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Current Biology

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

Highlights

- Bdelloid individuals of the genus *Adineta* exchange DNA within and between species
- Signatures of exchanges are scattered among genomic regions evolving asexually
- Such patchwork pattern is better explained by horizontal gene transfer than by sex

Authors

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In Brief

Debortoli et al. analyze patterns of allele sharing to delineate cryptic species in the bdelloid rotifer *Adineta vaga*. They find evidence of inter- and intraspecific genetic exchanges interspersed with chromosome regions bearing signatures of asexual evolution, suggesting that bdelloids exchange DNA horizontally rather than via meiotic sex.

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

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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 genetic exchanges scattered among regions evolving asexually. Our findings suggest that bdelloids evolve asexually but exchange DNA horizontally both within and between species.

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 and diversification 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]. Consistently 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, which are 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

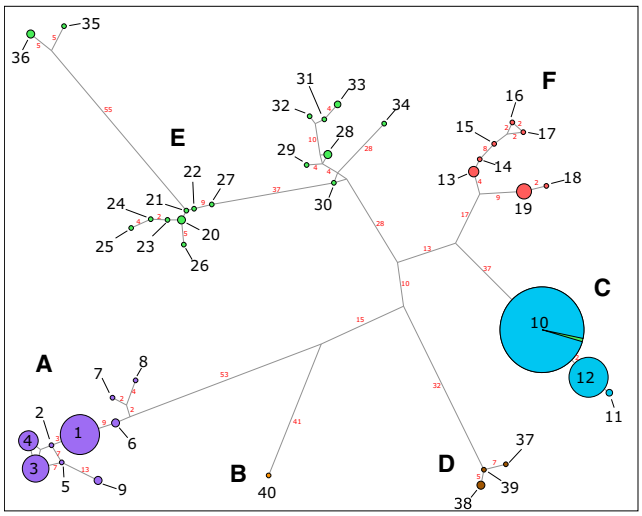

Figure 1. Median-Joining Haplotype Network of the COI Sequences Obtained from the 576 *A. vaga* Individuals Collected

The frequencies of the 40 COI haplotypes identified are proportional to the circle size. Each individual is colored 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 E according to the conspecificity matrix (Figure 2) harbored a COI sequence (haplotype 10) attributable to species C.

alpha and beta gametes can cross, heterozygosity is maintained within the population (hence the term "permanent translocation heterozygosity" [PTH]). The consequence of this atypical meiosis is that chromosomes are non-recombining along most of their length, and the extremities 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 occurs predominantly by selfing, although hybridization between PTH forms may occur (resulting in new PTH forms akin to species) [12–14]. In their study of bdelloid rotifers of the genus *Macrotrachela*, Signorovitch et al. [11] observed matching allele-sharing patterns among three bdelloid isolates at four genomic loci (ranging in size from 2.8 to 9.7 kb) and hypothesized that a 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.

To examine whether the observation of Signorovitch et al. [11] 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. 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 along 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 631-bp portion of the mitochondrial cytochrome *c* oxidase 1 gene (COI) was successfully sequenced in all these 576 individuals, yielding a total of 40 distinct haplotypes (Figure 1). Eighty-two individuals representative of the different COI haplotypes and distinct sampled patches were selected for further sequencing of four independent nuclear markers: 28S, EPIC25, EPIC63, and Nu1054. The first of these markers was successfully sequenced for all 82 individuals, whereas amplification and/or sequencing of the three others failed for 3, 1, and 17 individuals, respectively. Haploweb analyses of each nuclear marker yielded six (28S), 14 (Nu1054), 15 (EPIC63), and 18 (EPIC25) fields for recombination (FFRs), i.e., putative species (see Figure 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 along the diagonal; each of the blocks corresponded to a group of individuals sharing common allele pools for their different markers, i.e., a species according to the criterion of mutual allelic exclusivity (labeled A–F in Figure 2) [16]. As an independent confirmation using a different, tree-based approach, we applied the generalized mixed Yule-coalescent model [18–20] on the COI dataset, an approach used in previous studies of bdelloid rotifers [1, 3] to identify independently evolving lineages akin to species. The result (Figure S2) was identical: the same six species A–F were delineated as with the conspecificity matrix approach. The COI network and the haplowebs of each nuclear marker were colored according to the delimitation obtained from the conspecificity matrix, revealing either perfect (28S) or high congruence (COI, Nu1054, EPIC63, EPIC25) with the six molecularly defined species (Figures 1 and 3). All the nuclear genotypes of the 12 individuals harboring the COI haplotype 12 (Figure 1) were identical (aside from one individual harboring a single SNP on the 28S marker), which was consistent with a clonal mode of reproduction.

Interspecific DNA Transfers

Six individuals (Ind1, 5, 21, 23, 58, and 66) were assigned to distinct species according to different markers, as indicated by the off-diagonal framed rectangles on the conspecificity matrix (Figure 2). The species assignment of each individual according to each genetic marker is summarized on Figure 4: in this figure, the 82 individuals analyzed are connected to the genetic sequence retrieved for each of the five markers; both the individuals and their sequences are color-coded as in the haplowebs, and the species are labeled A–F. In total, 76 individuals were assigned to a single species (subdued links) congruently by all markers. By contrast, five individuals (Ind5, Ind21, Ind23, Ind58, and Ind66) had genetic markers attributed to two distinct species (bright colors), e.g., Ind5 had COI, 28S, EPIC63, and Nu1054 sequences from species A but harbored EPIC25 sequences from species C. These five individuals were homozygous at the loci that were incongruent. In addition, one individual

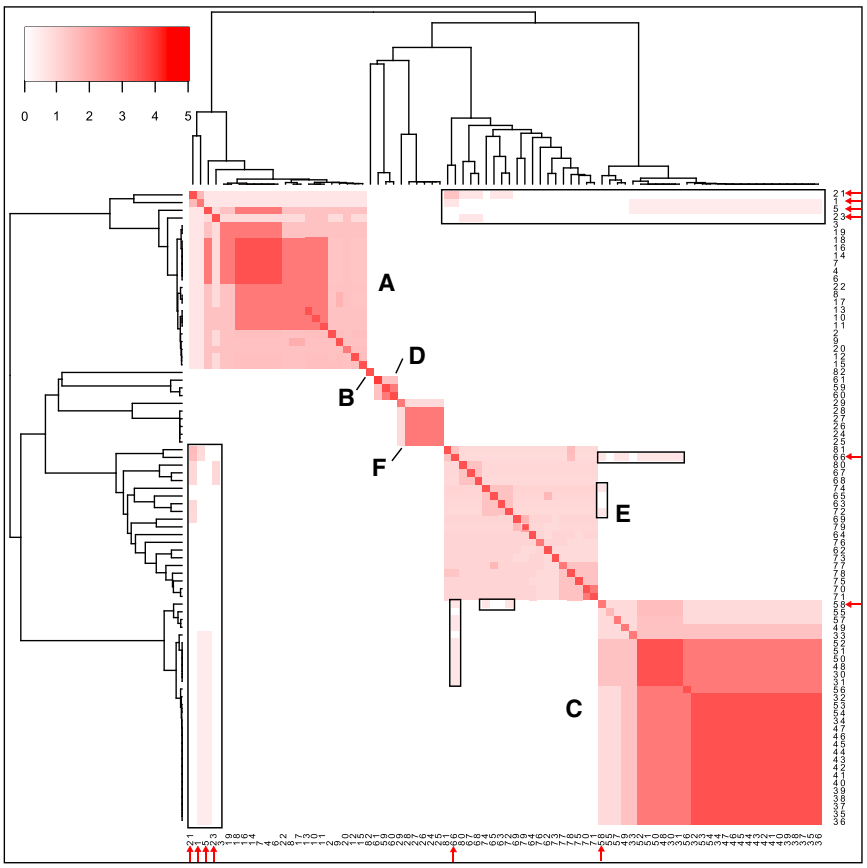

Figure 2. Conspecificity Matrix Highlighting the Congruence between the Five Markers, Resulting in the Delineation of Six Species, A–F, among the 82 *A. vaga* Individuals Sequenced

This matrix was obtained by computing, for each pair of individuals, a conspecificity score equal to the number of independent markers supporting the hypothesis of their conspecificity (i.e., for which the two individuals belong to the same field for recombination; see also Figure S1) 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 (0 out of 5) 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 12 individuals (Ind38-47, 54, and 55) isolated from the same patch and harboring the same COI haplotype 12 form one dense red block at the bottom right corner of the matrix.

(Ind1) assigned to species A harbored genetic markers belonging to two other distinct species (C for EPIC25 and E for EPIC63, Figure 4); this individual was heterozygous for these two latter markers (Figures 3C and 3D).

Intraspecific Haplotype Sharing

We observed two cases of EPIC25 haplotypes being shared by heterozygous individuals in a cyclic fashion (Figure 3D), and one such case with the Nu1054 marker (Figure 3B). Each of these cycles comprised three haplotypes, and we therefore refer to them as "haplotype trios" (displayed as red links on 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 Figure 3D) were separated by four to eight point mutations, making convergence unlikely. In the second trio (species A of marker EPIC25, Figure 3D), a 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 until reaching a total 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 5–10.5 kb around each of our nuclear markers (Figure 5). Although the posterior probabilities of our haplotypes reconstructed using PHASE were rather low (ranging from 0.245 to 0.999), 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, 1,865 and 1,031 bp on the contigs harboring the EPIC25 marker but also one recombining region of 4,547 bp on the contigs harboring the EPIC63 marker (Figure 5, gray areas). In contrast, no recombination was detected in the Nu1054 region.

The maximum-likelihood trees built from each region showed three contrasting patterns (Figure 5). First, the phylogeny obtained

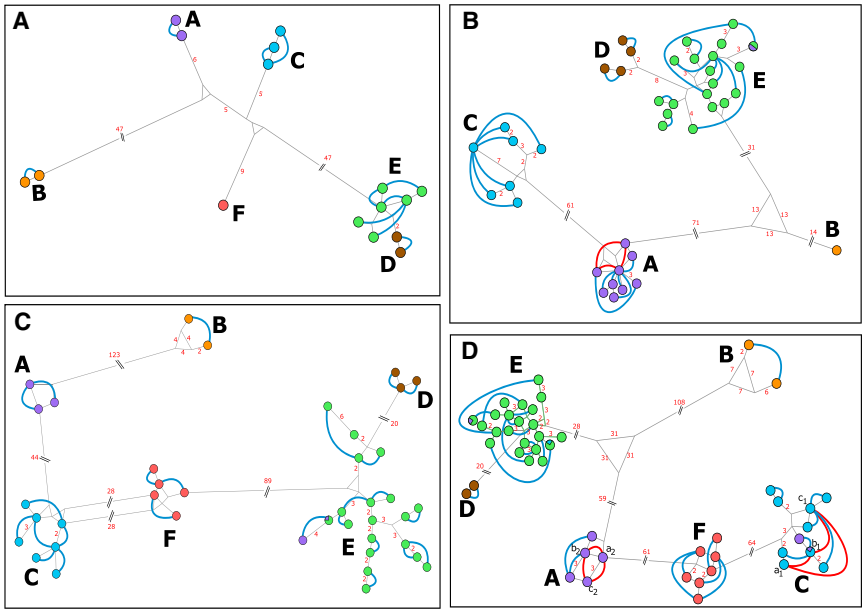

Figure 3. Haplowebs of the Nuclear Markers Amplified from 82 *A. vaga* Individuals

(A) 28S rDNA, (B) Nu1054, (C) EPIC63, and (D) EPIC25 are shown. 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 colored according to its assignment to one of the six species (A–F) determined by the conspecificity matrix (Figure 2). One individual from species C had the same sequence than individuals from species E for the Nu1054 marker. 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. Two individuals assigned to the A and C species according to the conspecificity matrix harbored 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. Red links highlight haplotype trios and represent signatures of genetic exchange. The number of mutations 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. See also Figure S1.

for the Nu1054 region was consistent with expectations for asexually evolving nuclear alleles: the alleles of each individual are diverging independently in the absence of recombination and therefore fell into two separate clades [21]. Second, other regions (2,831–4,695 and 8,110–9,141 bp on EPIC25 and 2,574–7,121 bp on EPIC63) presented signatures of inter-individual genetic exchange: the sequences fell into three clades, each of which comprised sequences from two different individuals. 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 homolog in the same individual (resulting in a clade made up of the two sequences of this individual). All three types of signatures were observed next to each other in the longest contig assembled around the EPIC25 marker (10,539 bp): it showed a patchwork of signatures of asexual evolution, recombination, and gene conversion, with the conversion events having occurred within individual 42 on both sides of the exchanged region (Figure 5).

For the three individuals (Ind 31, 42, 51) of species C whose genomes were sequenced, we also assembled contigs from the heat shock protein (hsp) and histone cluster (his) regions (for which allele sharing had been previously reported within the genus *Macrotrachela* [11]). The hsp assembly (1,399 bp) produced a tree indicating genetic exchange, whereas the hisA and hisB regions (1,642 bp when aligned) presented a pattern consistent with asexual evolution (alone for the hisA region and together with gene conversion for the hisB region; Figure 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 this morphospecies [22] as well as in other bdelloid and monogonont rotifer species (see [20, 23, 24]). Although most of the genetic exchanges we detected occurred within these six cryptic species, some individuals combined marker sequences attributed to different species (Figures 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 patchwork pattern of regions exhibiting signatures of asexual evolution, inter-individual recombination, and/or gene conversion (Figure 5). Such pattern is unlikely to arise in cases 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 (Figure 6) [25]. Hence, and since our previous analysis of the genome structure of *A. vaga* showed that conventional meiosis is impossible to occur [6], the observed patterns here are most likely explained by horizontal genetic exchanges among bdelloid rotifers (Figure 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 these genetic exchanges occur along the entire genome of bdelloids 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) are distributed all across the ten largest scaffolds (1.08–0.93 Mbp, ~4.1% of the total genome) of the *A. vaga* draft genome (Figure S4). This suggests that HGTs in the bdelloid *A. vaga* occur across their entire genome, and, since the interkingdom HGTs are up to 24,204 bp long (Figure S5), it seems plausible that the intra- and interspecific genetic

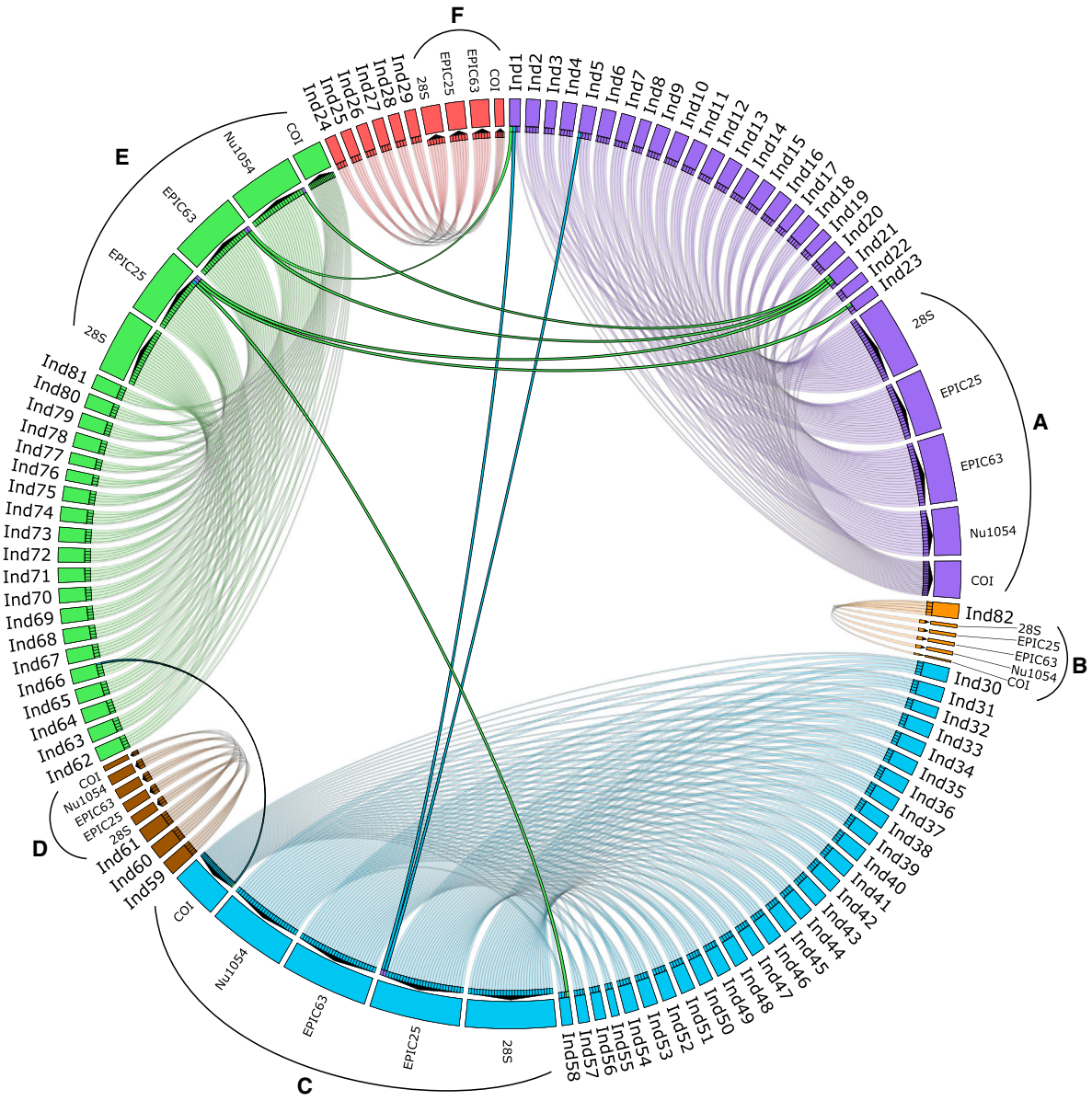

Figure 4. Circular Plot Summarizing the Species Affiliation of Each of the 82 *A. vaga* Individuals According to Each Marker

Individuals/markers combinations for which amplification and/or sequencing did not work are not displayed. The colors correspond to the six species (A–F) defined by the conspecificity matrix (Figure 2). Arrows point to the six individuals that are assigned to different species by different markers.

exchanges observed here and in Signorovitch et al. [11] also result from horizontal transfers.

The transfers observed in the bdelloids *A. vaga* and *Macrotrachela* [11] (integrating 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 plants, fungi, or bacteria [6], could be integrated during DSB repair by non-homologous end-joining mechanisms requiring no homology between the repaired fragments. In contrast, DNA exchanges between closely related individuals may be mediated by DSB repair through homologous recombination (HR), the frequency of which is strongly correlated with the degree of identity between the recombining DNA fragments and dramatically declines as the sequences diverge [26]. 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.

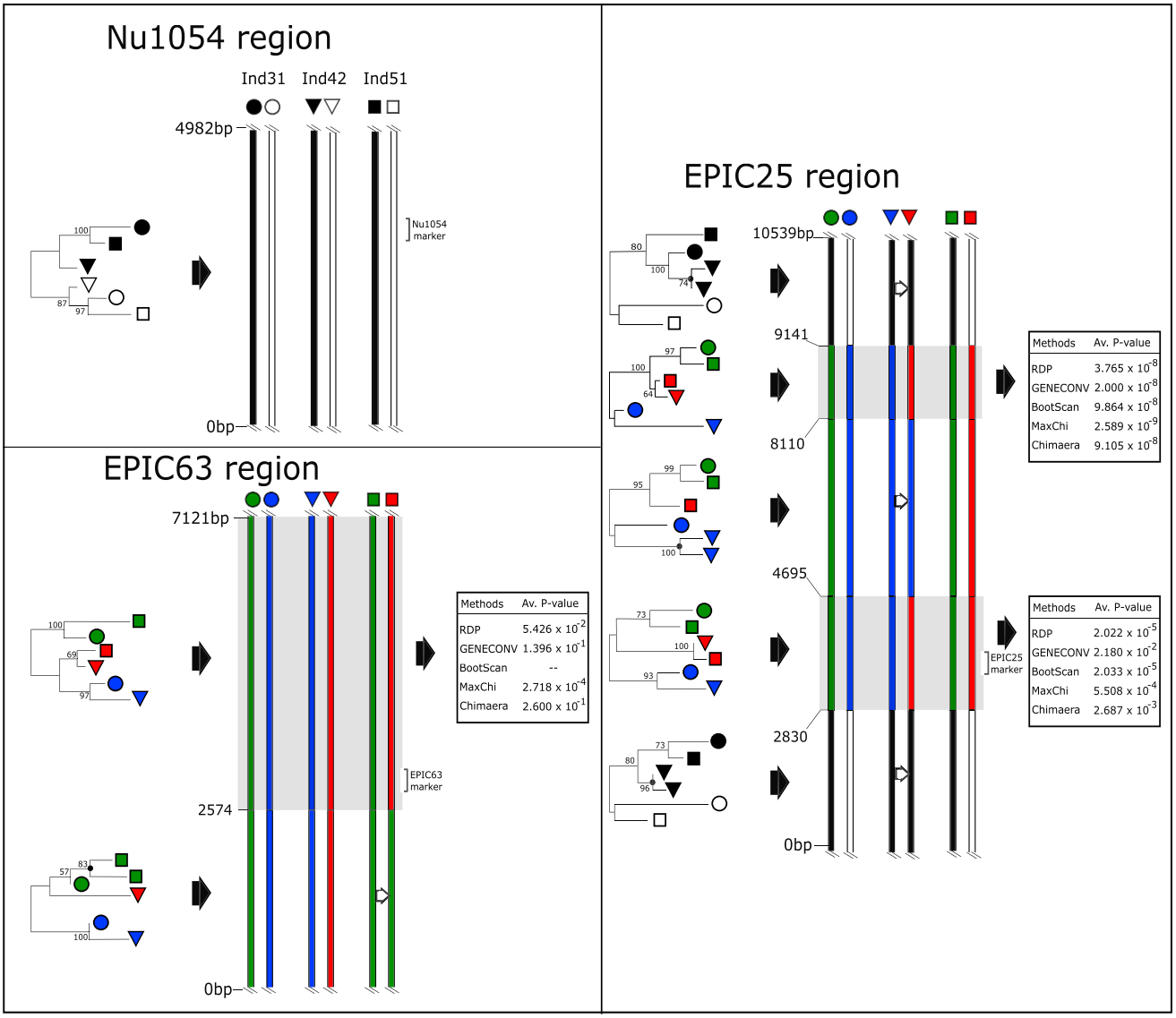

Figure 5. Representation of the Contigs Assembled around the Nu1054, EPIC25, and EPIC63 Markers for the Three Individuals Ind31, Ind42, and Ind51 Sequenced

The tables on the right show the posterior probabilities of the recombination breakpoints detected in RDP3 using five different methods. For each block delimited using RDP3, maximum-likelihood trees were generated in MEGA5 using the GTR+ Γ4+I model and rooted using orthologs from the reference *Adineta vaga* genome sequence [6]. Tree labels and contigs are shown in black and white for regions bearing a phylogenetic signature of asexual evolution, and in colors for regions indicating genetic exchanges among individuals. Gene conversion events are shown with black dots on the corresponding nodes of the trees and with white arrows between the contigs involved. See also Figure S3 for other genomic regions analyzed and Figures S4 and S5 concerning horizontally acquired genes.

Another intriguing observation is that we did not detect any *A. vaga* individual harboring 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 (Ind1) was heterozygous at the two transferred loci (EPIC25 and EPIC63) but presented for both of them 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].

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 (Figure 5), although no haplotype trio was observed in the haploweb for the EPIC63 marker (Figure 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 accumulation of

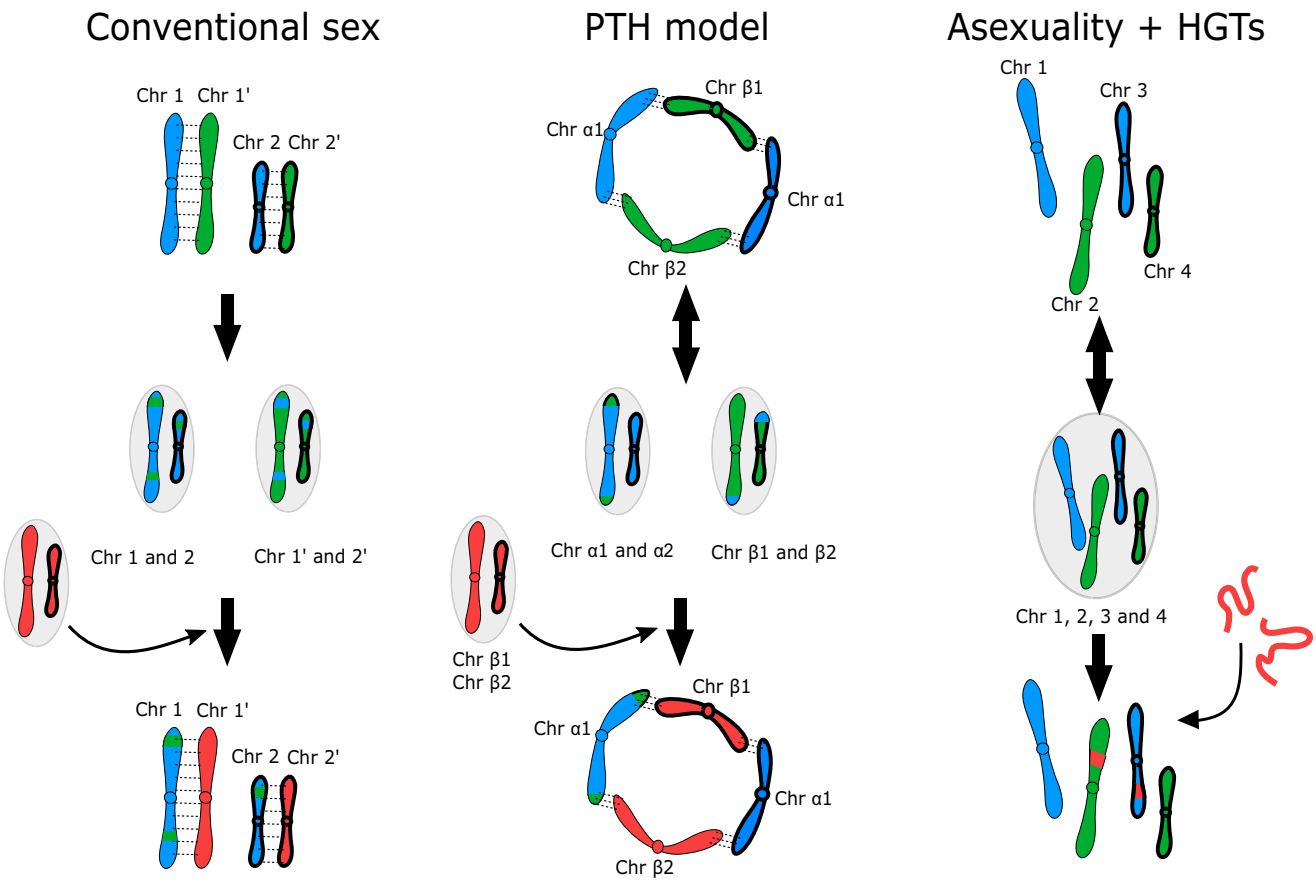

Figure 6. Comparison of Three Potential Mechanisms Leading to Allele Sharing

Conventional sex refers to classical meiosis during which paternal and maternal (blue and green) chromosome pairs segregate and form gametes with genome-wide exchanges. Colinearity is maintained here 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 groups (blue and green) that segregate entirely into two distinct gamete types; fertilization only occurs between opposing gamete types. As for *Oenothera*-like meiosis, asexuality does not require chromosome pairing or segregation. Frequent horizontal gene transfers (HGTs) between asexually diverging individuals lead to a patchwork pattern of signatures of allele sharing interspersed with other regions exhibiting asexual evolution. See also Figures S4 and S5.

mutations (Figure 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 genetic exchanges, as well as most individuals from species C (Figure 3D). An open cycle is also found among species A at the Nu1054 marker, although here the individuals involved seem to have accumulated several mutations since the recombination events. These results suggest that the frequency of genetic exchange among 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 Figure 1). This may suggest that intraspecific horizontal exchange increases the fitness of the individuals that engage in it, as suggested by Signorovitch et al. [11]. One possible explanation for the persistence of species in bdelloids despite this selective advantage of recombinants could be niche differentiation [3]. To test this hypothesis, we analyzed 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 the Supplemental Information) revealed highly significant departures from randomness and significant phylogenetic clustering, supporting 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 favor 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, 27], 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. This brings support to our earlier hypothesis that the homogenizing and diversifying roles of sex have been replaced in bdelloids by gene conversion and horizontal DNA transfer, in an unexpected (and possibly unique) convergence

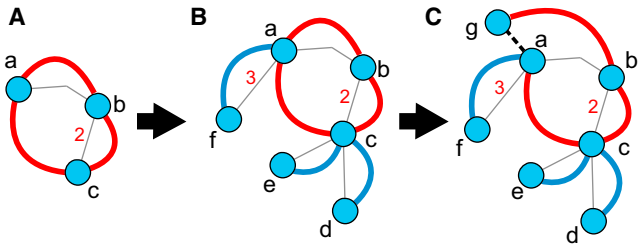

Figure 7. Hypothetical Evolutionary Scenario Explaining the Transition from Haplotypes Trios Indicating Allele Sharing into Open Cycles Indicating Past Recombination

(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.

of evolutionary strategy with bacteria [6]. 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 [28, 29]. We propose here to call "sapphomixis" (from the name of the Greek lesbian poetess Sappho and *mixis* "mingling") such ameiotic strategy of genetic exchange and recombination among asexual, morphologically female organisms.

Our data presented here add one more piece of evidence for the asexuality of bdelloid rotifers; others include the absence of 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 the sexuality of bdelloids much less likely than horizontal genetic exchange among them.

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 m^2 in Parc Louise-Marie, Namur, Belgium. The 36 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 hr were isolated (following the protocol of [22]). DNA was extracted from each *A. vaga* individual separately using the QIAamp DNA Micro kit (QIAGEN) according to the manufacturer's instructions.

Marker development, DNA amplification, and sequencing procedures are described in the Supplemental Information; the primers used as listed in Table S1. For the nuclear markers, we sequenced one individual per COI haplotype per patch (Table S2), in total 70 individuals. We also sequenced the whole set of 12 individuals isolated from patch A6 that harbored the same COI haplotype (Hap12), in order to verify whether the nuclear markers were identical across potential clones as expected in mitotic reproduction; the nuclear haplotypes of the total of 82 individuals for which we attempted to sequence them are summarized in Table S3. As the total amount of DNA obtained from single *A. vaga* individuals was very small, we performed whole-genome amplification using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Amersham Biosciences). The resulting amplicons were then used as template for further PCR amplification.

Phasing of Nuclear Markers from Direct Sanger Sequencing and Species Delimitation

For each nuclear marker, the haplotypes of heterozygotes were directly reconstructed from the patterns of double peaks in the forward and reverse chromatograms [30] using Champuru 1.0 [31], SeqPHASE [32], and PHASE [33].

Species delimitation was performed using two DNA taxonomy methods: haplowebs (based on shared alleles [16]) and the generalized mixed Yule-coalescent model (GMYC; based on variation in branching rates in phylogenetic trees [17, 18]). Haplowebs 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 approach to species delimitation rests on the assumption that intraspecific branching follows the neutral coalescent model, whereas interspecific branching follows a Yule (pure birth) model [18].

For investigation of allele sharing, median-joining haplotype networks [34] were constructed using the program Network, exported into PDF using Network Publisher (Fluxus Technology) and turned into haplowebs using Inkscape [35]. The allele-sharing information from all markers was then integrated into one "conspecificity matrix." 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. As the COI marker is haploid, we considered each haplotype as a distinct FFR for this analysis. 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" [36]. 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. [22] and six *Adineta* haplotypes from Birky et al. [37]. As outgroup in our tree, we used two sequences of the monogonont rotifer species *Brachionus plicatilis* (GenBank: AF266895.1 and AF266853.1; [38]). We constructed an ultrametric Bayesian tree using the program BEAST v.1.6.2 [39] with a single sequence for each haplotype as recommended by Tang et al. [40]. We chose the GTR+Γ4+I substitution model selected by jModelTest 3.8 [41] following the Bayesian Information Criterion [42]. The Markov chain Monte Carlo (MCMC) was run for 10^8 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 [39]. This ultrametric tree was used as input for the GMYC analysis using the R package "splits" (http://r-forge.r-project.org/projects/splits/; Figure S2). This method uses a maximum-likelihood approach to detect the shift in branching rate from interspecific branching to intraspecific branching [18, 19].

Genomic Sequencing, Assembly, and Analyses

We used the remaining gDNA (10–12 ng) of three individuals (Ind31, Ind 42, and Ind51) that shared alleles of marker EPIC25 for whole-genome sequencing on the Illumina HiSeq2500 platform (Genomics Core, UZ Leuven). Because of the very low amount of input material, we started by performing ten 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 the de novo assemblies of the reads were extremely fragmented (data not shown), we resorted to assemble targeted genome regions using the MITObim package [43], which runs MIRA [44] iteratively, using our Sanger-sequenced genetic markers as baits. Each assembled contig was then scrutinized for SNPs using SAMtools [45] and Tablet [46]. The contigs were aligned

in MAFFT (E-INS-i method [47]) and phased using SeqPHASE [32] and PHASE [33].

The phased sequences were then analyzed with different methods (RDP, GENECONV, Chimera, MaxChi, and Bootscan) implemented in RDP3 [48] 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 [49] following the GTR+Γ4+I model chosen by jModelTest [50] following the Bayesian Information Criterion [42].

Community Analyses

To find out whether our genetically based species delimitations were supported by ecological evidence, we used Fisher's test [51] and an analysis of the phylogenetic structure of communities (using the R package "Picante" [52]). Fisher's test was performed on a 2 × 2 contingency table summarizing the number of individuals belonging to the same or different species and present or not in the same patch (Table S4). Phylogenetic diversity was inferred using the COI dataset from the 576 individuals locally sampled in the 36 patches: first, we calculated the mean pairwise distance (MPD) between each individual in each patch and the mean nearest taxon distance (MNTD) separating each species in the community. We then calculated standardized effect sizes for MPD and MNTD to check whether communities were phylogenetically over-dispersed (positive values) or clustered (negative values); the detailed results are shown in Table S5.

ACCESSION NUMBERS

The accession numbers for all of the new sequences reported in this paper are GenBank: KU860573–KU861170.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.01.031.

AUTHOR 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 performed the ecological analyses. N.D., J.-F.F., and K.V.D wrote the core of the manuscript. K.V.D. acquired the funds for this study.

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