species that rare species can go through the bottlenecks despite low population densities. In the first case, this would explain how bdelloid species can adapt and diversify through time as the dominant species would stay for several generations in a given habitat patch, having time to adapt over generations to slowly changing conditions. In the second case, one species dominating a community at a given timepoint may not be the dominant after the bottleneck and community composition would fluctuate from year to year, limiting species adaptation and diversification. In that scenario, diversification would be the result of a stochastic process by which one random species dominating the community at a given timepoint is favoured by permissive conditions and thus expand long enough for adaptation to play its role.

Additional knowledges on the ecological response of bdelloid rotifers are compulsory to fully understand the dynamics responsible for their evolutionary success. Controlled lab experiments targeting species interactions and niche differentiation should be planned in future work. Co-culturing distinct *Adineta* species and monitor the reproductive output of each species could reveal competition for resources. Similarly, adding distinct food resources may highlight distinct preferences across species and thus the possibility for co-existence over the long term. Studying other bdelloid families could also be informative as Philodinidae and Habrotrochidae (filtering the water column) present feeding behavior different from the ones observed in Adinetidae (scratching the substrate), eventually facilitating inter-families co-existence. Furthermore, precisely defining the correlation between geographic distance and frequency of immigration could be helpful to understand how communities are susceptible to changes in species composition. In addition, conducting a similar experiment than the one we performed on the roof tops by letting open Petri dishes exposed to immigration throughout the study could be done again, but this time allowing immigration for only a restricted period (*e.g*. closing the dish after a few days). This could demonstrate if rare species arriving during early colonization can expand when dominant local species are not present. Overall, this could help to understand more precisely the dynamics of bdelloid communities. Finally, wider sampling around Belgium and other countries to estimate the

global species diversity for genus *Adineta* could provide data about the rate of diversification of those asexual organisms and provide insights on how they adapt to distinct habitats in spite of sex.

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ANNEXES

Chapter 5: Eco-evolutionary dynamics along gradients of environmental anthropogenic disturbances

This chapter is part of a wider study led by Prof. Luc De Meester entitled "SPatial and environmental determinants of Eco-Evolutionary DYnamics: anthropogenic environments as a model" or SPEEDY project. This project aimed at understanding the impact of human activities on natural environment and their consequences at every biodiversity levels, from genes to ecosystems. Predicting the eco-evolutionary responses to environmental changes is essential to elaborate new conservation strategies (Lankau et al, 2011). The scheme of stratified sampling design of our field campaign covering a wide range of organisms consist in a unique experimental setup to study how spatial configuration of habitat patches influence eco-evolutionary dynamics (Kozak et al, 2008). The dispersal capacity of organisms and their regional abundance impact their dispersal rates, while habitat configuration affects the identity of the immigrants. Furthermore, the strength of local responses to environmental changes may vary across taxa that widely differ in reproductive mode and generation times. In this proposal, we tried to take into account those distinct eco-evolutionary dynamics, bringing together a consortium that has the capacity to tackle this multifaceted problem. I took part in this project, collaborating with Dr Diego Fontaneto, by working on the genetic diversity of bdelloid rotifers along gradients of anthropogenic disturbances.

A first paper focusing on body size variations along a gradient of anthropogenic disturbances is currently under review. Body size is central to species interactions such as food web dynamics (Woodward et al, 2005) and intrinsically linked to metabolic rate (Brown et al, 2004). Urban areas being characterized by increased temperatures (Oke, 1982) are predicted to result in higher metabolic costs and oxygen stress with the consequence to lead to smaller body sizes (Scheffers et al, 2016). Urbanization, however, is associated with habitat fragmentation (Alberti et al, 2017) increasing the selection for highly dispersible phenotypes. In this first study, a shift of communities towards generally smaller species is shown, except for three taxa presenting a positive size-dispersal correlation among which bdelloid rotifers. This highlights the contrasting effects of urbanization on body size and the role of dispersal. Since body size is key to inter-specific relationships (Cheptou et al, 2017), such shifts may impact urban ecosystem functioning.

Those results are accepted for publication in *Nature*:

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Bdelloid community structure are not affected by landscape urbanization but rather shaped by biotic interactions among rotifer species

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Introduction

Human expansion over the last century results in an unprecedented detrimental impact on biodiversity from genes to ecosystems (Butchart et al, 2010; Barnosky et al, 2011). Depletion and fragmentation of natural habitats resulting from intensive land use are considered as major drivers of biodiversity loss. In contrast, human-altered environments can turn out to be favourable for a subset of organisms as new biotic and abiotic conditions emerge (Hobbs et al, 2006; Møller 2010). Thus, predicting the impact of human activities may be tricky for a wide variety of taxa.

The novel conditions and habitats created by those worldwide environmental modifications linked to anthropogenic activities imply strong selection regimes that may change ecological and evolutionary dynamics. Evolutionary responses to environmental changes can occur at the same temporal and spatial scales and thus directly impact ecological responses (Kinnison and Hairston, 2007). Eco-evolutionary dynamics are increasingly recognized as being of key importance for predictions about population and community responses to non-natural changes, such as climate change (De Meester et al, 2011; Urban et al, 2012) or landscape modifications (Cheptou et al, 2008). However, such predictions are mandatory to develop efficient strategies for biodiversity monitoring and conservation (Lankau et al, 2011). Spatial variation probably plays a major role in eco-evolutionary dynamics as local populations and communities may be influenced by the arrival of pre-adapted genotypes or species from the regional pool (Urban et al, 2012). In addition, anthropogenic disturbance exerts selection pressures that offer opportunities to study how organisms react and adapt to new and rapid environmental challenges.

The present study aimed at characterizing the response of a group of microscopic animals with high dispersal capacities (Fontaneto and Ricci, 2006; Wilson, 2011), namely the bdelloid rotifers, to a gradient of urbanization. The collected data were used to test our hypothesis that selection pressures due to urbanization impact microscopic animal communities composition and populations. We applied this hypothesis at two biodiversity levels: community composition and genetic diversity within the focal genus *Adineta*. For the first one, we studied assemblages of bdelloid species identified morphologically whereas for the genetic diversity, we focused on one genus widely spread in the communities and renown to comprise multiple cryptic species, namely *Adineta*. Our expectation is that, if an effect of urbanization is visible in bdelloids, a reduction in species richness and in heterogeneity between assemblages should be found at the community level in more urbanized sites; similarly, a reduction in genetic diversity could be hypothesized for the focal genus

Adineta sampled across the same urbanization gradient. We tested this prediction on different metrics of diversity on bdelloid communities found in lichens across an urbanization gradient in Belgium.

Material and methods

Plot, subplot and site determination

A polygon delimited by the cities of Antwerp, Gent, Brussels and Leuven was used as area within which we determined gradients of urbanization (semi-natural, rural and urban). To select sites over a gradient in urbanization in a standardized way for the heterogeneous group of aquatic and terrestrial target organisms, a GIS-based site selection was organised with 27 plots of 3 by 3 km characterized as semi-natural, rural or urban based on percentage of built-up area. Each of these plots was divided into 200 by 200 m subplots, which were characterized according to the same three levels of urbanization. Plot and subplot selection was done by UGent (Figure 1; see details in Supplemental methods). The sampling scheme was developed within the SPEEDY ("SPatial and environmental determinants of Eco-Evolutionary DYnamics: anthropogenic environments as a model") project whose overall objective is to obtain integrated insight into the responses of communities to urbanization for a wide variety of taxonomic groups ranging from bacteria to birds.

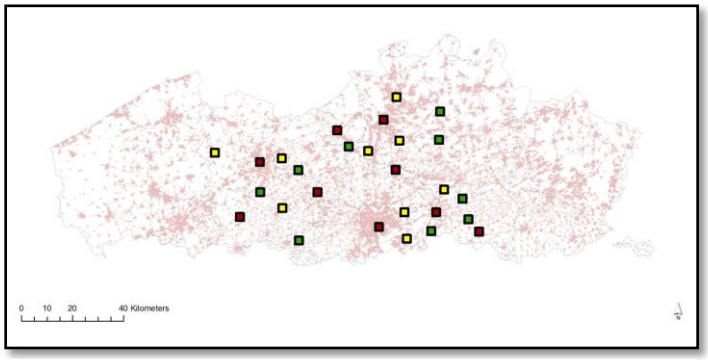

Figure 1 : Map of Flanders showing the 27 plots (3km x 3km). Green: 0-3% built up ratio (semi-natural); yellow: 5-10% built up ratio (rural); red: >15% built up ratio (urban).

One subplot of each type (semi-natural, rural and urban) was selected within each of the 27 plots leading to a total of 81 sampling sites. Because suitable sampling sites vary among the organism groups depending on their characteristics, different sites were selected depending on the target group. For bdelloid rotifers one lichen patch (≈10cm²) of *Xanthoria* was sampled per subplot and the type of substratum on which the patch grew was recorded (tree bark versus concrete). The sampling campaign took place in spring and summer 2013. We then collected two distinct subsamples (≈2cm²) from each patch to conduct two different analyses. One subsample was used to determine community structure based on morphological identification (work done by Dr. D. Fontaneto). The second subsample was treated at UNamur for population genetics analyses focusing on the *Adineta* spp.

Individual isolation, identification and genotyping

All bdelloids found in the subsample of each lichen patch used for morphological analyses were isolated and identified to species level according to the current taxonomical knowledge and nomenclature (Donner, 1965; Segers et al., 2012).

In the other subsamples used for genetic analyses, each individual morphologically identified as *Adineta* spp. was isolated by pipetting and washed serially in sterile SPA® water drops. Each clean

rotifer was placed in an autoclaved 1.5mL tube, briefly centrifuged to pellet the individual and checked under a binocular to make sure that a single individual was present. All individuals found within each subsample were used for DNA extraction according to the Chelex® protocol described in Chapter IV. When more than 24 individuals were sampled, we randomly selected 24 animals for DNA extraction as previous experience with genetic diversity of the focal species in Belgium suggested that the same mitochondrial lineage composed the population retrieved from a lichen patch (Debortoli et al, 2016). We amplified a fragment of the mtCOI gene (605bp) using universal primers (HCOI: 5' - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3' and LCOI: 5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3'; Folmer et al, 1994). The PCR conditions were the same as in Debortoli et al (2016), except that the quantity of gDNA used was 5µL and that the number of cycles was set to 60. The forward and reverse chromatograms resulting from Sanger sequencing (GENEWIZ, UK) were assembled and edited in Sequencher4 and each sequence was then aligned in MAFFT (E-INS-i method; Katoh and Standley, 2013) and visualized in MEGA5 (Tamura *et al*, 2011).

Phylogenetic reconstruction and species delimitation

The obtained haplotype dataset was combined with all the sequences retrieved from other projects around Namur (Belgium) and the mtCOI data available on GenBank (see Supplemental Table 1). A unique sequence was then selected for each distinct haplotype leading to a final dataset of 364 COI sequence of 589bp, which was used for ultrametric Bayesian tree reconstruction in BEAST v1.6.2 (Drummond and Rambaut, 2007). The details about the parameters used to generate the phylogeny were the same as those previously applied on other studies on bdelloid rotifers (Tang *et al*, 2014; Debortoli *et al*, 2016). To avoid confinement of the MCMC chain in a local optimum, we run the analysis three times independently and combined them with the LogCombiner software of the BEAST package. The resulting ultrametric tree was used as input for the General-Mixed Yule Coalescent method for species delimitation using the R version 3.1.1 (R Core Team, 2014) package "splits" (Pons *et al*, 2006; Fujisawa and Barraclough, 2013).

We used an additional method, namely the Automatic Barcode Gap Discovery (ABGD), which relies on the distribution of pairwise genetic distance among haplotypes to delimit species. A "barcode-gap" can often be observed between the distribution of distances among haplotypes belonging to a same species and the distance between haplotypes from distinct species (Puillandre *et al*, 2011). All the mtCOI sequences retrieved for the focal genus *Adineta* were aligned in MAFFT (E-INS-i method; Katoh and Standley, 2013) and the alignment was submitted to ABGD in command line mode using default parameters (software download at http://wwwabi.snv.jussieu.fr/public/abgd/).

Community parameters and structure analyses

To test if the degree of urbanization had an impact on community structure, we calculated, for each patch, the total abundance of individuals sampled, the species richness and Pielou's index of species evenness (Pielou, 1966). We expected that patches from urbanized plots/subplots would harbor less even communities due to a better tolerance of a few species. In addition, we computed the community dissimilarities across space using Bray-Curtis index under the R package "vegan 2.3-5" (Oksanen *et al*, 2007). We expected that communities from a same plot/subplot type would be more similar than communities from distinct plots/subplots type since probably the same pool of species will tolerate higher degree of urbanization. We performed those computations for both the morphological species dataset and the genetic-based delineation of *Adineta* dataset. Note here that

for the focal genus *Adineta*, only a subset of 24 individuals was genotyped eventually biasing the actual community structure. We then performed ANOVA tests and linear models (R software) to analyze the community response to the plot/subplot type (rural, semi-natural and urban) and to the type of substrate on which patches grew.

We also generated one clustered species matrix for each dataset in order to minimize the distance between patches based on the frequency of each species dwelling in it using the R package "heatmap3" (Zhao *et al*, 2014). We then calculated if there was a significant correlation between plot/subplot type and community composition using the Fisher's exact test.

Finally, we computed an AMOVA analysis under Arlequin version 3.5.1.3 (Excoffier and Lischer, 2010) on the *Adineta sp.* using the mtCOI dataset generated through the present study. Although AMOVA analyses rely on the decline in heterozygosity due to subdivision within a population, they have been widely applied at the community level in bacteria, probably because genetic mixing is limited (Roesh et al, 2009; Fridman et al, 2012; Zhao et al, 2012). As bdelloid rotifers reproduce clonally, we decided to apply the AMOVA (1000 permutations) on four different community structures to calculate fixation indices: we regrouped the lichen patches by location, plot type and subplot type.

Results

Sampling and diversity retrieved in the communities

In total, we were able to collect lichen patches (≈ 10cm²) in 80 out of 81 sites visited, identified as *Xanthoria sp*. In 65 cases, the patches were extracted from concrete substratum; all other 15 lichen patches were on tree barks. Regarding the results from the subsamples used for morphological identification, 4918 bdelloid rotifers were isolated and identified as 21 distinct morpho-species. The mean species richness in each patch was 2.43 (range 0-6) and the mean number of individuals isolated was 61.7 (range 0-362) whereas the mean Pielou's index of species evenness was 0.71 (range 0-1) (Figure 2). Even though the abundance seemed higher in the rural plots and subplots,

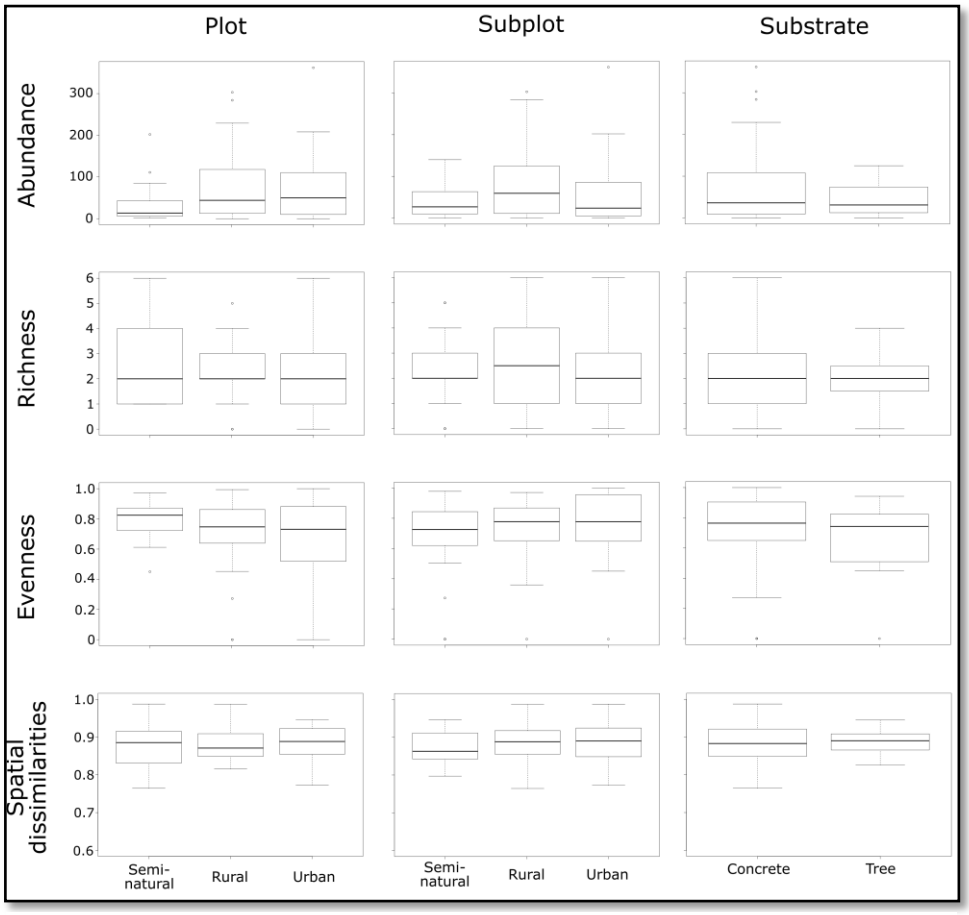

Figure 2 : Boxplot indicating the metrics calculated for each plot, subplot and substrate type for the morphology based determination. The total number of rotifer individuals sampled, the species richness, Pielou's index of evenness and community dissimilarities across space is for each patch collected is indicated.

there was no significant differences according to the ANOVA analyses, neither was the difference in species richness and evenness between the distinct plots/subplots (Table 1).

Community_metric	Variable	F.stat	df	p.value
Individual sampled	Plot	2.900	2 and 77	0.061
	Subplot	2.309	2 and 77	0.106
	Substrate	0.603	2 and 77	0.549
Richness	Plot	0.121	2 and 77	0.877
	Subplot	0.713	2 and 77	0.493
	Substrate	0.850	2 and 77	0.431
Evenness	Plot	1.389	2 and 60	0.257
	Subplot	0.739	2 and 60	0.481
	Substrate	0.342	2 and 60	0.712
Spatial	Plot	0.178	2 and 72	0.838
	Subplot	0.576	2 and 72	0.565
	Substrate	0.278	2 and 72	0.758

Table 1: Results of the ANOVA analyzing the response of each metrics to the plot, subplot and substrate type for the morphologically determined bdelloid communities.

We isolated 521 individuals identified as *Adineta* spp. from the 32 subsamples for DNA analyses where this genus was found. Interestingly, the number of individuals identified as *Adineta* spp. in the two subsamples originating from the same lichen was highly correlated but in six cases out of 32 (18.8%) we could find *Adineta* spp. in only one of the two subsamples. In five of the six cases, the abundance of individuals of the focal species was indeed low (≤ 4 individuals), yet in one case (lichen P9SR) we could isolate 56 individuals in the subsample used for morphological identification but none in the corresponding subsample used for genetic analyses suggesting that communities could be highly structured within a lichen patch. As a maximum of twenty-four individuals were used for each patch in order to reduce the costs, genotyping represented only 274 of the 521 (52.6%) individuals isolated, resulting in a total of 43 distinct mtCOI haplotypes. Both the GMYC analysis and the ABGD method congruently delineated 17 distinct species from this dataset, the only exception being that ABGD further split species 15 into two species when the prior value for intraspecific divergence was set below 0.0129 (Supplemental Figure 1). The two most abundant species (species 14 and 15, representing respectively 29.9 and 42.3% of the individuals genotyped), where also found in other region of Belgium but were less frequent in those regions (Figure 3). On the contrary, the most abundant species sampled on the UNamur roofs or Park Louise-Marie (species 9 and 13) were present but rarer over the area covered by the SPEEDY project (18 individuals in total distributed in four patches for species 9 and 3 individuals in a single patch for species 13). Finally, five other species were sampled during the SPEEDY project as well as in other places in Belgium but were rare in all areas (species 6, 16, 19, AZ and BR). Haplotypes sampled in other European countries also clustered with sequences sampled in the present study. This is the case for five species: species 14 in Slovenia, K in Serbia, N in the UK and Ukraina, BR in France and Sweden and CC in Italy, Poland and the UK. The remaining species were singletons that did not cluster with any of the available mtCOI haplotypes.

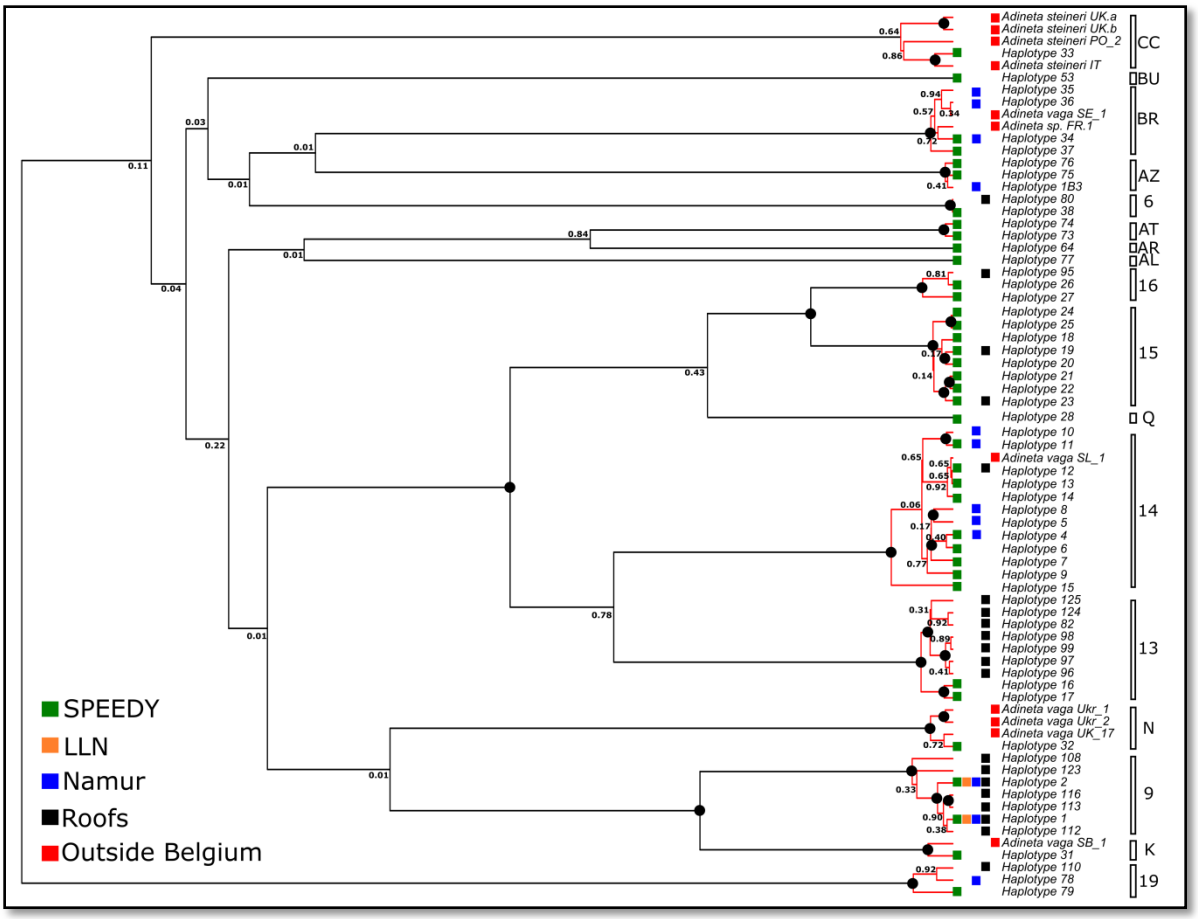

Figure 3 : Pruned ultrametric built from our local mtCOI dataset and *Adineta vaga* sequences available on GenBank. Only the 17 GMYC-defined *Adineta* species that were collected during the SPEEDY project are represented (boxes) in this simplified version of the ultrametric tree (Chapter IV, Supplemental figure 3). The posterior probabilities that support each node are indicated when lower than 0.95, otherwise black dots represent highly supported nodes. Haplotypes that cluster within a same evolutionary entity are linked by red branches. Green squares indicate the haplotypes from the SPEEDY dataset whereas blue, orange, black and red squares highlight the haplotypes that were retrieved from other studies and were sampled in Namur, Louvain-La-Neuve (LLN), roofs (Namur) and outside Belgium, respectively.

The mean number of individuals isolated from the 33 subsamples containing *Adinetida* spp. was 15.8 individuals (range 1-77). The average cryptic species richness in each of those 33 patches was 1.63 (range 1-3 species) and the mean Pielou's index of species evenness was 0.67 (range 0.21-1) (Figure 4).

Plots, subplots and substrate impact on communities

The clustered matrix generated from the matrix of abundance of each morpho-species in each patch did not show any clear differences in relation with the community present in a given patch and its location within a certain plot or subplot type and even with the substrate (Figure 5). Six species were frequent over all the lichen patches sampled: *Macrotrachela ehrenbergii*, *Adineta vaga*, *Philodina vorax*, *Mniobia russeola*, the not fully determined *Mniobia sp1* and *Habrotrocha pulchra*, present in 41.74 to 24.69% of the patches sampled. Those six morpho-species were often present in the same patches (in 47 cases) at high densities (mean 68.81 individuals; range = 5 – 303). The remaining 15 morpho-species were less frequent (in 1 to 10 patches only) and at much lower densities except for *Pleuretra lineata*, which was present in seven patches but represented by a total of 598 individuals.

The number of individuals sampled, the species evenness and the community dissimilarities across sample patches did not vary significantly with the degree of urbanization of the plot or subplot whichever the level of species identification (morphology or the genetic). In contrast, *Adineta* species richness varied significantly across plots type (ANOVA; F-stat = 5.368; df = 2, 30; p = 0.011; Table 2) with rural plots presenting globally less species than the semi-natural and urban plots. The clustered matrix generated from the matrix of abundance of *Adineta* species in each patch (Figure 6) showed that semi-natural plots tended to be colonized by the two most frequent species (species 14 and 15; 1.58 species per patch) but rarely by other *Adineta* sp., whereas rural plots were often colonized by a single species (1.13 species per patch), frequent or not. Similarly, urban plots were often colonized by several species (1.92 species per patch) whichever the species was. However, Fisher's exact test indicated that there was no significant correlation between plot or subplot type and colonization by the two dominant species or not (p-value = 0.724). Interestingly, the clustered matrix suggested that species 14 and 15 could co-exist within a patch but that the other species were rare when those two species were present.

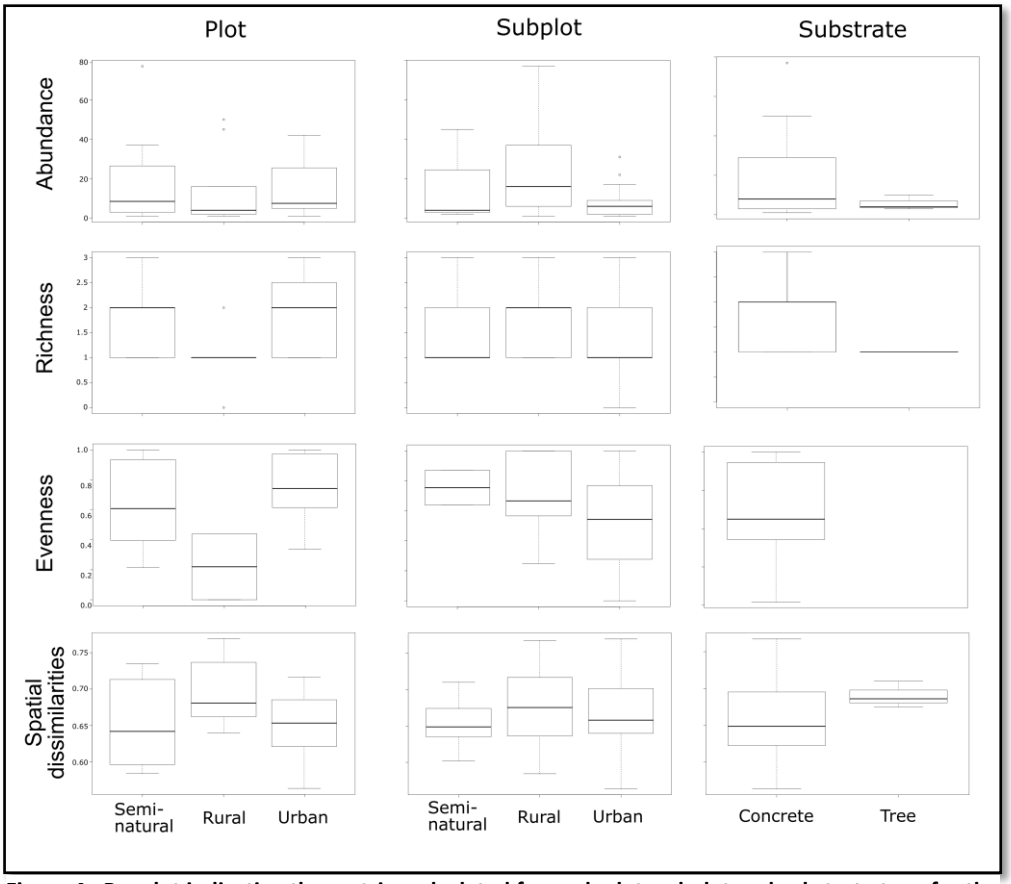

Figure 4 : Boxplot indicating the metrics calculated for each plot, subplot and substrate type for the genetically-based species determination of the focal genus *Adineta*. The total number of rotifer individuals sampled, the species richness, Pielou's index of evenness and community dissimilarities across space is for each patch collected is indicated.

Community_metric	Variable	F.stat	df	p.value
Individual sampled	Plot	0.136	2 and 30	0.873
	Subplot	2.030	2 and 30	0.149
	Substrate	0.627	2 and 30	0.541
Richness	Plot	5.368	2 and 30	0.010**
	Subplot	0.544	2 and 30	0.586
	Substrate	8.838	2 and 30	0.001***
Evenness	Plot	2.966	2 and 15	0.082
	Subplot	0.913	2 and 15	0.422
	Substrate	0.039	2 and 15	0.846
Spatial	Plot	2.425	2 and 30	0.106
	Subplot	0.176	2 and 30	0.839
	Substrate	0.592	2 and 30	0.559

Table 2 : Results of the ANOVA analyzing the response of each metrics to the plot, subplot and substrate type for the genetically based species delineation of the focal genus Adineta.

Even if the plot and subplot in which we sampled the patches did not seem to have an important impact on communities, the substrate type on which we collected each lichen patch significantly influenced the community structure. Indeed, we found less *Adineta* spp. on lichen collected on tree bark than on lichen extracted from concrete walls (ANOVA; F-stat = 8.838; df = 2, 30; p < 0.001). This was corroborated by the fact that

communities in which species 14 and 15 were rare or absent significantly correlated with patches collected from tree bark (Figure 6; Fisher's exact test p-value = 0.019).

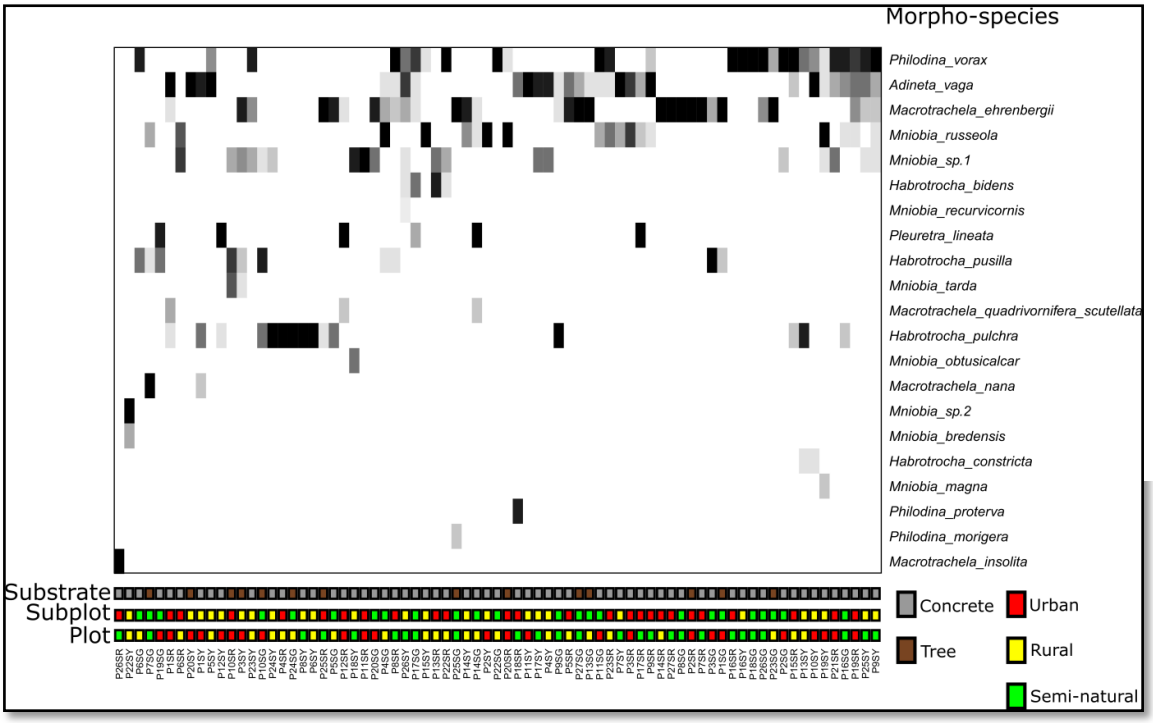

Figure 5: Clustered community matrix for the morphospecies dataset. The matrix was built from the presence/absence of each species in each non-empty community collected and the communities of each sample were then clustered hierarchically according to the species composition. Colored boxes indicate the plot, subplot or substrate type in which each plotted community was sampled.

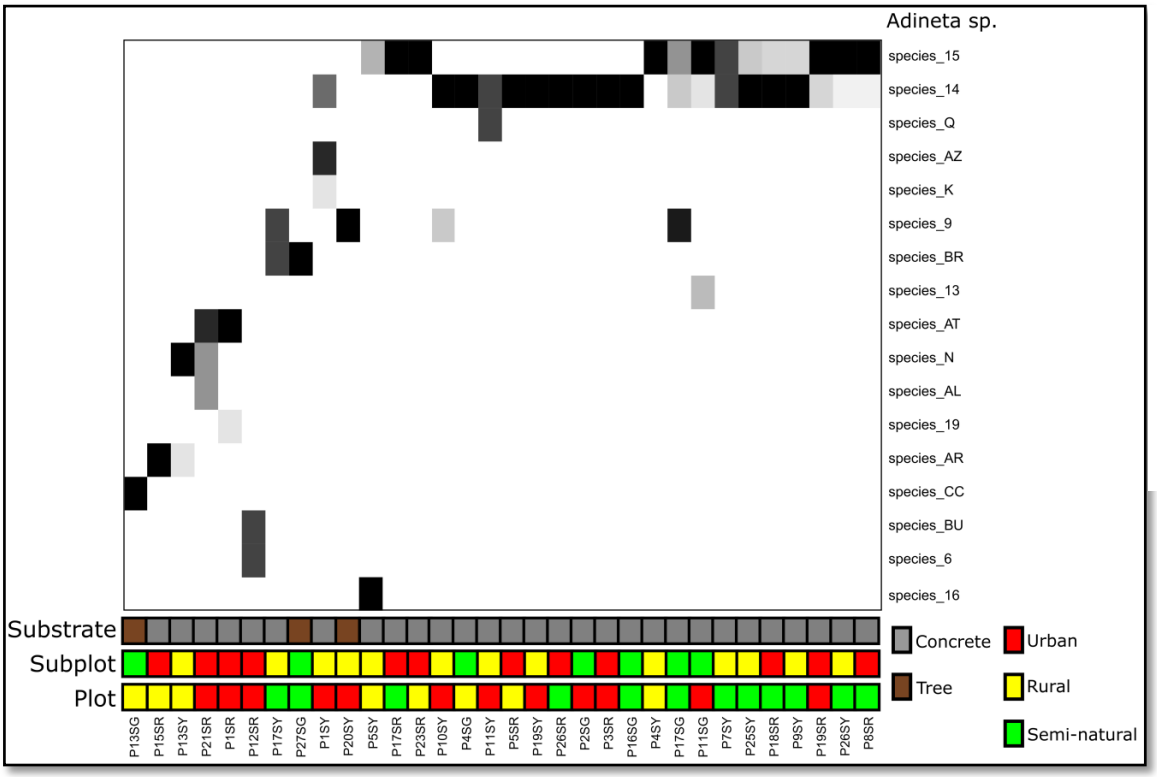

Figure 6: Clustered *Adineta* community matrix. The matrix was built from the presence/absence of each species in each non-empty community collected and the communities of each sample were then clustered hierarchically according to the species composition. Colored boxes indicate the plot, subplot or substrate type in which each plotted community was sampled.

We performed an AMOVA analysis on the individuals identified as *Adineta* since we got molecular data for a subset of those individuals. We grouped the lichen patches by plot type, subplot type, substrate type and geographical location and in each case, found sensitively similar values: FST

ranged from 27.0 to 28.3 (p-value = 0.0), FSC ranged from 66.0 to 84.7 (p-value = 0.0) and FCT ranged from -12.9 to 6.9 (p-value > 0.05) (Table 3).

Discussion

Absence of variation in community assemblages along the gradient of urbanization

Overall, the degree of habitat urbanization did not seem to influence bdelloid rotifers communities since none of the community structure parameters analyzed varied with the type of plot/subplot (semi-natural, rural or urban), except for richness in cryptic *Adineta vaga* Interestingly, even the AMOVA analyses suggested that plot and subplot type had little impact on the genetic diversity. Indeed, most of the genetic diversity (FSC: 66.0 – 84.7; p-value = 0.0) retrieved was explained when comparison among patches was performed no matter if they were grouped by plot or subplot type. This highlighted the fact the genetic diversity is rather explained by the presence of distinct species in the sampled lichens than the existence of specific preferences to plot/subplot type. Similarly, the differences in species composition between groups of plot/subplot type was low and non-significant indicating the lack of real structure along the gradient of habitat urbanization (FCT: -12.9-6.9; p-value > 0.05). Finally, the rest of the genetic diversity observed resulted from the diversity found within each individual patch (FST: 27-28.3; p-value = 0.0). This community structure was also suggested by the clustered species matrix (Figure 6) on which the most abundant species 14 and 15 were present in 71.9% of the patches harboring Adinetidae, independently of the plot/subplot type (Fisher's exact test: p-value = 0.724). Furthermore, the other *Adineta* species were often retrieved only once (species 6, 13, 19, K, Q, AL, AZ, BU and CC) or twice but in distinct plot/subplot type (*e.g.* species 9 and BR in rural and semi-natural subplots or species N and AR in urban and rural subplot).

Table 3 : Analysis of molecular variance (AMOVA) across communities regrouped by plot, subplot or substrate type.

Grouped.by	Source.of.variation	df	Sum.of.squares	Variance.components	Percentage.of.variation	Fixation.indices	p.value
Plots	Among groups (FCT)	2	687.60	1.48	6.48	0.060	0.073
	Between lichens within groups (FSC)	30	3662.09	15.05	66.03	0.760	0.000***
	Within lichens (FST)	241	1509.90	6.27	27.49	0.710	0.000***
Subplots	Among groups (FCT)	2	382.24	−0.51	−2.33	−0.020	0.549
	Between lichens within groups (FSC)	30	3967.44	16.37	74.01	0.720	0.000***
	Within lichens (FST)	241	1509.90	6.27	28.32	0.720	0.000***
Substrates	Among groups (FCT)	2	595.66	1.59	6.86	0.069	0.173
	Between lichens within groups (FSC)	30	3754.02	15.35	66.14	0.730	0.000***
	Within lichens (FST)	241	1509.90	6.27	27.00	0.710	0.000***
Regions	Among groups (FCT)	23	3435.11	−2.86	−12.85	−0.130	0.706
	Between lichens within groups (FSC)	9	914.56	18.84	84.70	0.720	0.000***
	Within lichens (FST)	241	1509.90	6.27	28.15	0.750	0.000***

Similarly, bdelloid communities did not seem to be impacted by habitat urbanization when morphological species were considered either. Six morpho-species (*Macrotrachela ehrenbergii*, *Adineta vaga*, *Philodina vorax*, *Mniobia russeola*, *Mniobia sp1* and *Habrotrocha pulchra*) were frequent throughout the plots/subplots sampled and abundant representing on average 68.81 individuals in the patches they colonized. The other morphospecies seemed to appear sporadically independently of the plot/subplot type. The ANOVA analyses did not present any significant correlation between plot/subplot type and any of the community metrics tested (number of individuals, species richness, Pielou's index of species evenness and community dissimilarities across space).

This lack of community structure could be the result of rare and random immigration from the metacommunity species pool. Bdelloids capacity to tolerate dry period by entering a dormant stage that turn into propagules for dispersal by wind may promote such random immigration. The fact to retrieve a few species that were already described in other Belgian areas and the five cases in which we found specimens that clustered with individuals from other countries underlined the possibility for medium to long range dispersal from the metacommunity pool (Figure 3). In addition, bdelloid rotifers seem to present significant but weak degree of habitat specificity which may result in low species-sorting processes (Fontaneto et al, 2011). Thus, the absence of barriers to dispersal and the low habitat specialization may explain why bdelloid rotifer show patchy distribution at local scale, unrelated to urbanization but wide distribution at the regional to global scale. Such explanation was already proposed by Fontaneto et al (2006) who observed that there was no hierarchical structure in local assemblages of bdelloid rotifers and diversity in a single Italian valley was surprisingly high with respect to the known worldwide diversity.

Ubiquitous morphospecies are complexes of habitat-specific cryptic species

Interestingly, there was a significant correlation between the presence of dominant *Adineta vaga* (species 14 and 15) and the absence of other rarer *Adineta* species. It is hard to determine if those rare species were able to colonize a few patches because of habitat specialization or just because dominant species were absent or at low density. However, this may either reveal that bdelloid could present ecological requirement that we were not able to describe or that habitat are harder to colonize once already monopolized by a given species. Thus, competition may play a role in species assemblages of this taxon. Inversely, we could not find any correlation between the presence of the six most frequent species and the absence of rarer species when we considered them at the morphological level. This could indicate that bdelloid species are actually complexes of cryptic species which have distinct habitat preferences but that those preferences are invisible when species are considered at the morphological level. Such hypothesis is corroborated by the description of multiple species complexes across bdelloid species (Ricci et al, 1989; Fontaneto et al, 2007; Fontaneto et al, 2008; Birky Jr et al, 2011; Debortoli et al, 2016).

It would make sense that competition among cryptic species is higher than competition between morphologically distinct species since feeding behavior and life cycle are similar if not identical within species complexes. This could explain why several morphospecies were often co-occuring within the same patches while *Adineta* species 14 and 15 seemed to limit the presence of other *Adineta vaga* or that both species 14 and 15 were abundant within a same patch.

Conclusions

According to our results, the degree of landscape urbanization does not seem to affect bdelloid rotifers communities. We could not detect habitat preferences in morphologically determined species. However, some cryptic species of the focal genus *Adineta* seemed to mutually exclude each other, eventually through a competition process.

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Chapter 6: Comparative genomics of distinct bdelloid families, how dynamic are bdelloid genomes?

In 2013, the first bdelloid genome has been published presenting features not compatible with conventional meiosis, *i.e.* pairing of homologous chromosomes (Flot et al, 2013). The peculiar genomic structure has since then been referred to as an evidence for the long-term asexuality of *Adineta vaga*, if not all other bdelloid species (Fontaneto and Barraclough, 2015; Debortoli et al, 2016). However, the clonal lineage used for the whole-genome sequencing (originally named AD008) was first isolated and cultured in lab conditions approximately thirty years ago by Claudia Ricci (Ricci, 1983) and may thus be a lab artifact. Here, we do no doubt the degenerate tetraploid structure as it had been observed in other bdelloid species (Hur et al, 2009), but rather the peculiarities that are not compatible with classical meiosis (collinearity breakpoints between allelic regions and palindromes, see introduction). Indeed, recent re-assemblies of the *A. vaga* genome involving multiple methods (PacBio reads were assembled with Falcon Unzip; Illumina® reads were assembled with BWISE and Illumina® reads obtained through contact genomics were assembled with GRAAL) enabled to recover fifteen scaffolds, ten of which have approximately the expected chromosomes sizes. Those ten scaffolds formed clear allelic pairs that extended to the whole length (allelic pair 1 to 5 on Figure 1). Interestingly, one additional scaffold was slightly smaller (dot chromosome) but appeared palindromic (allelic pair 6 on Figure 1). Finally, four shorter scaffolds could not

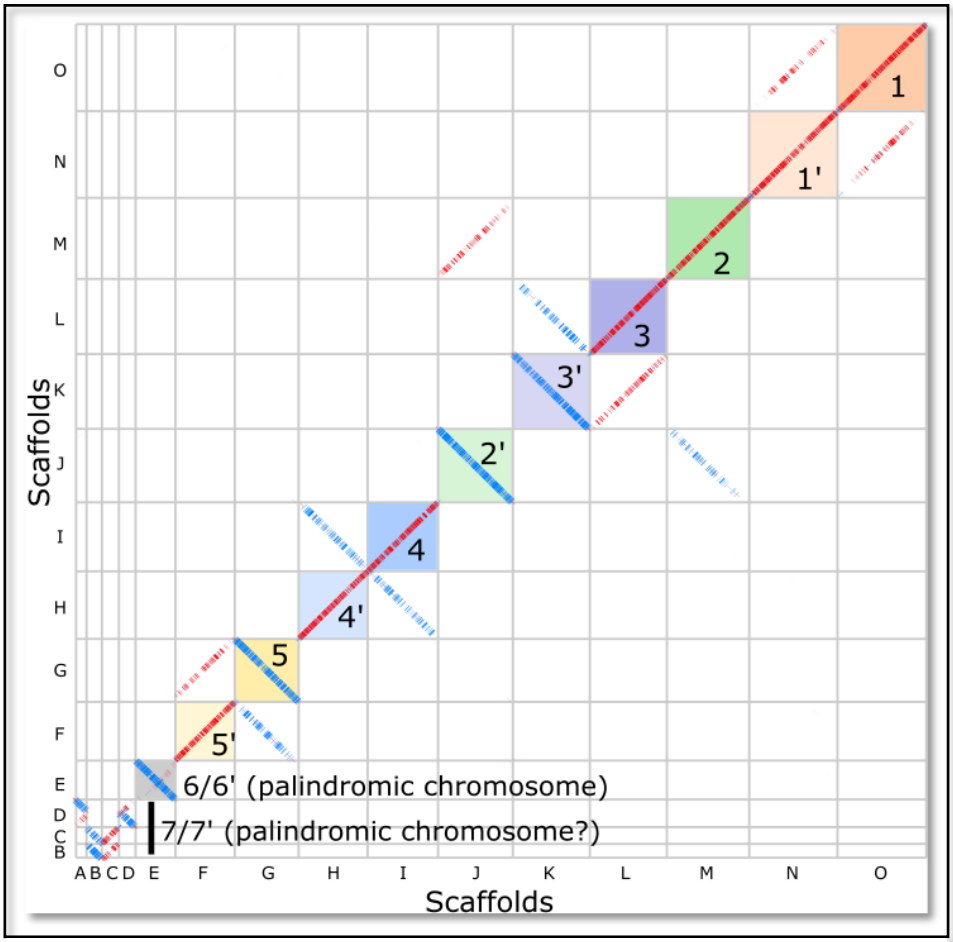

Figure 1 : Alignement of the fifteen scaffolds assembled (A-O) with themselves. The red and blue dots indicate sequence match (threshold of 2% divergence) in same and reverse orientation, respectively. Scaffolds F to O form clear allelic pairs (1 to 5), *e.g.* scaffold O perfectly aligns with itself and with scaffold N forming chromosome pairs 1/1'; similarly, scaffold K and L aligns perfectly together (forming pair 3/3') but are in different orientation.

be further assembled but may constitute the last chromosome which also presented palindromic regions (allelic pair 7 on Figure 1). Even though this new assembly seems to fit the observation that *A. vaga* karyotype was constituted of 12 chromosomes (J. Virgo, personal communication; Mark Welch *et al*, 2009) assembly of the fragmented scaffolds (A-D) and palindromes confirmation needs to be done.

Another question we wanted to address, if we confirm the genomic structure found in the reference genome, is whether those peculiarities were established early in the evolution of class Bdelloidea. To answer those questions, we isolated several individuals identified as Adinetidae, Habrotrochidae and Philodinidae from patches collected around Namur (Belgium) in September 2013. Each isolated individual was then cultured in separated Petri dishes until the clonal population (≈30 000 individuals *i.e.* adults, juveniles and eggs) was large enough for the extraction of approximately 1µg of genomic DNA (QIAGEN DNeasy kit). In addition, we received from Birky a few specimens of the fourth bdelloid family, Philodinavidae, but could not maintain them in lab cultures. We sequenced the whole genome of one *A. vaga* strain (named COI3B) that was closely related to the reference genome (Flot et al, 2013) and one Habrotrochidae in November 2015 (2x250bp Illimuna® reads, Genomics Core, UZ Leuven, Belgium). As our Philodinidae cultures grow slower, we did not gather enough genomic DNA for WGS yet.

We tested several assemblers (Platanus, Discovar De Novo, MIRA…) with the help of Marie Cariou and Jitendra Narayan (both LEGE members) and checked the output of each method carefully. Even though we could not assemble the genomes of the two strains sequenced yet, due to unsuitable software (Platanus and Discovar De Nova) or extensive computation time (MIRA), we spent a few months checking the outputs. It appeared that Platanus and Discovar De Novo could not phase the allelic regions appropriately, resulting in almost diploid genomes with low N50 unsuitable for comparative genomics. I wanted to highlight this part even though no interesting results were obtained yet as it represented a considerable amount of work (weekly cultures maintenance and assembly checks) during which I gathered a collection of about 300 isolates in total that are now used in distinct project of the LEGE lab (MIS, ERC, RADseq, optimization of BWise,…).

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