Chapter 10
Evidence for Ancient Horizontal Gene Acquisitions in Bdelloid Rotifers of the Genus *Adineta*

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Abstract Until recently, obligate asexuality was often considered an evolutionary dead end. However, recent advances suggest that conventional sexual reproduction, defined as the alternation of meiosis and fertilization, is not the only sustainable eukaryotic lifestyle. Moreover, different modes of asexual reproduction are observed in nature, raising the question of the diverse mechanisms responsible for the long-term survival and adaptation of strict asexuals. One possible way to study the molecular-genetic consequences of the loss of meiotic recombination is to scrutinize the genomes of asexuals of ancient and more recent origins. The first genome draft of an ancient asexual species, the bdelloid rotifer *Adineta vaga,* was recently made available, revealing a peculiar genomic structure in which allelic regions were massively rearranged and sometimes found on the same chromosome. Such genome organization devoid of homologous chromosomes appears incompatible with meiotic pairing and segregation, and represents therefore a compelling genomic signature of asexuality. Besides, the genome of *A. vaga* contains around 8 % of genes of apparent nonmetazoan origin, a percentage much higher than observed in most eukaryotes. Interestingly, a similar percentage of genes of nonmetazoan origin was independently inferred from a large-scale transcriptome analysis of the bdelloid rotifer *Adineta ricciae*. In this chapter, we conducted a comparative study between these two closely related species using reciprocal best blast hits, followed by functional annotation using the GOANNA pipeline. Around 10 % of all the orthologs identified between the two species were putatively acquired by horizontal gene transfer and lots of them were associated to hydrolases (18 %) and oxidoreductases (16 %) functions. We hypothesize that these acquisitions may have helped bdelloids to adapt to multiple food sources and

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to develop enhanced resistance to desiccation. Furthermore, comparisons with sequences available for the monogonont rotifer *Brachionus plicatilis* suggest that some nonmetazoan genes were acquired by rotifers before the separation of bdelloids and monogononts.

10.1 Introduction

Sexuality is considered the dominant mode of reproduction in the animal kingdom. The supremacy of sex among metazoans is usually explained by invoking its supposed long-term evolutionary advantages. Sex brings together genetic material from different individuals during fertilization, and shuffles it through genetic recombination during meiosis. This mixing provides novel allelic combinations that subsequently undergo selection, enhancing adaptation to the environment (Maynard Smith 1978; Lesbarrères 2011; Lodé 2011). Asexuals, on the contrary, lack meiosis and are therefore expected to exhibit less genetic diversity (hence lower adaptation rates). Moreover, genomes that do not experience sexual recombination have been described to fall prey to an irreversible, stochastic accumulation of deleterious mutations known as Muller's ratchet (Muller 1932; Felsenstein 1974). It is mainly because of these two reasons (low genetic diversity and accumulation of deleterious mutations) that obligate asexuality is often considered an "evolutionary dead end". Asexual populations of the model crustacean *Daphnia pulex* illustrate well the rapid evolutionary turnover of asexual animals. In *D. pulex*, obligate asexuality arises when a male transmits a dominant meiosis-suppressing allele to its offspring (Lynch et al. 2008). Interestingly, all current asexual populations of *D. pulex* bear "a meiosis-suppressing element" that originated only 1250 years ago, and the populations themselves appear even younger (Tucker et al. 2013). This suggests that asexual *Daphnia* populations do not survive in the long-term and appear doomed to extinction.

Nevertheless, some asexual metazoans have apparently been highly successful for millions of years: the so-called 'ancient asexuals'. The most notorious ones are darwinulid ostracods, oribatid mites, *Meloidogyne* root-knot nematodes (RKNs) and bdelloid rotifers (Danchin et al. 2011). The latter group, comprising more than 400 morphospecies, has apparently been able to diversify without sexual reproduction and was labeled for this reason by Maynard Smith as "something of an evolutionary scandal"(Maynard Smith 1986). Beside these asexual metazoans, many asexuals have also been inventoried in other eukaryotic groups, adding up to 20 % of all fungi for instance (Judson and Normark 1996; Seidl and Thomma 2014). These examples suggest that the alternation of meiosis and fertilization events is not the only sustainable eukaryotic life cycle. This implies the existence of alternative mechanisms that may prevent mutation accumulation and generate genetic diversity on the long term, thus making up for the absence of sex.

10.2 Genomic Insights into Ameiotic Evolution

One possible way to study the molecular-genetic consequences of the loss of meiotic recombination is to tap the genomes of asexuals, both of ancient and recent origins.

A comparative whole-genome analysis of sexual and asexual lineages of *D. pulex* was recently carried out (Tucker et al. 2013). This study, as mentioned in the introduction, indicates that asexual *D. pulex* lineages are much younger than sexual ones. Interestingly, heterozygosity does not seem to increase in asexual *Daphnia* as expected under the so-called Meselson effect, i.e., the accumulation of differences between alleles that do not experience recombination (Birky 1996). On the contrary, gene conversion and gene deletion events appear to be more common than point mutations. *Timema*, another interesting example, is a genus of plant-feeding insects among which at least five independently derived asexual lineages have been described (Schwander et al. 2011; Henry et al. 2012). The estimated ages of these asexual lineages range from 100,000 to over 1 million years. The occurrence of both sexual and asexual species in *Timema* makes it an ideal model to study the origin and evolution of asexuality over time. Comparing allelic variation between two *Timema* sister species revealed a higher level of nuclear allelic divergence in the asexual line (Schwander et al. 2011) consistent with the Meselson effect. Interestingly, in a few asexual *Timema* groups, only one allelic copy of each gene was found to accumulate deleterious mutations. Therefore, these asexuals appear to have the capability to make up for the functional loss of one copy from each of their genes (Schwander et al. 2011), thereby evolving toward haploidy. This trend is also visible at chromosome scale in one species (*Timema genevievae*) whose karyotype is devoid of pairs of homologous chromosomes (Schwander and Crespi 2009). The evolution towards haploidy observed in asexual *Timema* species contrasts with the hypothesis that polyploidy may be favored as it slows down Muller's ratchet: hence, studying several independent examples of asexuals appears necessary to understand all the possible genomic consequences of a switch from sexual towards asexual reproduction.

The genome sequences of sexual (*Meloidogyne hapla*) and asexual (*Meloidogyne incognita*) species of RKNs have provided a powerful system to improve our understanding of these consequences. A striking difference is that the size of the genomic assembly of *M. incognita* is 86 Mb, almost twice the haploid size suggested by flow cytometry (50 Mb), whereas for its sexual relative *M. hapla* the assembly size (54 Mb) agree with the haploid size estimate (Abad et al. 2008). This is because 64 % of the genome assembly of *M. incognita* appears to be made up of highly divergent allelic regions (with an average nucleotide divergence between two copies of 7 %) that were assembled separately (Castagnone-Sereno and Danchin 2014). Despite this separate assembly of allelic regions, there were no pairs of large scaffolds completely colinear to each other (Castagnone-Sereno and Danchin 2014). Such absence, first described in the bdelloid rotifer *A. vaga* (Flot et al. 2013; see below), is considered a signature of long-term ameiotic evolution, and is consistent with previous reports of a diversity of chromosomes numbers in *M. incognita* (Triantaphyllou 1981). Initially, the high level of nucleotide divergence observed in *M. incognita* was considered an instance of the Meselson effet (Castagnone-Sereno and Danchin 2014), which may result in alleles becoming functionally divergent (as paralogs do). However, subsequent phylogenetic analyses revealed that alleles did not cluster according to morphological species, suggesting that asexual RKN lineages are actually allopolyploids (Lunt 2008; Castagnone-Sereno and Danchin 2014).

Although the first bdelloid genome became available 5 years after *M. incognita*, it was this genome that provided the most thorough breakthrough in our understanding of the genomic peculiarities of asexual animals. Bdelloid rotifers comprise more than 460 morphospecies, among which no sign of sexuality has ever been observed: there are no male organs, and cytological studies by Hsu (1956a, b) on two distinct bdelloid species reported that oogenesis proceeds in a mitotic way, without meiotic pairing nor reduction in chromosome numbers. However, these observations were not sufficient to exclude that bdelloid rotifers engage in some rare, cryptic mode of sexual reproduction. Indeed, their sister group the monogononts are cyclical parthenogens that only produce males when the environmental conditions start to deteriorate. The recent publication of the genome draft of a bdelloid rotifer species, *A. vaga,* has brought strong new support to the hypothesis of their asexuality by revealing a peculiar genomic structure in which allelic regions are massively rearranged and sometimes found on the same chromosome, in most cases in a palindromic fashion (Flot et al. 2013). The absence of homologous chromosomes is incompatible with meiotic pairing (as mentioned above in the case of *M. incognita*), whereas the physical link between allelic regions co-occurring on the same chromosome precludes meiotic segregation: hence, it appears very unlikely that this bdelloid lineage is able to perform meiosis. The average divergence between allelic regions in *A. vaga* is 4.4 %, less then in *M. incognita*. Although relatively high among metazoans, this average heterozygosity fall within the upper range observed for sexually reproducing species (Leffler et al. 2012). About one fifth of the genes of *A. vaga* are present in four copies, indicating that it is an ancient tetraploid that has lost already many of its duplicated genes.

In addition to shedding light on the peculiar genome structure of *A. vaga*, the sequencing of the genome of this species also revealed genetic signatures of frequent gene conversion between homologous regions (Flot et al. 2013). The inferred lengths of the conversion tracks ranged from tens of bases up to hundred thousand bases (in which case the near-identical copies collapsed during genome assembly, resulting in regions of double coverage depth). Gene conversion was hypothesized to play an important role in the long-term survival of bdelloids in the absence of sexual recombination: first, because gene conversion slows down Muller's ratchet (Connallon and Clark 2010; Flot et al. 2013); and second, because gene conversion either exposes recessive deleterious mutations to selection by turning them into homozygous state or removes them by overwriting them with the other allele, in both cases decreasing the mutational load of the genome (Khakhlova and Bock 2006; Flot et al. 2013).

10.3 Mechanisms Enhancing Genomic Plasticity

The genomic data available on asexual animal lineages suggest several mechanisms that can enhance genomic plasticity in the absence of meiotic recombination. Both the genomes of *A. vaga* and *M. incognita* contain numerous synteny breakpoints (Flot et al. 2013; Castagnone-Sereno and Danchin 2014), suggesting a dynamic genome structure: such genomic plasticity may be one of the keys to understand how asexuals adapt to their environment. In the asexual phytopathogenic fungus *Verticillium dahliae* a variety of large genomic rearrangements have also been observed, in contrast with the low degree of genome-wide nucleotide diversity of this species (de Jonge et al. 2013). These rearrangements lead to the individualization of lineage-specific genomic regions enriched in genes involved in adaptation to the host plant, thereby increasing the virulence of this asexual fungus. These results are the first compelling evidence that genome rearrangements facilitate adaptation in asexual organisms, as was also hypothesized to be the case for some cancerous lineages (chromothripsis; cf. Stephens et al. 2011).

Transposable elements (TEs) have been proposed as important mediators of genomic plasticity (Seidl and Thomma 2014). TEs are genomic DNA segments that are able to move around a genome in a copy/paste or cut-and-paste fashion, inducing mutations and chromosomal rearrangements (Gladyshev and Arkhipova 2010). Multiple major chromosomal rearrangements induced by TEs have been described in plants (Zhang et al. 2011). In *Saccharomyces cerevisiae*, TEs have been described to induce rearrangements allowing fast adaptation to the environment (Crombach and Hogeweg 2007). Interestingly, variation in genomic structure through chromosomal rearrangements was associated to increased fitness during asexual growth of *Schizosaccharomyces pombe* (Avelar et al. 2012). In the asexual fungus *V. dahliae*, rearranged portions of chromosomes are also mostly flanked by retrotransposons and repetitive sequences (de Jonge et al. 2013). Comparative genomic analyses between sexual and asexual nematodes detected a higher proportion of TEs and repetitive sequences in *M. incognita* (Abad et al. 2008) than in its closest sexual relative *M. hapla* (36 vs. 12 %, respectively). Interestingly, a putative full-length Tm1 transposase has been detected in the genomes of the mitotic parthenogenetic species *M. javanica* and *M. incognita* (Gross and Williamson 2011). By contrast, no functional Tm1 transposase sequence was detected in *M. hapla*. These observations suggest that functional TEs are present in the genomes of asexual nematodes and may play a role in their genomic plasticity. However, the maintenance of a high fraction of TEs in an asexual genome also carries a risk of unrestrained genomic expansion that could cause the extinction of the lineage (Arkhipova and Meselson 2005). Indeed Arkhipova and Meselson (2005) hypothesized that sexual reproduction can limit the expansion of TEs through ectopic crossing-over and homologous recombination. In contrast, in the absence of sex, TEs are hypothesized to multiply indefinitely, leading to population extinction (Arkhipova and Meselson 2005). Ancient asexuals are therefore likely to possess genome defense mechanisms that prevent TE expansion. A recent comparison of TE content in sexual and asexual wasps suggests that reality is even more complex than the theoretical considerations above (Kraaijeveld et al. 2012). In this study, TE content was compared in a sexual lineage of the parasitoid wasp *Leptopilina clavipes* and in another lineage in which *Wolbachia* bacteria had induced parthenogenesis. Despite the presence of TEs in both sexual and asexual wasp lineages, there was no evidence of an overall increase in copy number for all TEs in the asexual populations. However, one group of TEs (the *gypsy*-like LTR elements) was overrepresented in the asexual lineage. This could be caused by the manipulation of the host genome by *Wolbachia* rather than by a direct impact of asexuality (Kraaijeveld et al. 2012). Only 3 % of the genome of the asexual bdelloid species *A. vaga* is made up of TEs, despite the high diversity of bdelloid TE families (255 in total), each of which is present in very low copy numbers (Flot et al. 2013). Moreover, most detected TEs appear to be recent arrivals and the protein families involved in the epigenetic silencing of TEs are substantially expanded in the genome of *A. vaga*, suggesting that incoming TEs are quickly reduced to silence (Flot et al. 2013; Arkhipova et al. 2013). Despite their low abundance, a role of TEs in promoting copy number variation in *A. vaga* has been suggested based on the observation of expanded gene families surrounded by TE footprints (Arkhipova et al. 2013).

In bdelloid rotifers, exposure to frequent desiccation events in their temporary habitats has been hypothesized to play a role in promoting structural genomic rearrangements and gene conversion. Bdelloids are highly adapted to semi-terrestrial environments: at any stage of their life cycle, when their habitat dries out, they can enter a metabolically quiescent state of desiccation for a prolonged period of time. Recently, it was demonstrated that desiccated *A. vaga* individuals accumulate DNA double-strand breaks (DSBs) through time and start repairing them once they become rehydrated (Hespeels et al. 2014). Interestingly, desiccation-induced DNA DSBs may promote gene conversion through mitotic recombination during DSB repair, which could in turn to prevent the accumulation of deleterious mutations in this asexual lineage (Flot et al. 2013). Moreover, the repair of DNA DSBs induced by desiccation may promote genome rearrangements and copy number variation. Copy number variation could be more frequent than point mutation, as in *C. elegans* where it is two orders of magnitude higher (Lipinski et al. 2014), and may therefore play an important role in generating genome variation. As a result of such duplications, some facultative asexual lineages of aphids and *Daphnia* present numerous duplications or even expansion of gene families, resulting in a number of gene more than twice the arthropod average (International Aphid Genomics Consortium 2010; Colbourne et al. 2011). Genes present in multiple copies can diverge and eventually acquire novel functions, enhancing in turn genomic plasticity (Castagnone-Sereno and Danchin 2014). Further studies on the evolutionary dynamics of gene conversion, gene duplications, and functional divergence in asexual lineages will be required to find out whether these mechanisms enhancing genomic plasticity may be sufficient to allow the adaptation and long-term persistence of asexual animals.

10.4 Horizontal Gene Transfers in Asexual Metazoans

Horizontal gene transfer (HGT) used to be considered relevant to prokaryotes only. Indeed, there seems to be some important barriers to HGT in eukaryotes. First, foreign DNA needs to pass both the eukaryotic cell membrane and the nuclear envelope. Second, metazoan germline cells are usually segregated from the rest of the body and not in contact with the outside world, reducing the likelihood that genes acquired from the environment are passed down to the next generation. Third, in contrast to prokaryotes where three distinct pathways of HGT have been well-characterized (conjugation, transformation, and transduction; Ochman et al. 2000), no general mechanisms allowing HGT has been described in eukaryotes.

The recent increases in analytical power and in the number of sequenced eukaryotic genomes have dramatically changed this view. Although only a few metazoans have been screened for HGTs, the percentage of genes of suspected HGT origin in their genome was consistently around 0.5–1 %, and much higher in a few species (Table 10.1). Most of these genes seem to be functional and encode proteins involved in metabolic processes. One of the sources of HGTs to eukaryotic organisms are their symbionts. For instance, the fruit fly *Drosophila ananassae* has integrated nearly the whole genome of its bacterial endosymbiont *Wolbachia* (living in close association with the host gonadic tissues): in total, 8 % of the genes of *D. ananassae* originated from *Wolbachia* (Hotopp et al. 2007). However, the functional significance of these integrated *Wolbachia* genes is not known. HGTs from *Wolbachia* genes into the genome of its host were also observed in other insects and nematodes known to be infected by this bacterial genus. Despite these examples, integration of symbiont DNA is not the only source of HGT: for instance, the aphid *Acyrthosiphon pisum* and the cnidarian *Hydra magnipapillata* harbor nonmetazoan genes that did not originate from their identified symbionts (Nikoh et al. 2010; Chapman et al. 2010).

Interestingly, numerous HGT events have been reported in nematodes. These foreign transfers have apparently played a key role in the acquisition of parasitic capacities by some nematode species. In *Bursaphelenchus xylophilus*, for instance, the transferred genes seem to be involved in adaptation to pine-tree parasitism (Kikuchi et al. 2011). *Pristionchus pacificus*, a self-fertilizing nematode, has a complex life cycle inside a beetle host: the first part of the larval development occurs when the host is alive, then development stops and resumes once the host is dead. The decaying body of the beetle becomes a complex ecosystem containing bacteria, fungi, and unicellular eukaryotes, providing ample sources for HGTs. Indeed, 2.1 % of the genes of *P. pacificus* are putative HGTs from diverse phyla. Interestingly, a strong enrichment in genes of apparent insect origin was reported, suggesting that the intimate physical contact between the nematode and its host promotes HGT. Like *A. vaga*, *P. pacificus* presents an enrichment in TEs in the genomic neighborhood of horizontally transferred genes (Rödelsperger and Sommer 2011). Multiple HGTs have also been reported in RKNs of the genus *Meloidogyne* and are suspected to have favored the emergence of plant parasitism

Table 10.1 List of metazoan species screened for horizontally transferred genes

Species name	%HGT	%TE	Discovery method	Reproductive mode	Reference
Acyrthosiphon pisum (pea aphid)	0.06	40.2	Phylogeny	Sexual/asexual	Nikoh et al. (2010); Moran and Jarvik (2010)
Bursaphelenchus xylophilus (nematode)	0.13	/	Phylogeny	Sexual	Kikuchi et al. (2011)
Bombyx mori (silkmoth)	0.29	45	Phylogeny	Sexual	Zhu et al. (2011)
Hydra magnipapillata (hydrozoan)	0.36	57	Phylogeny	Sexual/asexual	Chapman et al. (2010)
Drosophila melanogaster (fruit fly)	0.6	15	h_U	Sexual	Boschetti et al. (2012)
Ciona intestinalis (tunicate)	0.66	/	Phylogeny	Sexual	Ni et al. (2012)
Brachionus plicatilis (monogonont rotifer)	1.8	/	h_U	Sexual/asexual	Boschetti et al. (2012)
Caenorhabditis elegans (nematode)	1.8	12	h_U	Sexual/asexual	Boschetti et al. (2012)
Pristionchus pacificus (nematode)	2.10	<15 %	Codon usage, phylogeny	Self-fertilization	Rödelsperger and Sommer (2011)
Meloidogyne incognita (nematode)	3.19	6.3	Phylogeny	Asexual	Paganini et al. (2012)
Adineta vaga (bdelloid rotifer)	8	3	AI	Ancient asexual	Flot et al. (2013)
Adineta ricciae (bdelloid rotifer)	9.6	/	h_U	Ancient asexual	Boschetti et al. (2012)
Drosophila ananassae (fruit fly)	8	/	PCR	Sexual	Hotopp et al. (2007)

Most studies used phylogenies to uncover HGT: in this approach, horizontally acquired genes are detected because they show up in a different, unexpected part of the phylogeny. Other commonly used methods include the HGT index h_U, equal to the difference between the highest nonmetazoan and the highest metazoan bitscores (Boschetti et al. 2012), and the “alien index” (AI), computed as the log-ratio of the BLAST E-values for the best metazoan hit and the best nonmetazoan hit for a given gene (Gladyshev et al. 2008). In the fruit fly *D. ananassae*, *Wolbachia* sequences were detected by direct PCR amplification (Hotopp et al. 2007) within this genus (Danchin et al. 2010; Haegeman et al. 2011). HGTs are found in both sexual and asexual *Meloidogyne* species but the most virulent RKNs are the asexual ones (*M. incognita*, *M. javanica,* and *M. arenaria*, together responsible for 90 % of worldwide agricultural plant damage). A majority of the HGTs in *M. incognita* are enzymes involved in the degradation or modification of plant cell walls and were acquired from bacteria sharing the same ecological niche as RKNs. Compared with other genes, HGTs in *M. incognita* are characterized by their higher degree of autonomy, i.e., they do not require synergy with other genes to yield a function and are therefore more easily transferable horizontally than genes involved for instance in the regulation of metabolic processes. In addition, many of the genes acquired horizontally apparently experienced duplication after their acquisition. This process of gene duplications facilitates the emergence of multigene families where each copy can yield a better or divergent function through neo-functionalization (Paganini et al. 2012).

In bdelloid rotifers, a first transcriptomic study of the species *A. ricciae* submitted to desiccation revealed that around 10 % of the genes expressed under hydrated or desiccated conditions were potentially acquired by HGT; by contrast, in monogonont rotifers, the percentage of HGT is only 1.8 % (Table 10.1) (Boschetti et al. 2012). Most of these acquired sequences (80 %) appear to be involved in enzymatic reactions such as toxin degradation and antioxidant production. Similarly to *M. incognita*, these data are consistent with the hypothesis that genes that can act independently are more easily transferred and/or retained in the recipient genome than genes integrated in a pathway and requiring other proteins to function. Multiple independent acquisitions from up to 533 distinct source organisms (bacteria, fungi, plants,…) are suspected to have happened in *A. ricciae* (Boschetti et al. 2012). A similar level of abundance of HGTs was observed in the genome of *A. vaga*, where an estimated 8 % of the geneset present strong signatures of nonmetazoan origin. As 20 % of these foreign genes are present in quartets, it was hypothesized that these transfers occurred before the establishment of tetraploidy and therefore before the separation of the bdelloid families (Hur et al. 2009; Flot et al. 2013). However, 60 hypothetical genes acquired horizontally in *A. vaga* had no intron, were present in one copy, and presented a GC content markedly different from the genome average, suggesting that they were acquired quite recently (Flot et al. 2013). Hence it appears that horizontal gene acquisition in bdelloid rotifers is still ongoing.

The availability of both a transcriptomic dataset of *A. ricciae* and a genome draft of *A. vaga* allowed us to use for the first time a comparative analysis to answer some key questions related to HGT in bdelloids. How many genes are shared by both species? What are their functions? How many orthologs shared between the two species were potentially acquired by HGT prior to their divergence? Can they inform us about past events in the evolutionary history of the genus *Adineta* and of rotifers in general?

10.5 Looking for Orthologous Genes Between *A. vaga* and *A. ricciae*: Genomic Evidence for Ancient Horizontal Gene Transfers in Bdelloids

Orthologs, a special class of homologs, are defined as genes originating in a common ancestor species and having evolved independently in different species following speciation. Orthologs usually share similar functions, in contrast to paralogs (homologs originating from a gene duplication event) that often diverge in function following duplication (Fitch 1970; see Koonin 2005 for review). Here, we focus our comparative analysis on orthologs of *A. vaca* and *A. ricciae* of putative nonmetazoan origin, in order to determine whether HGT acquisition occurred before or after the separation of these two lineages and how these genes became apparently "domesticated" in bdelloids.

The bdelloid genus *Adineta* is composed of 14 morphological species (Segers et al. 2007). However, molecular data suggest that cryptic species are frequent within this genus (Fontaneto et al. 2009, 2010). Here, we compared the draft genome of one lineage of the morphospecies *A. vaga* originating from Matthew Meselson's laboratory at Harvard University (Flot et al. 2013) with cDNA libraries from *A. ricciae* (Boschetti et al. 2012). The latter species, discovered in a dry pond in Australia, presents a morphology close to *A. vaga* with a few differences: *A. ricciae* has eyes in frontal position that are absent in *A. vaga* and the two species have different numbers of mastax teeth (Segers and Shiel 2005). Both species were selected for high-throughput sequencing because they are easy to culture in vitro (in contrast to most bdelloids collected from the wild) and are therefore amenable to molecular biology analyses requiring thousands of individuals.

Reciprocal best blast hits (RBBHs), also known as best bidirectional hits (Hulsen et al. 2006), provide a simple and reliable method to detect orthologs between two species (Altenhoff and Dessimoz 2009). This approach considers that two genes are orthologous if they are each other's best hit when aligning the geneset of one species against the one of the other and vice versa (Moreno-Hagelsieb and Latimer 2008). The RBBH compares well in term of accuracy with more complex approaches (Tekaia and Yeramian 2012; Altenhoff and Dessimoz 2009). Indeed, RBBH results are characterized by low rates of false positives. This method was therefore adopted here to find orthologs between the fully annotated genome of *A. vaga* and the partial sequences from the *A. ricciae* transcriptome.

We used the complete geneset of *A. vaga (*49,300 predicted genes) and 28,965 partial cDNA sequences obtained from hydrated and desiccated *A. ricciae* retrieved from Genbank. BLASTALL (TBLASTX with an E-value cutoff of 10^{-10}) was used as an alignment algorithm, yielding detection of 10,506 orthologs in total between *A. vaga* and *A. ricciae*. In order to detect orthologs of likely nonmetazoan origin, we used the Alien Index (AI) approach of Gladyshev and Meselson (2008) with the threshold set to 45 as in Flot et al. (2013). We found 955 orthologs with AI $\geq$ 45 and therefore considered them as putative HGT candidates shared by both *Adineta* species. The presence of 10 % shared foreign genes between *A. ricciae* and *A. vaga* suggests that numerous HGTs happened already before these two lineages separated (unless some of them were acquired first by one lineage, then transferred horizontally to the other).

The genome of *A. vaga* is an ancient tetraploid in which 40 % of the genome is still organized in quartets of four homologous regions A1, A2, B1, and B2 (with A1-A2 and B1-B2 being allelic pairs and As being homologous to Bs; Flot et al. 2013). The nucleotide divergence in an allelic pair is low (average 3.8 %) whereas it is much higher in an ohnologous pair (average 26.4 %) (Flot et al. 2013). Interestingly, 47 orthologs with AI $\geq$ 45 were present in 4 copies, suggesting that HGT was already occurring before tetraploidy became established in bdelloids. When aligning (using BLASTX with a similarity threshold of 10^{-10}) these 47 orthologous genes against the transcriptome of the monogonont rotifer *B. plicatilis* (52,772 EST sequences available in GenBank; Denekamp et al. 2009; Suga et al. 2007), we found 3 hits (FM924334.1; FM931385.1; FM908274.1) suggesting that 3 genes had potentially been acquired by HGT in rotifers even before the separation of the bdelloid and monogonont clades. This hypothesis was confirmed when aligning these sequences against the whole GenBank database since the top five hits for each of them were bacteria or fungi (Table 10.2). The acquisition of foreign DNA by rotifers may therefore have started at the dawn of their evolutionary history.

After their integration in an eukaryotic genome, horizontally transferred genes of bacterial origin frequently acquire introns. Orthologs with AI $<$ 45 were characterized by a high proportion of introns: 93 % of them contained at least one intron and 57 % had more than three introns (Fig. 10.1). On the contrary, 32 % of the genes with AI $\geq$ 45 did not contain any intron and 26 % contained only 1 intron (Fig. 10.1). Out of the 47 orthologs with AI $\geq$ 45 present in 4 copies, only 7 did not contain any intron. These results suggest that intron acquisition is comparatively slower in bdelloids than in *M. incognita*, in which all putative HGT genes contained at least 1 intron.

10.6 Origins and Functions of Ancient Bdelloid HGTs: Emerging Results

A first functional analysis of the genes shared by *A. vaga* and *A. ricciae* was performed using the GOANNA pipeline (McCarthy et al. 2006). Since only partial sequences of *A. ricciae* were available, all subsequent analyses were done on the geneset of *A. vaga*. Briefly, orthologs were aligned using default parameters against the Swissprot database of manually curated protein sequences (comprising on 01/04/2014 542,258 sequences with 6,957,756 annotations). Each ortholog was attributed the gene ontologies (GO) of its top three hits in Swissprot. At the end of this process, 95 % of the orthologs with AI $<$ 45 and 86 % of the orthologs with AI $\geq$ 45 had received at least one GO term. To reduce the information content and gain a broader overview, each GO was replaced with its parent term in the PIR

Table 10.2 Top 5 best BLAST hits (using BLASTX) of 3 *B. plicatilis* EST homologous to *A. vaga/A. ricciae* sequences putatively acquired by HGT

Best BLASTX hits	E-value	%Identity	Taxonomic origin
FM931385.1			
Lactate dehydrogenase-like oxidoreductase (*Alistipes* sp. CAG:29)	2×10^{-46}	53	Bacteria
2-Hydroxyacid dehydrogenase (*Alistipes onderdonkii*)	2×10^{-46}	53	Bacteria
Lactate dehydrogenase-like oxidoreductase (*Alistipes finegoldi*i CAG:68)	8×10^{-46}	51	Bacteria
2-Hydroxyacid dehydrogenase (*Bacteroidetes bacterium* oral taxon 272)	1×10^{-45}	53	Bacteria
2-Hydroxyacid dehydrogenase (*Bacteroides coprosuis*)	1×10^{-45}	51	Bacteria
FM908274.1			
Trp repressor binding protein (*Mucor circinelloides* f. *circinelloides* 1006PhL)	4×10^{-68}	58	Fungi
NAD(P)H:quinone oxidoreductase, type IV (*Mucor circinelloides* f. *circinelloides* 1006PhL)	$7x10^{-68}$	60	Fungi
Hypothetical protein RO3G_01460 (*Rhizopus delemar* RA 99-880)	$5x10^{-66}$	55	Fungi
NAD(P)H:quinone oxidoreductase, type IV (*Rhizopus delemar* RA 99–880)	$3x10^{-65}$	58	Fungi
NAD(P)H:quinone oxidoreductase, type IV (*Mucor circinelloides* f. *circinelloides* 1006PhL)	$4x10^{-65}$	56	Fungi
FM924334.1			
Sodium/potassium-transporting ATPase subunit alpha (*Mucor circinelloides* f. *circinelloides* 1006PhL)	$2x10^{-53}$	46	Fungi
Hypothetical protein MYCFIDRAFT_163458 (*Pseudocercospora fijiensis* CIRAD86)	$2x10^{-49}$	41	Fungi
Hypothetical protein PFL1_01803 (*Pseudozyma flocculosa* PF-1)	$3x10^{-49}$	44	Fungi
Hypothetical protein PPL_00295 (*Polysphondylium pallidum* PN500)	$4x10^{-49}$	44	Fungi
Hypothetical protein BC1G_04401 (*Botryotinia fuckeliana* B05.10)	$1x10^{-48}$	41	Fungi

slim ontology using GOSlimViewer from AgBase (McCarthy et al. 2006). The ten most abundant functions associated to orthologous genes with AI ≥ 45 were compared with their respective abundance in orthologous genes with AI < 45 (Fig. 10.2). Interestingly, orthologs with AI ≥ 45 were enriched in putative oxidoreductase genes (+9 %) and hydrolases (+6 %). By contrast, protein-binding functions were underrepresented among orthologs with AI ≥ 45 compared with orthologs with AI < 45 (15 % vs. 7 %). Protein-binding genes are involved in selective and noncovalent interactions with proteins or protein complexes (GO:0045308). These observations are in agreement with the previous observation that genes acquired horizontally and remaining functional in the new host are more often involved in specific enzymatic reactions than in regulatory processes and complex protein-protein interactions (Boschetti et al. 2012).

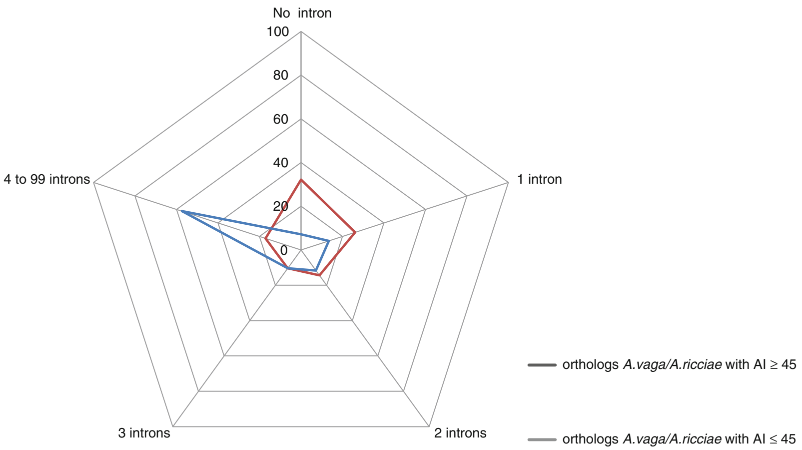

Fig. 10.1 *Distribution of the number of introns found in A. vaga/A. ricciae orthologs*. Introns were annotated in the *A. vaga* geneset by mapping RNAseq data (see Flot et al. 2013). Numbers represent percentages of orthologs

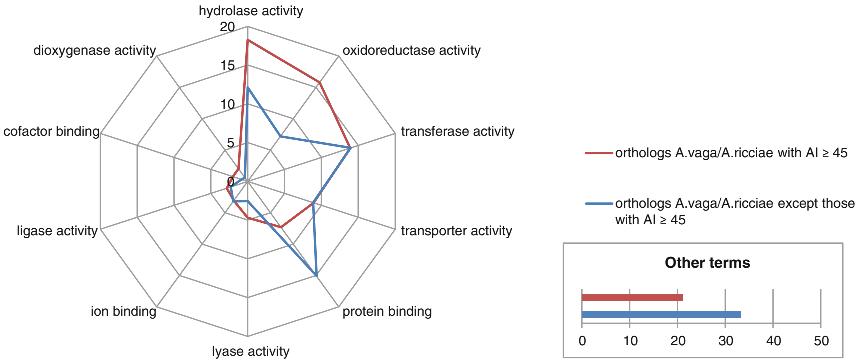

Fig. 10.2 *Top 10 most abundant GOSlim descriptions of the molecular functions of orthologs with AI ≥ 45 and with AI < 45*. Numbers represent percentages of orthologs. The inlet "other terms" represents the percentage of rare GOSlim terms not shown in the main graph

The abundance of hydrolase domains among genes of putative nonmetazoan origin can be explained by the high abundance of CAZymes (carbohydrate-related enzymes) in the genome of *A. vaga*, including a large number of glycoside hydrolases (Flot et al. 2013). At total of 299 orthologous CAZymes were found in both *A. vaga* and *A. ricciae*, including GH (glycoside hydrolases), GT (glycosyl transferases), PL (polysaccharide lyases), CE (carbohydrate esterases), and CBM (carbohydrate-binding modules). Of these 299 CAZymes shared between the two species, 99 appear to have been acquired through HGT (AI ≥ 45). Carbohydrates are a widely distributed source of energy and carbon. Therefore, the integration of

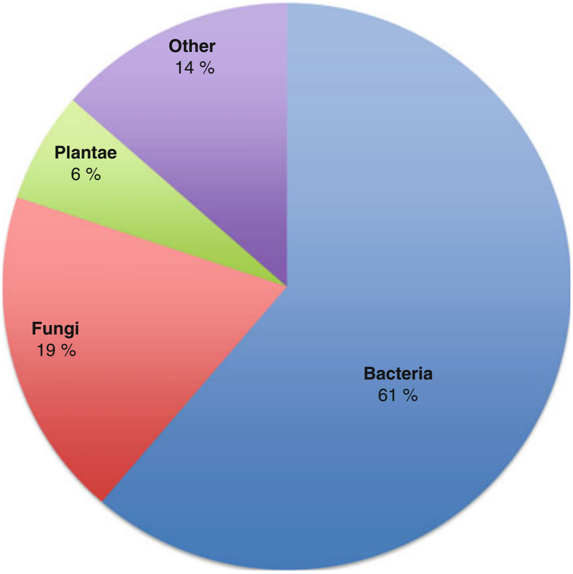

Fig. 10.3 *Pie chart of the putative donor groups of the horizontally acquired genes shared by A. ricciae and A. vaga*. Numbers represent percentages of orthologs. The most abundant donor group is bacteria, followed by fungi and plants. The donor group for each sequence was determined by reference to its best BLAST hit (smallest E-value) in GenBank

horizontally acquired CAZyme genes in bdelloids might have opened them access to new ecological niches by contributing to their adaptation to multiple food sources, as already observed in the case of human gut microbionts (Hehemann et al. 2012).

Oxidoreductases are involved in protection against oxidative damage. Several enzymes able to detoxify toxic free radical molecules were found among the *A. vaga/A. ricciae* orthologs, including nitric oxide dioxygenase, nitrilotriacetate monooxygenase, and nitroalkane oxidase. We screened orthologs for key antioxidant genes previously annotated in *A. vaga* (Flot et al. 2013): 30 % of these genes were found to have orthologs in the *A. ricciae* transcriptome. The 10 antioxidant gene families previously annotated were shared between the two *Adineta* species. In total, 19 % of the orthologs from three gene families were of putative non-metazoan origin: GST (glutathione S-transferase); GR (glutathione reductase) and AKR (aldo-keto reductase). By manual screening, we detected 6 orthologs with AI $\geq$ 45 that were putative homologs of trypanothione synthase, a key antioxidant in Kinetoplastida highlighted in Boschetti et al.'s transcriptome study. Several orthologs of three families of the antioxidant methionine sulfoxide reductase (MSR) were also found. As in *A. ricciae* (Boschetti et al. 2012), we were not able to detect any metazoan homolog of MSR in *A. vaga*. The metazoan homolog of another oxidoreductase acquired by HGT, stearoyl-CoA desaturase, was also absent in *A. ricciae* (Boschetti et al. 2012) and in *A. vaga*. This confirms the previous suggestion that HGTs can complement the absence of the corresponding metazoan gene (Boschetti et al. 2012). By contrast, screening the *B. plicatilis* transcriptome revealed the presence of a stearoyl-CoA desaturase expressed sequence (FM934536.1) of apparent metazoan origin, indicating that the replacement of the metazoan stearoyl-CoA desaturase by a horizontally acquired homolog occurred in *Adineta* spp. after the divergence from monogononts but before the separation of the two species sequenced. In the case of MSR, each homologous sequence found in *B. plicatilis* was of apparent nonmetazoan origin as no metazoan hit was found in GenBank (using TBLASTX/BLASTX with a threshold of 10^{-10}). These observations support the hypothesis that the replacement of MSR by a non-metazoan homolog occurred in rotifers even before the separation of bdelloids and monogononts.

As a last analysis, a putative origin was assigned to each ortholog with AI $\geq$ 45 based on its best hit in GenBank. We found that 61 % were of apparent bacterial origin, 19 % of fungal origin, and 6 % from plants. Other orthologs were of diverse origins (Fig. 10.3). This predominantly bacterial origin of HGTs in rotifers had already been suggested in previous studies of *Adineta* (Boschetti et al. 2012; Flot et al. 2013).

10.7 Conclusions

In this chapter, the first comparison between the genes of two bdelloid species was performed to search for ancient acquisitions of nonmetazoan genes. The RBBH approach identified 10,506 orthologs between the partial transcriptome of *A. ricciae* and the predicted geneset of *A. vaga*. Among these orthologs, 9 % had an AI $\geq$ 45 and were therefore likely horizontal acquisitions. The presence of these genes in both species suggests that they were acquired prior to the divergence of *A. vaga* and *A. ricciae*. Furthermore, some of these genes of nonmetazoan origin are present in four copies in the genome of *A. vaga*, suggesting that HGTs were already happening before the tetraploidization event shared by all extant bdelloids. It is therefore highly probable that these genes will also be detected in other, more distantly related bdelloid species. Furthermore, we screened the transcriptomic data available for the monogonont *B. plicatilis* (a cyclical parthenogen) and found homologs to several bdelloid HGT candidates. This result supports the hypothesis that the acquisition of foreign genes predates the loss of sex in bdelloid rotifers. However, only 1.8 % of the genes of *B. plicatilis* seem to be of nonmetazoan origin (Boschetti et al. 2012): this is much lower than the 8–10 % reported in *A. vaga* and *A. ricciae*, suggesting that horizontal gene acquisitions are more frequent in rotifers that lack sex than in cyclical parthenogens (who do perform sex occasionally).

As mentioned previously, HGTs have been described in multiple metazoans and no consensus exists about how this foreign genetic material is acquired. Indeed, we speculate that these events are highly specific to the lifestyle of each organism. For example, most HGTs of *M. incognita* seem to have originated from bacteria living in the rhizosphere, in close proximity with this parasitic nematode (Castagnone-Sereno and Danchin 2014). Living in temporary habitats, bdelloids typically undergo multiple cycles of desiccation during their life as they are able to withstand desiccation at any developmental stage. Adult monogononts, on the contrary, lack such ability but produce specific resting eggs during their sexual cycle that are protected against desiccation. Given that the genome of dehydrated bdelloids becomes shattered into multiple fragments that are subsequently repaired after rehydration (Hespeels et al. 2014), the frequent desiccation cycles experienced by bdelloids could facilitate their integration of foreign genes from the environment (Gladyshev and Meselson 2008; Hespeels et al. 2014). If such a mechanism at cellular level looks plausible, how extraneous DNA finds its way into the bdelloid germ line remains unclear. One possibility could be that foreign DNA present in the intestinal tract of bdelloids diffuses into the adjacent ovaries (the "you are what you eat" hypothesis; Doolittle 1998; Castagnone-Sereno and Danchin 2014) or perhaps even diffuses directly from the environment (Overballe-Petersen et al. 2013). Finally, how genes acquired from bacteria become associated with an eukaryotic promoter region (a prerequisite for expression) remains an intriguing question for which no satisfying hypothesis has been proposed yet.

As theoretically predicted and experimentally confirmed in our and previous studies, successful lateral gene transfers are more frequent for genes involved in metabolic processes than for those involved in DNA replication, transcription and translation (Boschetti et al. 2012; Whitaker et al. 2009; Jain et al. 1999). Indeed, a significant proportion of the shared HGTs of *A. vaga* and *A. ricciae* are involved in sugar metabolism, in antioxidant production and in detoxification, three processes essential to survive desiccation. The acquisition of these genes could therefore have been important events enhancing the desiccation resistance of bdelloids. This is in accordance with the hypothesis that HGT accelerates evolution and adaptation by extending the metabolic capabilities of the organisms, by improving their resistance to stress or by increasing their parasitic ability (Gogarten et al. 2002).

It has been hypothesized that, in the absence of meiosis and recombination, bdelloids and bacteria accumulate deleterious mutations. However, recent data from prokaryotes suggest that HGTs—known to occur frequently in bacteria and in bdelloids—can prevent Muller's ratchet from clicking by restoring genes inactivated by mutation and may therefore be an important prerequisite for the long-term maintenance of prokaryotic lineages (Takeuchi et al. 2014). A similar evolutionary role could be hypothesized for HGT in bdelloids. This is supported by the observation that the metazoan stearoyl-CoA desaturase has been replaced by its HGT equivalent in two *Adineta species*. (whereas the metazoan gene is still present in the monogonont *B. plicatilis*). Interestingly, the replacement of methionine sulfoxide reductase by its HGT homolog appears to have taken place in the common ancestor of bdelloids and monogononts prior to their divergence; however, additional phylogenetic studies are needed to confirm this hypothesis. Therefore, HGT could have a double role in bdelloids: first, speeding up evolution and adaptation, and second, counteracting Muller's rachet (with the help of gene conversion; cf. Flot et al. 2013).

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