Evidence for "inter- and intraspecific horizontal genetic transfers" between anciently asexual bdelloid rotifers is explained by cross-contamination

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Abstract

Bdelloid rotifers are a diverse group of microscopic invertebrates that are believed to have evolved for tens of millions of years without sexual reproduction. They have attracted the attention of biologists puzzled by the maintenance of sex and recombination among nearly all other eukaryotes. Bdelloid genomes show a high proportion of non-metazoan genes, acquired by horizontal transfer. This well-substantiated finding has invited speculation that homologous horizontal transfer between rotifers also may occur, perhaps at rates sufficient to "replace" the functions of sex in bdelloids. In 2016, Debortoli and colleagues claimed to supply evidence for this hypothesis (*Current Biology* 26, 723-32). They sampled individuals of the bdelloid genus *Adineta* from natural populations, extracted DNA and sequenced five marker loci. For several samples, the assignment of haplotypes to species was incongruent among loci, which the authors interpreted as evidence of "interspecific horizontal genetic transfers". Here, we use sequencing chromatograms supplied by the authors to demonstrate that samples treated as single individuals actually contained mitochondrial and ribosomal haplotypes from two or even three animals. We also show that the putatively transferred DNA molecules share only 75% sequence identity, which is not compatible with known mechanisms of homologous recombination, or with established features of bdelloid genomes. We argue that these and other patterns are parsimoniously explained by cross-contamination of animals or DNA between sample tubes, and therefore that the study offers no reliable evidence for the hypothesis that genes are transferred either within or between these bdelloid species. In light of this and other recent cases, we recommend that work considering horizontal gene transfer in microscopic animals be conducted and evaluated with special caution in future, owing to the risk of contamination.

35 **Background**

36

37 The maintenance of sexual reproduction is a fundamental evolutionary problem. In theory, an
38 obligately asexual population will grow at twice the rate of a competing sexual population, half of
39 which are males that cannot produce eggs (Maynard Smith 1978). Despite this and other costs
40 (Lehtonen et al. 2012), sex is nearly universal among eukaryotes (Speijer et al. 2015), whereas
41 obligately asexual lineages are rare and typically short-lived (Bell 1982; Burt 2000). Various genetic
42 and ecological hypotheses have been proposed to explain this paradox (Kondrashov 1993; Hartfield
43 & Keightley 2012), but definitive tests are lacking, in part because it is challenging to identify
44 appropriate study systems (Lehtonen et al. 2012; Meirmans et al. 2012).

45

46 One approach is to investigate groups that seem to have evolved over extended timescales without
47 sex (Judson & Normark 1996; Normark et al. 2003). Whatever mechanism maintains sex ought to be
48 absent or unusually mitigated in these exceptional groups, whose genetics and ecology may thus
49 help illuminate the rules that maintain sex everywhere else. However, that approach first requires
50 that the remarkable claim of longstanding asexuality be rigorously established. Only a handful of
51 'ancient asexual' candidates have been identified, and some are subject to ongoing doubt (Lunt
52 2008; Schurko et al. 2009), especially when extant populations continue to invest in males (Palmer &
53 Norton 1991; Smith et al. 2006; Schwander et al. 2013). Many are difficult to study owing to long
54 generation times and other obstacles to culture (Van Doninck et al. 2002). Attention has therefore
55 focused on rotifers of the Class Bdelloidea, which are tractable in the laboratory and field (Ricci
56 1984; Mark Welch & Meselson 1998a; Fontaneto et al. 2008; Wilson & Sherman 2010, 2013).

57

58 Bdelloid rotifers are microscopic filter-feeding invertebrates, about 100-500µm long (Donner 1965).
59 They live in nearly every freshwater habitat worldwide, however tiny or ephemeral, thanks to their
60 tolerance for extreme conditions, including complete desiccation (Ricci 1987). Bdelloidea comprises
61 nearly 500 described species (Segers 2007), though molecular surveys suggest the number of cryptic
62 taxa may be orders of magnitude higher (Fontaneto et al. 2009, 2011; Robeson et al. 2011). The
63 primary indication of longstanding asexuality is the absence of evidence for males in any species
64 despite centuries of observation (Hudson & Gosse 1886; Mark Welch et al. 2009; Birky 2010).
65 Cytology provides further suggestive evidence: *Philodina roseola* has 13 chromosomes, including
66 three without apparent morphological homologs, and oogenesis does not involve chromosomal
67 reduction or synapsis (Hsu 1956; Mark Welch & Meselson 1998b).

68

Several features of bdelloid genomes have attracted attention as possible evidence for longstanding asexuality, or as potentially important in that theoretical context. Bdelloids have far fewer vertically transmitted retrotransposons than other animals (Arkhipova & Meselson 2000; Flot et al. 2013). This is predicted by selfish DNA theory under long-term asexuality, which curtails passage of these elements to new genetic backgrounds (Hickey 1982). Without recombination, alleles on formerly paired chromosomes were predicted to diverge in sequence (Birky 1996), and deeply divergent gene copies in certain bdelloid species were initially interpreted as former alleles that had been evolving independently for millions of years (Mark Welch & Meselson 2000). However, up to four gene copies were found rather than the two predicted for anciently asexual diploids. Later work clarified that bdelloids are ancestrally tetraploid (Mark Welch et al. 2008). Ancient polyploidisation or hybridisation seems to have produced four copies of each gene, which now form two pairs of closely related sequences (Hur et al. 2009). Each pair diverges deeply from the other pair, and the two parallel gene lineages no longer seem capable of genetic exchange (e.g. homology is 86% for two copies of the heat-shock gene *hsp82* in *P. roseola*). By convention, a typical gene has a closely related 'homolog', and a pair of distantly related 'ohnologs' (Flot et al. 2013). Within pairs, homologous copies are similar or identical, which implies some ongoing homogenising mechanism whose nature is unclear. It has been speculated that homologs serve as reciprocal templates for the repair of DNA double-strand breaks (DSB) after desiccation, and that break-induced replication, recombination and gene conversion mediate concerted evolution between them (Gladyshev & Meselson 2008; Mark Welch et al. 2008; Flot et al. 2013; Hespeels et al. 2014).

Another remarkable feature of bdelloid genomes has caused particular excitement, as it involves sharing of genetic material, and may thereby relate to sex. Bdelloids have a very high proportion of genes of non-metazoan origin, which indicates exceptional rates of horizontal gene transfer (HGT) from foreign sources (Gladyshev et al. 2008). Initial claims of this kind in other animals (e.g. Boothby et al. 2015) have been rejected as artefacts of contamination after more careful analysis (Delmont & Eren 2016; Koutsovoulos et al. 2016; Richards & Monier 2016). However, several independent lines of evidence consistently support the hypothesis of massive inter-kingdom HGT in multiple bdelloid species (Gladyshev et al. 2008; Boschetti et al. 2012; Flot et al. 2013; Eyres et al. 2015). Even stringent analyses recover a proportion of foreign genes far higher than other metazoans examined (Crisp et al. 2015). The majority appear bacterial in origin, but fungal, plant and archaeal genes also are represented (Boschetti et al. 2012). Animal genes have probably been acquired horizontally too, but these are not readily distinguished against a metazoan background.

How and why bdelloids have accumulated so many foreign genes remains unclear. One hypothesis is that environmental DNA is incorporated during recovery from desiccation, when cell membranes are compromised and extensive DNA damage is repaired (Gladyshev et al. 2008; Hespeels et al. 2014). However, at least one desiccation-tolerant tardigrade shows no such accumulation (Hashimoto et al. 2016), whereas desiccation-intolerant bdelloids seem to go on acquiring foreign genes (Eyres et al. 2015). Regardless of mechanism, the absolute rate of stable incorporation of foreign genes is estimated to be low: on the order of ten events per million years (Eyres et al. 2015).

When first describing HGT to bdelloids from non-metazoans, Gladyshev et al. (2008) speculated that "there may also be homologous replacement by DNA segments released from related individuals", and thus "bdelloid rotifers may experience genetic exchange resembling that in sexual populations". This attempt to link two striking genetic traits has a superficial appeal, but difficulties arise in light of evolutionary theory. Sex has genome-wide consequences each generation via segregation, crossing-over, independent assortment, outcrossing and sexual selection. These effects underpin a plethora of formal models for the maintenance of amphimixis (Kondrashov 1993). Local horizontal transfer, homologous or otherwise, has very different population genetic outcomes (Redfield 2001; Narra & Ochman 2006; Agrawal 2009). Even bacterial HGT is not a simple analog of meiotic sex (Redfield 2001; Croucher et al. 2016). Despite these issues, efforts have been made to confirm the idea that HGT occurs regularly between bdelloid rotifers, and is sufficient to "replace" sex (e.g. Flot et al. 2013). Here, we discuss some recent work purporting to supply evidence for that view.

The study of Debortoli et al. (2016)

Debortoli et al. (2016) collected lichen and soil from five trees in a small area and looked for rotifers of the cosmopolitan bdelloid genus *Adineta* (Hudson & Gosse 1886). They moved individuals to 576 tubes, from which they extracted DNA. They amplified a 0.6kb region of mitochondrial cytochrome oxidase I (mtCO1) by PCR. Using this common molecular barcode (Hebert et al. 2003), the authors delineated six molecular taxa (Pons et al. 2006), which they call "*Adineta vaga* Species A-F" (we follow this usage for convenience). A subset of 82 samples was selected to represent a range of mtCO1 haplotypes from these “cryptic species”. The DNA in these tubes was subjected to whole-genome amplification, then four nuclear marker loci were further amplified by PCR and sequenced.

A majority of samples yielded sequences characteristic of a single species at all five loci. However, for six samples (7.3%), sequences at different loci matched two or even three different species. The

137 authors interpret this incongruence as "strong evidence" of "multiple cases" of "interspecific
138 horizontal genetic transfers" from "donor species" to "recipient individuals." They conclude that
139 they have discovered "an unexpected (and possibly unique)...ameiotic strategy of genetic exchange
140 and recombination among asexual, morphologically female organisms", which they "propose here to
141 call 'sapphomixis' (from the name of the Greek lesbian poetess Sappho and mixis 'mingling')."
142
143 This dataset features some surprising patterns that seem to require further clarification. In every
144 case where incongruence was reported, the inferred "donor species" was recovered from the same
145 maple or plane tree as the "recipient individual" at the time of sampling (Debortoli et al. 2016; Table
146 S3). This is quite unexpected, since the genus *Adineta* subsumes vast cryptic diversity; even *A. vaga*
147 comprises at least 36 independently evolving entities (Fontaneto et al. 2011; Robeson et al. 2011).
148 Rotifers of this genus have high dispersal potential (Fontaneto et al. 2008; Wilson & Sherman 2013)
149 and the lifespan of a patch of lichen is short in evolutionary terms. If genetic exchange occurs so
150 promiscuously among such diverse and mobile animals, why should every “donor species” happen to
151 be sampled in the same small area as the recipients at the same time? Even more striking, every
152 case of incongruence involves a haplotype whose sequence is identical to a haplotype found
153 'natively' in one of the other 81 rotifers sampled, enabling the authors to construct a perfectly self-
154 contained circular representation of the “transfers” (their Figure 4).
155
156 We hypothesised that the evidence interpreted as "interspecific genetic exchanges" might instead
157 result from accidental cross-contamination of rotifers or rotifer DNA between tubes during sample
158 preparation. This would explain the hermetically self-referencing pattern of incongruent haplotypes.
159 Specifically, while isolating 576 individuals, more than one animal may occasionally have been added
160 to a tube, or loose DNA fragments may have been introduced in the gut or on the surface of a focal
161 individual. From personal experience, we find it can be technically challenging to isolate *Adineta* to
162 Eppendorf tubes individually. The animals are small even when extended; if disturbed, they contract
163 rapidly into tiny, motionless, transparent spheroids that stick tenaciously to plasticware and have
164 about the same refractive index. These technical issues are discussed further in the Supplementary
165 Material. If the isolation protocol used by Debortoli et al. (2016) resulted in cross-contamination of
166 a subset of samples presented as single individuals, it would give the misleading appearance of
167 "inter- and intraspecific genetic exchanges". Here, this alternative hypothesis is tested using two
168 independent and complementary sources of evidence: original Sanger sequencing chromatograms
169 provided by the authors, and alignments of genetic and genomic data from public repositories.
170

Results and Discussion

Experimental determination of the effects of cross-contamination on chromatograms

If samples showing "interspecific horizontal genetic transfers" contained DNA from more than one animal, additional haplotypes ought to be evident in chromatograms produced by Sanger sequencing of PCR amplicons. In particular, previous sequencing of thousands of rotifers indicates that each individual has only one mtCO1 haplotype (e.g. Fontaneto et al. 2009), therefore to find double peaks in mtCO1 chromatograms would provide evidence for contamination.

We conducted an experiment to determine the pattern of mtCO1 chromatogram peaks when two animals are present in one tube. We chose two bdelloid clones from our cultures: '*A. vaga* (AD008)', which supplied the reference genome for *A. vaga* (Flot et al. 2013), and '*Adineta* sp. (AD006)'. We prepared replicate Eppendorf tubes, either with a single individual, or deliberately contaminated with two individuals, one from each species (Supplementary Material). We extracted DNA and amplified the mtCO1 marker using the methods and primers described by Debortoli et al. (2016). Bidirectional chromatograms were generated by direct Sanger sequencing with the PCR primers.

The phred quality scores of the chromatogram files (Ewing & Green 1998) were only slightly and not significantly lower for tubes with two animals versus one (Q20; 90% vs. 92%, N=38, t=1.13, P = 0.26). Even when two animals were present, the vast majority of base calls (97.9-99.6%) matched a single species (Supplementary Material). The additional animal did not manifest as obvious double peaks at the expected polymorphic sites, but as small, subtle minority peaks, typically hidden in baseline noise (Kronick 1997) and sometimes missing entirely. Perhaps this is unsurprising: double peaks are seldom equal in height even when amplifying alleles from diploid heterozygotes (Kronick 1997). PCR is non-linear, and if two animals contribute substantially divergent templates, large biases in final amplicon representation may arise from small initial differences in numbers of cells or mitochondria, or the efficiency of lysis, DNA extraction, primer binding, denaturation, etc. (Mullis et al. 1994).

We developed a simple quantitative method to test whether the identity of the additional animal in our deliberately contaminated samples could be recovered from the pattern of minority peaks (Supplementary Material). We refer to this as ConTAMPR (Contingency Table Analysis of Minority Peak Ranks). Briefly, chromatograms were aligned bidirectionally with the known majority sequence and that of the contaminant *Adineta* clone. At each site where the two haplotypes differed, we

205 manually ranked the heights of the fluorescence trace lines corresponding to the three minority
206 nucleotide bases, and recorded the rank for the base fitting the contaminant haplotype. Contingency
207 table analysis was used to test whether the distribution of peak height ranks differed from the
208 expectation if chromatogram noise were random. For additional rigor, we measured the distribution
209 of minority peaks fitted to a control species (*A. ricciae,* Segers & Shiel 2005) that was not present in
210 any sample, but whose sequence identity to both AD006 and AD008 at mtCO1 was equal (87.5%).
211
212 In uncontaminated single-animal samples with *A. sp*. (AD006), the rank distributions of minority
213 peaks matching *A. vaga* (AD008) and *A. ricciae* did not differ significantly from the null expectation
214 or from each other (Figure 1A; χ^2 = 3.32, d.f. = 4, P = 0.51). In deliberately contaminated samples,
215 however, minority peaks fitted the known contaminant species better than the null expectation
216 (Figure 1B; χ^2=174.54, d.f. =2, P < 2.2 x 10^{-16}) or the control species (χ^2=22.54, d.f. =2, P = 1.27 x 10^{-5}).
217 The analysis correctly detected that tubes contained two animals, identified the second haplotype
218 and differentiated it from other candidates. Similar results were obtained over multiple biological
219 and technical replicates (Supplementary Material). This approach works because PCR amplicons are
220 directly sequenced, leaving visual evidence of minority sequences. Cloning-based methods might
221 easily miss minority haplotypes unless very many clones are sequenced for each reaction (Mullis et
222 al. 1994). Debortoli et al. (2016) also employed direct sequencing, and so the same method could be
223 applied to test whether their samples were cross-contaminated with DNA from multiple animals.
224
225 **Evidence of cross-contamination in samples treated as individuals by Debortoli et al. (2016)**
226
227 To test for the signatures described above, we requested original chromatogram files from Debortoli
228 and colleagues. We were provided with 36 out of at least 1152 mtCO1 chromatograms, including
229 files for the six samples where "interspecific genetic exchanges" were claimed. We also received 483
230 out of at least 656 chromatograms for 28S ribosomal DNA, and 133 out of at least 158 files
231 corresponding to the EPIC25 (exon-primed intron-crossing) nuclear marker. We appreciate the open
232 and collaborative spirit in which the authors shared their data.
233
234 Sample B11 was the first alphabetically to show "interspecific recombination". It was interpreted by
235 Debortoli et al. as a single animal (Individual 21) belonging to *A. vaga* Species A, with one mtCO1
236 haplotype (Hap6 [A]), and one 28S ribosomal haplotype (Hap1 [A]). However, at other nuclear loci,
237 haplotypes were putatively transferred from Species E: EPIC25 Hap35, EPIC 63 Hap16 and Nu1054
238 Hap22. As discussed above, all three haplotypes also were found 'natively' in other animals sampled

during the study. Indeed, Table S3 of Debortoli et al. (2016) shows that they all occurred together in a single animal: Individual 81 [E]. Contamination of Sample B11 with a second animal similar to this would explain the apparently incongruent signal. If so, the mtCO1 chromatograms for Sample B11 ought to show evidence of a second haplotype that looks like Hap31 [E], as seen in Individual 81.

We tested this prediction using ConTAMPR (Figure 1C). The fit of minority peaks to Hap31 [E] was significantly better than the null expectation (χ^2=127.95, d.f. =2, P < 2.2 x 10^{-16}) or the fit to *A. ricciae* (χ^2=25.39, d.f. =, P = 3.07 x 10^{-6}). We attempted to match the minority peaks to multiple control sequences, including the other five species (B, C, D, E, F) reported by Debortoli et al., as well as *A. ricciae* and the *A. vaga* reference genome (Figure 2). Hap31 [E] matches the chromatograms much better than any other species (χ^2=39.2, d.f. =12, P = 9.73 x 10^{-5}). Like other animals, individual rotifers are not known to have two substantially divergent mtCO1 haplotypes. This evidence suggests that Sample B11 contained a second animal belonging to Species E, very similar to Individual 81 [E]. A parsimonious inference is that this rotifer supplied the incongruent Species E haplotypes at other loci, where "interspecific genetic exchanges" were claimed.

Although mtCO1 was amplified directly from genomic DNA, Debortoli et al. performed PCR for the 28S, EPIC25, EPIC63 and Nu1054 nuclear markers after whole-genome amplification (WGA) of the samples via an Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Amersham Biosciences). When DNA from two animals was present, haplotypes were very unequally represented among our PCR amplicons (Figure 1B). Interposing another nonlinear amplification step introduces further opportunities for bias: some haplotypes might be dropped entirely, or a majority of amplicons might be generated from a minority template. In particular, this kit has a known bias in favor of templates with lower guanine-cytosine (GC) content (Han et al. 2012), which we suggest may be an important factor in selective loss of haplotypes (Supplementary Material). For these reasons, it was less clear whether nuclear loci would show evidence of a second animal. However, we attempted to conduct the same analysis, first at the ribosomal 28S marker. The putative second animal was predicted to show a 28S haplotype characteristic of Species E.

As predicted, the fit of minority peaks to Species E differed significantly from the null expectation, more than any other species (Figure 3). Species E fitted significantly better than Species F (χ^2=22.45, d.f. = 2, P = 1.3 x 10^{-5}), or B (χ^2=12.75, d.f. = 2, P = 0.0017), or C (χ^2=12.24, d.f. = 2, P = 0.0022) or *A. ricciae* (χ^2 = 11.13, d.f. = 2, P = 0.0038). These differences remain significant even if a conservative Bonferroni correction is applied (α =0.0083). Species E was also a better fit than Species D and the

A. vaga reference genome, but this was not significant because these species are nearly identical at the highly conserved 28S marker. Overall, the match to the secondary peaks recapitulates a phylogenetic tree based on 28S (Figure 3), with species more closely related to E fitting better. The evidence at 28S therefore is consistent with the evidence from mtCO1, indicating a second animal belonging to Species E. Because Debortoli et al. (2016) already reported Species E haplotypes at EPIC25, EPIC63 and Nu1054, evidence from all five sequenced loci is now brought into congruence, as predicted if Sample B11 contained an animal belonging to Species E, with haplotype combinations similar to those seen in Individual 81.

We used the same methods to investigate the other five samples where Debortoli et al. diagnosed "interspecific horizontal genetic transfers" based on incongruence of species assignment among loci. Where the relevant chromatograms were provided, we aligned them in both directions against the haplotype(s) reported by Debortoli et al., then analysed the pattern of minority peaks with reference to the haplotypes of other *Adineta* species reported in the study (Supplementary Material).

All six samples showed significant evidence of additional haplotypes that were not reported by Debortoli et al. (Table 1). Three samples (B11, B22, B39) each contained two clearly identifiable mtCO1 haplotypes, which is only consistent with DNA from more than one animal. Even if mtDNA itself could participate in interspecific horizontal transfers (as Debortoli et al. imply for Sample B39), intergenerational bottlenecking of mitogenomes (Mishra & Chan 2014) would still result in a single mtCO1 haplotype (J.-F. Flot, pers. comm), as seen in thousands of rotifers previously sequenced. In samples B11, B14 and B22, evidence of additional 28S haplotypes was found. This is not an expected outcome of HGT, because ribosomal DNA undergoes concerted evolution (Liao 2000) that is believed to preclude the maintenance of two substantially dissimilar 28S haplotypes in a single rotifer, even if one arrived horizontally (N. Debortoli, J.-F. Flot & K. Van Doninck, pers. comm.). Ribosomal markers have been amplified from hundreds of rotifers without finding copies that differ by more than a handful of bases (e.g. Tang et al. 2012). Debortoli et al. sequenced 82 individuals without finding more than three base differences between 28S copies in any animal (their Figure 3A). In contrast, the two haplotypes found in Samples B14 and B22 differ at over 40 positions in only the first 700bp fragment. As a consequence of multiple peaks from these additional haplotypes, quality scores for 28S chromatograms were significantly lower in samples where transfer was claimed than those where it was not (Figure 4; Mann-Whitney Test: N=122, W=373.5, P=0.015).

In two samples (B14 and B3B1), there was evidence of DNA originating in three different rotifers (from species A, C and E). The mtCO1 chromatograms for these samples were extreme outliers in quality scores (Figure 5), and too noisy to narrow down the minority peaks to just one candidate (Table 1, Supplementary Material). Samples B39 and D14 showed evidence of the expected 'native' haplotypes that were supposed to have been replaced by horizontal transfer. All loci in both samples are now brought into congruence; a parsimonious inference is that they represent typical animals with haplotype associations similar to conspecifics. The incongruent sequences arose either from WGA or PCR amplification of contaminating DNA fragments, or from a second animal whose haplotypes were dropped during amplification at other loci (Supplementary Material).

Consistent with biased or capricious amplification, the predicted 'native' haplotype in Sample D14 was almost equivalent in peak strength to the "interspecific" haplotype after one PCR, but absent among amplicons from a second PCR using the same tube of template (Supplementary Material). If the locus had only been amplified once, there might have been no evidence of the native sequence, and the case for "interspecific recombination" would have been harder to reject. A lack of minority peaks after the second PCR confirms an important message in this dataset: even when a sample has two different sequences, one may fail to appear in any given whole-genome amplification or PCR. A significant pattern of minority peaks is thus sufficient but not necessary to indicate a second haplotype, whereas the absence of such a pattern is necessary but not sufficient to exclude it.

The sequencing evidence confirms that a subset of samples prepared by Debortoli et al. (2016) were cross-contaminated with DNA from multiple animals belonging to different species. This may quite simply explain why incongruent haplotypes always matched a "donor species" that happened to be sampled from the same tree. Elsewhere in the publication, the authors also claim evidence for "intraspecific haplotype sharing". In light of the results, it seems parsimonious to infer that these patterns also arise from cross-contamination, this time involving individuals or DNA belonging to the same species, which would be far less obvious from chromatograms or other analyses. Interestingly, the incidence of proposed intraspecific transfer in species A and C was approximately 10%, and it now appears that at least 7% of tubes contained DNA from more than one animal. It would be surprising if all tube-sharing events in the study happened to involve individuals of different species.

“Interspecific recombination” events are not mechanistically compatible with genomic evidence

We believe mechanistic considerations independently falsify the hypothesis of "interspecific recombination" presented by Debortoli et al. (2016). According to the authors, transfers “may be mediated by DSB repair through homologous recombination (HR)". In their view, "this hypothesis is reinforced by the observation that...the transferred sequences replaced the original copies", and they "speculate that, after the integration of DNA, gene conversion promptly copied the integrated DNA on its homologous region". This last hypothetical step is critical because each gene in the degenerate tetraploid genome of *A. vaga* has a closely similar paired homolog, and the pairs undergo concerted evolution (Mark Welch et al. 2008; Flot et al. 2013).

In the context of HGT, Thomas and Nielsen (2005) define HR as "recombination that depends on extensive segments of high sequence similarity between two DNA molecules" (p. 714). As Debortoli et al. themselves remark, the frequency of HR "is strongly correlated with the degree of identity between the recombining DNA fragments and dramatically declines as the sequences diverge." A log-linear decline in HR with decreasing sequence identity is well established in bacteria (Watt et al. 1985; Zawadzki et al. 1995; Vulić et al. 1997; Majewski 2001; Thomas & Nielsen 2005); protists (Bell & McCulloch 2003); yeast (Datta et al. 1997); plants (Opperman et al. 2004; Li et al. 2006) and animals (Larocque & Jasin 2010; Do & LaRocque 2015). Datta et al. (1997) found that even a single mismatched base (99.7% identity) reduced HR rates fourfold in yeast. Reductions to 99% or 90% identity reduced HR rates by one and two orders of magnitude respectively. To this point, the relationship is largely governed by active mismatch repair systems, and HR does not decline so dramatically if these are abolished. However, when sequences fall below 90% identity, rates of HR decline exponentially even in mutants devoid of mismatch repair, indicating that the machinery of HR itself fails to engage when so many bases are mispaired (Datta et al. 1997). At 83.5% identity, HR is effectively absent in yeast (three recombinant cells per billion: Datta et al. 1997), and rates were too low to measure even for otherwise promiscuous *Bacillus* species (Zawadzki et al. 1995).

Following this logic, Debortoli et al. write that "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." Indeed, an extremely high frequency of HR is required to explain their data. Even at just the five loci examined, interspecific recombination was claimed for nearly 10% of the "individuals" sampled. The self-contained pattern of the results can only be accommodated if these events occurred so recently that all the putative donor species and recipient clones were still living together at the time of the study, with the transferred haplotypes remaining identical between them. Moreover, HR must have occurred at

least twice in each case: once when the exogenous DNA was first integrated, and again within the recipient genome, when "gene conversion promptly copied the integrated DNA on its homologous region". Given the sequence-based constraints on HR, the species involved would need to have a very high level of sequence identity, to recombine so readily, recently and frequently.

We tested this prediction for each case of "interspecific recombination" using a simple method. We aligned each incongruent haplotype from a putative donor species against the native haplotype it was supposed to have replaced, and measured pairwise identity between them (Supplementary Material). This recreates the genetic divergences across which Debortoli et al. posit multiple, recent HR events (Table 2). The mean identity between sequences undergoing putative interspecific recombination is just 74.8% (median 72.3%). This surprising figure was validated for a genomic region of approximately 10kb around the 400bp EPIC25 intronic marker (Supplementary Material). Identity values were the same or lower regardless whether we considered exons, introns or intergenic regions. These values are not compatible with current understanding of HR in the model systems discussed above. They also seem incompatible with current views of homologous exchange in the genome of *A. vaga* itself. A recent assembly (Flot et al. 2013) indicates that gene conversion and concerted evolution occur between sequence pairs that "are on average 96.2% identical at the nucleotide level (median = 98.6%)." Debortoli et al. (2016) claim "interspecific recombination" between sequences approximately an order of magnitude more divergent.

The tetraploid genome of *A. vaga* supplies a second critical benchmark: each homologous pair of genes has a pair of 'ohnologous' copies whose mean identity is "73.6% (median = 75.1%)" (Flot et al. 2013). This is very close to the value calculated for "interspecific horizontal genetic transfers", yet ohnologs have been evolving independently for millions of years within the same genomes, cells, individuals and clones, with "no conspicuous tracts of identity" between them (Hur et al. 2009). This feature of *A. vaga* militates strongly against the claim that molecules with 74.8% sequence identity could undergo frequent recombination or gene conversion within this genome, still less horizontally.

We considered the possibility that "interspecific recombination" might be mediated by some alternative mechanism with less stringent identity requirements than HR. For instance, microhomology-mediated end joining involves “the use of 5–25 bp microhomologous sequences during the alignment of broken ends before joining" (McVey & Lee 2008). We measured the degree of microhomology between two “transferred” EPIC25 regions at all scales from 1-40bp (Supplementary Material). They were no more similar than 7650 independently evolving ohnologous

pairs in the same genome, consistent with the results of global homology contrasts. One of the sequences even shared more microhomology with its own ohnolog (the EPIC63 region) than with its "transfer" partner. This seems to exclude the hypothesis that microhomology-based mechanisms mediate interspecific HGT. If that were so, ohnologous loci could not evolve independently, as they share at least as much microhomology at every scale, and their DNA must be more abundant and accessible in any genome, cell or individual than DNA arriving across horizontal barriers.

Integration of DNA over great genetic distances must be possible at least occasionally in bdelloid rotifers, since foreign genes with no homology to metazoan sequences are incorporated (Gladyshev et al. 2008). However, the mechanism in these cases would not require homologous substitution, nor does it appear sufficiently frequent or sequence-dependent to prevent ohnologs evolving independently (Eyres et al. 2015). It may involve a series of individually unlikely events or conditions, in which case direct demonstrations may be more challenging than anticipated.

Conclusions

Debortoli et al. (2016) claimed to supply "strong evidence that inter- and intraspecific DNA exchanges occur within the bdelloid rotifer genus *Adineta*." Sequencing data provided by the authors demonstrates that samples treated as individuals were cross-contaminated with DNA from multiple rotifers, whose identities we reconstructed in multiple cases. The probabilities that these patterns could emerge from random noise are vanishingly small (Table 1). Unintentional cross-contamination parsimoniously explains the results of Debortoli et al. (2016) without reference to extraordinary or novel genetic phenomena. We can reject the 'middle ground' argument that some fraction of transfers are real and some are artefacts, because genomic alignments show that interspecific recombination is not a mechanistically credible interpretation for any of the incongruent haplotypes the authors report. Sequence identities are not compatible with homology-based substitution even within the *A. vaga* genome, still less with HGT at the rates the dataset implies. Cross-contamination explains these patterns, and would remain the most plausible interpretation even had it been less evident in chromatograms. In our view, these analyses constitute clear evidence that the findings of Debortoli et al. (2016) are unreliable. The interesting hypothesis that "bdelloid individuals of the genus *Adineta* exchange DNA within and between species" may be true or false, but the work supplies no credible evidence to address that question.

The study appears to have met with broad acceptance (Krause et al. 2016; Ram & Hadany 2016; Sharp & Otto 2016; Tilquin & Kokko 2016), perhaps because bdelloid asexuality has long been considered problematic (Maynard Smith 1986), and the study seemed to confirm the enticing solution that sex is "replaced" in bdelloids by HGT (Gladyshev et al. 2008; Flot et al. 2013). In our view, current formulations of this hypothesis are simplistic and imprecise, lacking sufficient mechanistic or theoretical detail to assess evolutionary plausibility or to guide empirical evaluation.

The work of Debortoli et al. (2016) was criticised by Signorovitch et al. (2016), who did not identify the issues discussed here, but were concerned that the results seemed incompatible with their own prior claim of "a striking pattern of allele sharing consistent with sexual reproduction and with meiosis of an atypical sort" in the bdelloid *Macrotrachela quadricornifera* (Signorovitch et al. 2015). We believe one negative conclusion of Debortoli et al. (2016) remains valid, namely that "our observations do not support the hypothesis of an *Oenothera*-like meiosis" in *Adineta*. That mechanism was predicted to generate a distinctive pattern of exactly concordant haplotypes that is clearly absent from the *Adineta* dataset, cross-contamination notwithstanding. The study of Signorovitch et al. (2015) itself features some patterns and arguments that seem to require empirical clarification, and we join the authors in "awaiting full genome sequencing of the allele-sharing individuals of *M. quadricornifera*" (Signorovitch et al. 2016), which will shed further light.

Schwander (2016) commented on the work of Signorovitch et al. (2015) and Debortoli et al. (2016), in an associated Dispatch titled "*The End of an Ancient Asexual Scandal*". In the assessment of Schwander, "both papers...provide direct evidence for some form of genetic exchange between bdelloid rotifer individuals". In her view, "these two studies show beyond doubt that genetic exchange between individuals occurs in different bdelloid species." Schwander takes the position that "even small amounts of recombination and genetic exchange between individuals appear to be enough to provide all the benefits of sex". Schwander therefore judges that "bdelloids should no longer be considered as asexuals" and that "the starring role of the most notorious asexual scandals should be transferred to a different group." With the advantage of hindsight, we suggest that some of these rather strong assertions might now be considered premature.

In the words of Maynard Smith (1986), bdelloid rotifers "remain something of an evolutionary scandal." Molecular inquiries have revealed some truly extraordinary features in these tiny and unassuming creatures (Arkhipova & Meselson 2000; Gladyshev et al. 2008; Mark Welch et al. 2008; Boschetti et al. 2012; Hespeels et al. 2014), inviting speculation about links to their unusual mode of

reproduction. It is tempting to seek confirmation of these exciting ideas, and to expect further extraordinary discoveries. However, that approach opens the door to well-known biases, both when interpreting data and when evaluating the work of others (Nickerson 1998). A more incremental programme based on falsification may better facilitate firm progress, and we recommend that work touching the molecular genetics of bdelloid rotifers be treated with heightened scrutiny in future. Evidence relating to 'non-canonical' forms of genetic exchange appears particularly susceptible to contamination and misinterpretation (Boothby et al. 2015), and we join others in recommending even greater stringency in these cases (Richards & Monier 2016). A cautious approach will help exploit the unusual leverage that bdelloids offer on fundamental evolutionary and genetic questions.

Acknowledgements

We again sincerely thank N. Debortoli, K. Van Doninck and J.-F. Flot for freely sharing sequencing chromatograms to help us test the alternative hypothesis we had proposed. This open approach goes beyond standard practice for sharing sequencing data, which perhaps ought to be revisited. We have very much appreciated their transparent and scholarly conduct, and their thoughtful and collegial correspondence since we first communicated these issues in April 2016. We are grateful to M. Blaxter and G. Koutsovoulos for comments on the manuscript. This work was funded in part by a NERC Postdoctoral Fellowship (NE/J01933X/1) to C.G.W., and NERC grant NE/M01651X/1 to T.G.B.

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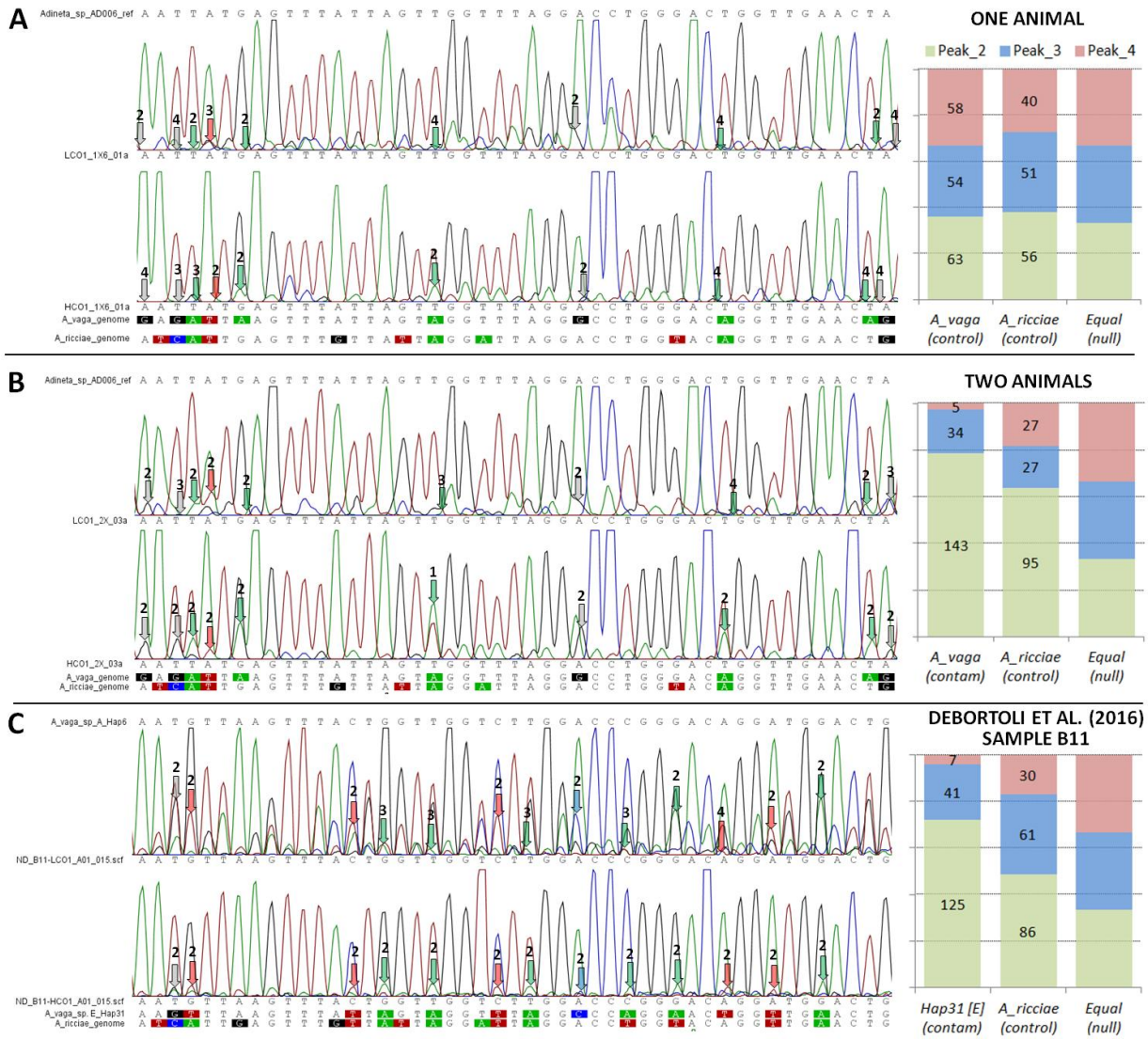
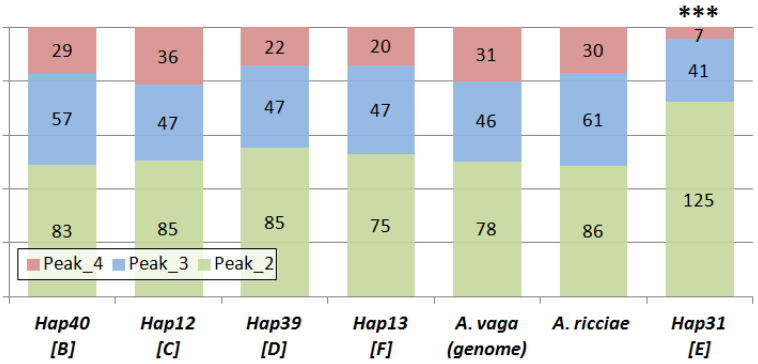
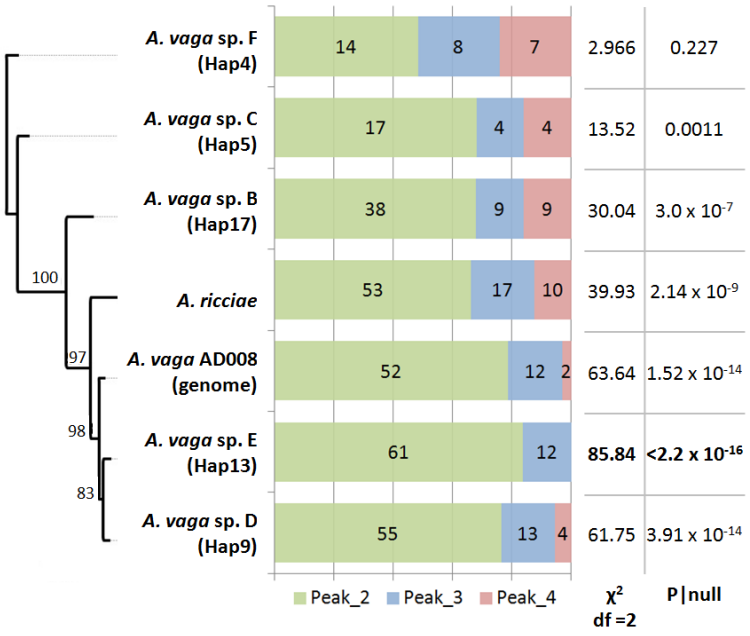


774 **Figure 1.** Contingency table analysis of minority peak height ranks (ConTAMPR), illustrated for
775 mtCO1 chromatograms. **A.** For a sample with a single animal, the height ranks of minority peaks
776 matching other rotifer haplotypes did not differ significantly from an equal distribution (χ^2 = 3.32,
777 d.f. = 4, P = 0.51). **B.** When two animals were deliberately added to a sample, the minority peaks
778 were a significantly better fit to the known haplotype of the second animal (*Adineta vaga*) than
779 either the null expectation (χ^2=88.15, d.f. =2, P <2.2 x 10^{-16}), or a control rotifer haplotype (*A. ricciae*;
780 χ^2=22.54, d.f. =2, P = 1.27 x 10^{-5}). **C.** For Sample B11 of Debortoli et al. (2016), minority peaks were a
781 significantly better fit to the predicted haplotype of a suspected second animal (*A. vaga* Species E
782 Hap31) than either the null expectation (χ^2=127.95, d.f. =2, P < 2.2 x 10^{-16}), or *A. ricciae* (χ^2=25.39,
783 d.f. =, P = 3.07 x 10^{-6}). Minority peaks matching the focal query sequence are pointed out for a short
784 illustrative region; numbers indicate peak height ranks. Bar graphs show the total peak height rank
785 distributions for each haplotype across the full length of the aligned sequences (~605bp), and the
786 null expectation if the peaks represent random noise.



787 **Figure 2.** The mtCO1 chromatograms of Debortoli et al. (2016) for Sample B11 ("Individual 21" [A])
788 indicate a second animal belonging to Species E. The minority peaks fit Hap31[E] significantly better
789 than six control haplotypes (***: χ^2=39.2, d.f. =12, P = 9.73 x 10^{-5}), which represent two reference
790 clones and the other four species reported by Debortoli et al. (2016), and do not have significantly
791 different distributions from each other (χ^2=6.66, d.f. = 10, P = 0.76).



792 **Figure 3.** Minority peaks in 28S chromatograms for Sample B11 of Debortoli et al. (2016) indicate a
793 second animal belonging to Species E. The probability of obtaining a peak rank distribution this
794 extreme given random noise is lowest for Species E and increases for more distantly related control
795 species, according to a neighbor-joining phylogeny of the 28S marker. When species distributions
796 are compared to each other rather than to the null expectation, Species E fits significantly better
797 than Species F (χ^2=13.61, d.f. = 2, P = 0.0011), B (χ^2=12.75, d.f. = 2, P = 0.0017), C (χ^2=12.24, d.f. = 2, P
798 = 0.0022) or *A. ricciae* (χ^2=11.13, d.f. = 2, P = 0.0038). Species D and *A. vaga* (genome) are very
799 closely related to E, and do not have significantly different distributions.

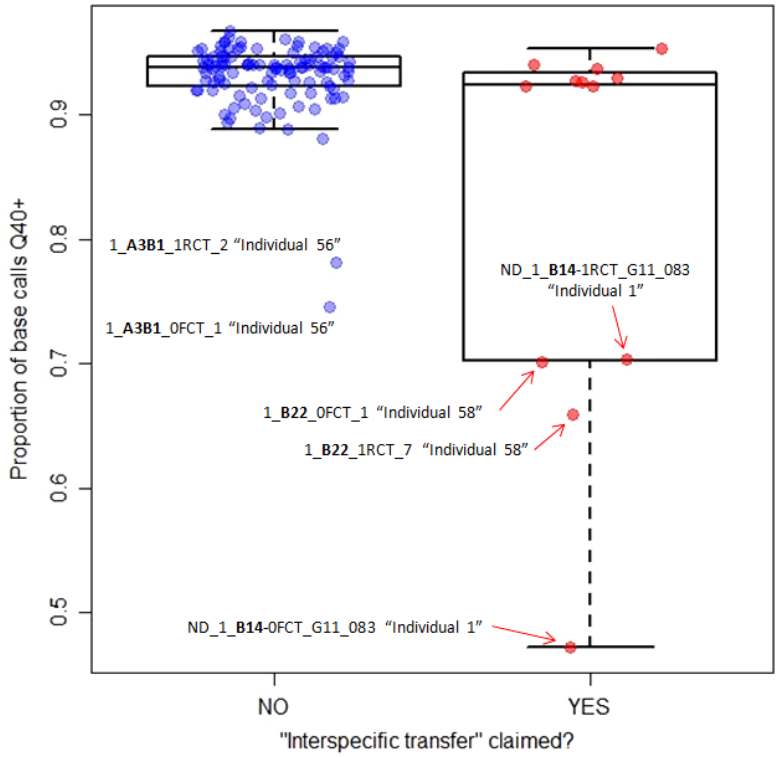


Figure 4. For samples where Debortoli et al. (2016) reported "interspecific horizontal genetic transfers", 28S chromatograms have a significantly different distribution of phred quality scores (Mann-Whitney Test: N=122, W=373.5, P=0.015), because multiple 28S haplotypes are more often evident in minority peaks. This evidence is consistent with DNA from multiple animals in these samples, and perhaps also in some samples where "transfer" was not reported (e.g. A3B1). Sample B14 represents an extreme datapoint with an especially prominent second haplotype, but even if this is removed from the analysis, the two groups still differ significantly (Mann-Whitney Test: N=121, W=373.5, P=0.04).

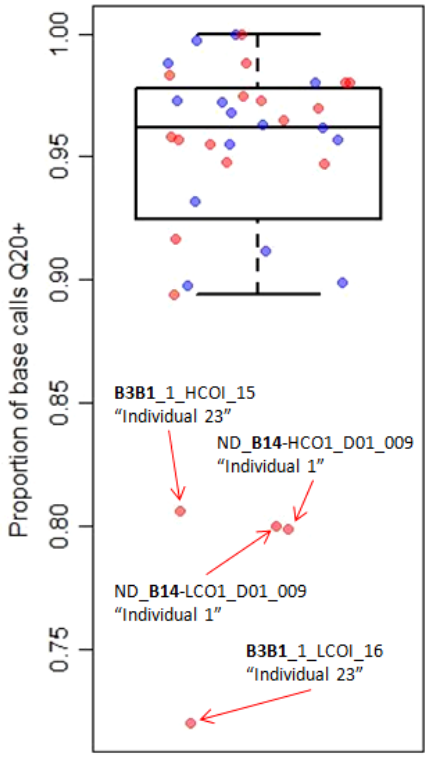


Figure 5. Phred quality scores for the subset of mtCO1 chromatograms provided by Debortoli et al. Red points represent samples where interspecific recombination was claimed; blue points represent samples where no such claim was made. The two groups do not have significantly different distributions (Mann-Whitney Test, N = 35, W = 127.5, P = 0.46), which matches the outcome we saw in experimental samples deliberately contaminated with two animals. However, extreme outliers in quality correspond to the only two samples with evidence of nuclear haplotypes from three different species (Table 1). We suggest DNA from three animals was present.

Sample code	Interpreted as	Haplotypes reported by Debortoli et al. (2016)					Additional haplotypes indicated by ConTAMPR	As seen also in	P (fit) given random noise
		mtCO1	28S	EPIC25	EPIC63	Nu1054			P (fit) versus {control sequences}
B11	Ind 21 [A]	Hap6 [A]	Hap1 [A]	Hap35 [E]	Hap16 [E]	Hap22 [E]	**mtCO1 Hap c.f. 31 [E]**	Ind 81 [E]	$<2.2 \times 10^{-16}$
									9.73×10^{-5} {B;C;D;F;Ar;Av}
							28S Hap c.f. 16 [E]	Ind 81 [E]	2.36×10^{-15}
									0.0085 {B&C;F}
B14*	Ind 1 [A]	Hap6 [A]	Hap1 [A]	Hap9-Hap10 [C]	Hap16-Hap20 [E]	Missing	**mtCO1 Hap c.f. 10 [C]**	Ind 50 [C]	9.1×10^{-4}
									0.187 {B;D;E;F;Ar;Av} n.s.
							28S Hap c.f. 16 [E]	Ind 81 [E]	$<2.2 \times 10^{-16}$
									3.0×10^{-4} {B;C;F;Ar}
							EPIC25 Hap c.f. 30 [E]	Ind 81 [E]	0.00374
									NA (only one direction available)
B22	Ind 58 [C]	Hap10 [C]	Hap5-Hap6 [C]	Hap37 [E]	Missing	Hap16 [C]	**mtCO1 Hap c.f. 36 [E]**	Ind 80 [E]	$<2.2 \times 10^{-16}$
									3.99×10^{-10} {A;B;D;F;Ar;Av}
							28S Hap c.f. 16 [E]	Ind 72 [E]	3.69×10^{-10}
									0.029 {A&B&F}
							EPIC25 Hap ≠ 37 [E]	Ind 72 [E]	NA (>7 self-evident polymorphisms)
B3B1*	Ind 23 [A]	Hap1 [A]	Hap1-Hap2 [A]	Hap35 [E]	Hap1 [A]	Missing	**mtCO1 Hap c.f. 11 [C]**	Ind 33 [C]	0.00158
									0.99 {B;C;D;E;F;Ar;Av} n.s.
							EPIC25 Hap c.f. 10 [C] (& Hap c.f. 6 [C]?)	Ind 47 [C]	$< 2.2 \times 10^{-16}$
									NA (no other species would align)
B39	Ind 66 [E]	Hap10 [C]	Hap11-Hap16 [E]	Hap30-Hap36 [E]	Hap16 [E]	Hap19 [E]	**mtCO1 Hap c.f. 31 [E]**	Ind 81 [E]	$<2.2 \times 10^{-16}$
									$<2.2 \times 10^{-16}$ {A;B;D;F;Av;Ar}
									2.2×10^{-11} {Hap29;32;33;34;35;36 [E]}
									0.0095 {Hap29 [E]; Hap34 [E]}
D14	Ind 5 [A]	Hap3 [A]	Hap1 [A]	Hap10 [C]	Hap1 [A]	Hap1 [A]	**EPIC25 Hap4 [A]**	Ind 6 [A]	$<2.2 \times 10^{-16}$
									NA (no other haplotype would align)

Table 1. Debortoli et al. (2016) reported six samples with incongruent species assignments among marker loci, which were interpreted as *Adineta* individuals that had acquired DNA horizontally from a "donor species". For all six samples, minority peaks in chromatograms provided by the authors revealed additional haplotypes closely comparable to those seen in other animals in their dataset. These either matched the putative donor species, or the 'native' sequence that was supposed to have been replaced, as expected if incongruence was caused by cross-contamination. The height rank distributions of minority peaks corresponding to additional haplotypes were recorded, and we calculated the probability of obtaining a fit at least this good given random noise (final column, upper values). Where possible, we also recorded the equivalent fit for control species or haplotypes, and calculated the probability that the fit of the focal haplotype shared the same distribution (final column, lower values). "Ar": *A. ricciae*; "Av": *A. vaga* (reference genome). Asterisks indicate two samples where haplotypes from three different species were recovered.

Sample	Interpreted as	Marker	Claimed replacement of haplotypes via HR	GC content difference (%)	Pairwise identity (%)
B11	Ind 21 [A]	Nu1054	Hap22 [E] rpl. Hap2 [A]	-21.2	**71.0**
		EPIC25	Hap35 [E] rpl. Hap1 [A]	-17.7	**72.3**
		EPIC63	Hap16 [E] rpl. Hap1 [A]	-13.6	**70.8**
B14	Ind 1 [A]	EPIC25	Hap9&10 [C] rpl. Hap1 [A]	+4.9	**80.2**
		EPIC63	Hap16&20 [E] rpl. Hap1 [A]	-13.6	**70.4**
B22	Ind 58 [C]	EPIC25	Hap37 [E] rpl. Hap10 [C]	-21.8	**68.4**
B39	Ind 66 [E]	mtCO1	Hap10 [C] rpl. Hap31[E]	-0.2	**88.4**
B3B1	Ind 23 [A]	EPIC25	Hap35 [E] rpl. Hap1 [A]	-17.7	**72.3**
D14	Ind 5 [A]	EPIC25	Hap10 [C] rpl. Hap4 [A]	+4.7	**79.8**
			Mean (median):	-10.7 (-13.6)	**74.8 (72.3)**

Table 2. Pairwise identity between sequences that have undergone extremely recent "interspecific genetic exchange" according to Debortoli et al. (2016). This degree of sequence divergence is not compatible with current understanding of homologous recombination, or with the tetraploid structure of the *A. vaga* genome. However, it is consistent with the alternative hypothesis of cross-contamination. Large divergences correspond to substantial GC content differences among species.